

Insect Development

Morphogenesis, Molting and Metamorphosis



Editor
Lawrence I. Gilbert



INSECT DEVELOPMENT

MORPHOGENESIS,
MOLTING AND
METAMORPHOSIS

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MORPHOGENESIS, MOLTING AND METAMORPHOSIS

EDITED BY

LAWRENCE I. GILBERT



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PREFACE

When Elsevier published the seven-volume series “Comprehensive Molecular Insect Science” in 2005, the original series was targeted mainly to libraries and larger institutions. While this gave access to researchers and students based at those institutions, it has left open the opportunity for a new volume, one tailored more specifically to aspects of insect development comprising morphogenesis, molting and metamorphosis. Such a volume would be of considerable value to an additional audience in the insect research community – individuals who either had not had access to the larger work or desired a more focused treatment of these topics.

As the creator and Editor-in-Chief of the original series, not to mention someone who has been involved with editing almost 30 volumes and who edited a major journal in the area for some 19 years, I had a paternal view of this volume. The chapters selected for the noted theme of development have previously been edited with vigor by me and my two co-editors (Kostas Iatrou and Sarjeet Gill) of the Comprehensive series and they are presented here as a strong combination of articles on development, newly available to the individual insect researcher. I felt that the volume should be diverse in the sense that it includes sample chapters from each volume of the series, and the major theme of Development was chosen in part because of my own research interests and experience but also because of the obvious interest of the biological and biomedical research communities in that topic. A significant portion of this new volume is involved with insect hormones that control many aspects of insect development. Only the major hormones are noted here, but the references in those chapters should prove invaluable to researchers and students in both the fields of development and its hormonal control.

Each chapter chosen for this volume has a developmental twist, some more obvious than others. The chapters on “Homeotic Genes,” “Drosophila Limb Development,” “Early Embryonic Development,” “Hormonal Control of Form and Function of the Nervous System,” and “Programmed Cell Death” deal primarily with embryonic development. Chapters on “The Prothoracicotropic Hormone,” “The Ecdysteroid Receptor,” “The Juvenile Hormones,” and “Circadian Organization of the Endocrine System” are endocrinological in nature, hormones being so very important for the control of post-embryonic morphogenesis, molting and metamorphosis. The latter contribution explores the circadian control of these processes, a subject of great relevance to almost every phylum of organisms on this planet. The chapters on “Transposable Elements for Insect Transformation,” “Chitin Metabolism in Insects,” “Cuticular Proteins,” and “Insect Cytochrome” are of critical importance because the chitinous exoskeleton is a major reason for the success of insects on this planet and because the molting process involves both the breakdown of the old exoskeleton and the synthesis of a larger or different type of exoskeleton leading to growth and/or differentiation. Chapters on “Insect G Protein-Coupled Receptors,” “Insect Transformation for Use in Control,” and “Insect Growth- and Development-Disrupting Insecticides” have been selected for their emphasis on the use of technologies affecting growth processes or phenomena at the cell and molecular levels.

Several years of effort was expended by me and my colleagues in choosing topics for the seven-volume series, in the selection of authors and in the editing of the original manuscripts and galley proofs. Each and

every chapter in those volumes was important, and even essential, to make it a Comprehensive series. Nevertheless, I feel strongly that having this volume with the accumulated material and many references on these important aspects of insect development will be of great help to professional insect biologists, to graduate students conducting research for advanced degrees and even to undergraduate research students contemplating an advanced degree in insect science.

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1 Insect Homeotic Complex Genes and Development, Lessons from *Drosophila* and Beyond

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1.1. The History and Early Genetics of the *Homeotic Complex Genes*

1.1.1. Introduction

The word homeotic has its origins in the Greek word *homoios*, meaning “same” or “similar.” William Bateson coined the term “homeosis” in 1894 to

describe a mutation that transforms one structure to the likeness of another, particularly with respect to repeated systems such as segments, petals/sepals/stamens, vertebrae, etc. In *Drosophila* genetics, the term is used to describe a mutation that causes one segment to lose its normal identity and take on the appearance of another segment. Two well-known



Figure 1 *Drosophila* homeotic transformation. *Drosophila melanogaster* hemizygous for three regulatory mutations in the *Ultrabithorax* region of the *Bithorax Complex*: *anterobithorax* (*abx*), *bithorax*³ (*bx*³), and *postbithorax* (*pbx*). The third thoracic segment is completely transformed to a second thoracic identity. The haltere, the dorsal appendage normally present on the third thoracic segment, is transformed to a wing, and the dorsal third thoracic cuticle develops the characteristics of the second dorsal thoracic cuticle. (Reproduced with permission from E.B. Lewis.)

examples are shown in **Figures 1** and **6c**. Pictured are a loss of function *Ultrabithorax* (*Ubx*) mutant, in which the halteres (balancing organs found on the third thoracic segment) are transformed to second thoracic wings, and a gain of function *Antennapedia* (*Antp*) mutant, in which the antennae are transformed to legs.

There are many homeotic mutations scattered throughout the *Drosophila* genome, but two regions on the right arm of the third chromosome are unique. These two clusters of homeotic genes, the *Antennapedia Complex* (*ANT-C*) and the *Bithorax Complex* (*BX-C*), are referred to jointly as the *Homeotic Complex* (*HOM-C*). *HOM-C* genes direct regional specification of the anterior–posterior body axis in insects as well as in other animals (in which they are called the *HOX* genes). The organization and expression patterns of these genes are collinear; in general, a gene’s domain of expression along the anterior–posterior body axis correlates with its genomic position within the complexes (**Figure 2**). This collinearity is also conserved across phyla.

1.1.2. Identification of the *Drosophila Bithorax* and *Antennapedia* Complexes

The first homeotic mutation identified in *Drosophila* was *bithorax*¹ (*bx*), isolated in Thomas Hunt Morgan’s laboratory in 1915 by C.B. Bridges. This mutation affects the third thoracic segment, transforming part of the haltere towards wing. Other homeotic mutations were isolated in the following years, but it was not until the work of Edward B. Lewis that the *HOM-C* was recognized as a complex of genes required for proper anterior–posterior patterning.

Lewis began his work on genes within the *BX-C* in 1946 because these genes appeared to be organized in a cluster of duplicated and diverged genes, rather than as a series of alleles of the same gene (Lewis, 1998) – the mutant loci within the cluster were functionally related and separable by recombination, while maintaining their mutant phenotypes. The isolation of a deficiency (*Df-P9*) deleting the entire *BX-C* allowed Lewis to characterize the functions of each mutation along the *BX-C* individually and together.

Lewis’ seminal review article (Lewis, 1978), describes his view on the organization and developmental function of the *BX-C*. Important was his observation that individual mutations within the complex cause transformations within specific, and sometimes exclusive, body regions. For example, the *bx* mutation effects only the anterior of the third thoracic segment, while the *bithoraxoid* (*bx_d*) mutation transforms the posterior portions of the third thoracic segment and the first abdominal segment towards a posterior second thoracic identity. Based on adult and larval phenotypes of *BX-C* mutations, Lewis proposed that at least eight genes existed within the complex, which “seem(s) . . . to control much of the diversification of the organism’s thoracic and abdominal segments.” He further suggested that these genes act “indirectly by repressing or activating other sets of genes which then directly determine the specific structures and functions that characterize a given segment.” Lewis also put forth the idea that such genetic changes explained evolutionary changes in body pattern – such as the change in insects from four wings, as in lepidopterans, to the two wings found in dipterans, through the development of what Lewis called “a haltere promoting gene.” Similarly, Lewis suggested that the reduction in insect leg number, from an early millipede-like ancestor, might be explained by the development of a “leg-suppressing gene.” Finally, Lewis also identified mutations that defined the *Polycomb* (*Pc*) gene as encoding a regulator of the *BX-C* genes. *Polycomb* is the founding member of a group of genes that are involved in maintaining proper homeotic gene expression patterns, and is further discussed below (see Section 1.3.2).

In 1980, using deficiencies and complementation analysis, Thomas C. Kaufman and colleagues identified a second cluster of homeotic genes on the right arm of the third chromosome (Kaufman *et al.*, 1980) (**Figure 2**). Like the *BX-C*, the genes in this cluster had the ability to cause homeotic transformations of one segment type to another, but this cluster of genes controlled the development of the head and anterior thorax, and was named the

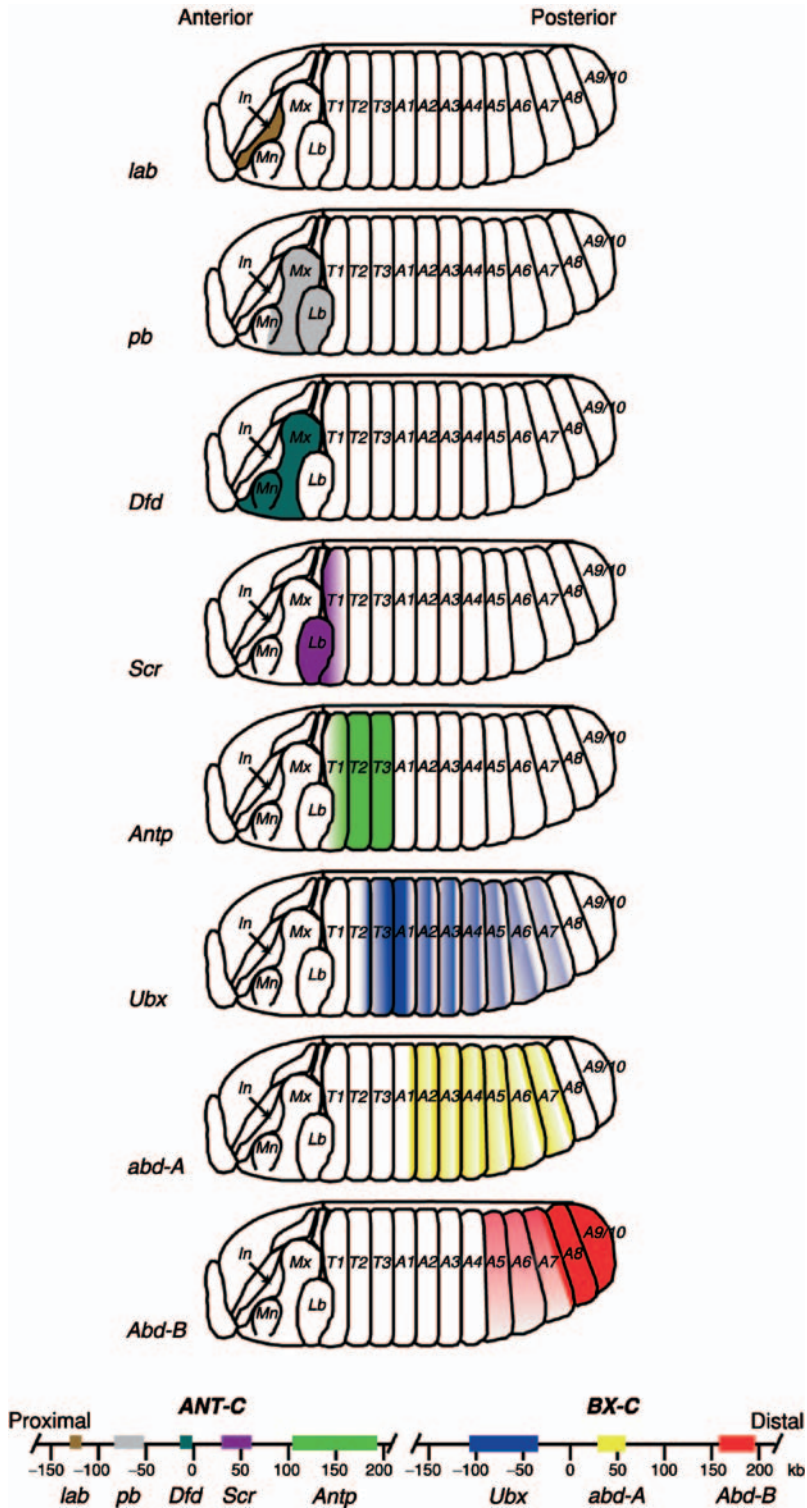


Figure 2 Regional HOM-C gene expression in *Drosophila* embryos. A simplified representation of the HOM-C genes' epidermal expression domains. Each gene's relative position within the ANT-C or BX-C is also shown. The collinearity of genomic location and expression along the embryonic axis is evident. The variation in the shading intensity approximates variations in the level of gene expression. Numbers along the complex are in kilobases. In, intercalary; Mn, mandibular; Mx, maxillary; Lb, labial; T#, thoracic segments; A#, abdominal segments. See text for references.

Antennapedia Complex (*ANT-C*) after its first characterized gene.

A number of diverse homeotic transformations correspond to mutations within the *ANT-C*, and many of the initially recovered mutations lead to viable, transformed adults. For example, one deficiency causes the reduction of male sex combs on the first thoracic leg, indicating that genes within this region are required for first thoracic leg identity. A gain-of-function mutation transforms the second and third thoracic legs to a first thoracic identity as evidenced by the presence of ectopic sex combs on the male second and third thoracic legs (Kaufman *et al.*, 1980). Saturating X-ray and ethyl methanesulfonate (EMS) mutagenesis allowed more precise characterization of the complex and the beginnings of the developmental mapping of the *ANT-C* genes (Lewis *et al.*, 1980a, 1980b; Kaufman *et al.*, 1990). The analogy to the *BX-C* suggested that larval recessive lethal mutations, resulting in homeotically altered larvae, ought to be recoverable from the *ANT-C*, and this proved to be the case (Wakimoto and Kaufman, 1981; Wakimoto *et al.*, 1984; Sato *et al.*, 1985).

Although genetic analyses demonstrated a requirement for the homeotic genes for the establishment of the correct body pattern of the fly, how homeotic genes accomplished this was also on the minds of these pioneering researchers. Both Lewis and García-Bellido predicted correctly that the homeotic genes encoded proteins that controlled the expression of other genes (García-Bellido, 1977; Lewis, 1978). García-Bellido referred to the homeotic genes as “selector” genes that encoded developmental switches sending the expressing cells down one or another developmental pathway. He proposed that “realizator” genes were the receivers of this message and that these encoded the products constructing specific segmental identities.

1.1.3. Cloning and Characterization of the *Antennapedia* and *Bithorax* Complexes

The *BX-C* and *ANT-C* regions were cloned at about the same time in different laboratories. The Hogness laboratory initiated a chromosome walk in the *Ultrabithorax* (*Ubx*) region and cloned the *BX-C* in 1983 (Bender *et al.*, 1983). Both Kaufman’s group and Walter Gehring’s laboratory cloned the *ANT-C* complex, also in 1983 (Scott *et al.*, 1983; Garber *et al.*, 1983). The next significant discovery, in 1984, was that of the homeobox. This 180 bp sequence was found to be present in all *HOM-C* genes (McGinnis *et al.*, 1984; Scott *et al.*, 1985) and supported Lewis’s original idea that the *BX-C* might represent a group of duplicated and diverged genes.

The *ANT-C* was found to contain one homeobox for each homeotic gene that was characterized, and for a few that were not located within homeotic loci. The *BX-C* was, however, found to have only three homeobox sequences – significantly fewer than the eight genes predicted to reside in this region (Regulski *et al.*, 1985). This discovery, along with further genetic analysis demonstrating the existence of only three lethal complementation groups in the *BX-C*, confirmed that only three genes reside within this region (Sánchez-Herrero *et al.*, 1985). The remaining “genes” proved to be *cis*-regulatory elements that control subdomains of *BX-C* gene expression (see Section 1.3.3).

One major difference between the *ANT-C* and the *BX-C* is the presence of intervening, nonhomeotic genes within the *ANT-C* (Figure 3). The *fushi tarazu* (*ftz*), *bicoid* (*bcd*), *zerknüllt* (*zen*), and *zerknüllt-2* (*z-2*) genes are all required during early embryogenesis, and all of these genes contain a homeobox. Interestingly, *bicoid*, *zerknüllt*, and *zerknüllt-2* are thought to have arisen through the duplication and divergence of a single ancestral *HOM-C* gene (Falciani *et al.*, 1996; Stauber *et al.*, 1999). Also present in the *ANT-C* is a cluster of cuticle synthesis genes, which are repeated elsewhere in the genome and do not appear to be required for development. Finally, *amalgam* (*ama*), encoding a protein involved in cell adhesion, is also present in the *ANT-C*.

In the years following, a vast amount of study has examined *HOM-C* gene function, regulation, and structure in detail. The complexes are conserved in virtually all animals, as are the axial patterning functions and collinear relationship between expression patterns and genomic arrangement. Studies have further characterized mutant phenotypes, expression, function, and the genomic structure of the *HOM-C* genes in *Drosophila* and other insects. In 1995, Edward Lewis, along with Eric Wieschaus and Christiane Nüsslein-Volhard, were awarded the Nobel Prize for Physiology or Medicine for their discoveries concerning the genetic control of early embryonic development. We will, in subsequent pages, attempt to present a comprehensive summary of *HOM-C* gene study in insects and will add results from other organisms where appropriate.

1.2. *HOM-C* Gene Function, Cytology, Expression, and Mutant Phenotypes

1.2.1. Introduction

Without doubt, *Drosophila melanogaster* is the most studied organism with regard to the *HOM-C* genes. These transcription factors were initially identified

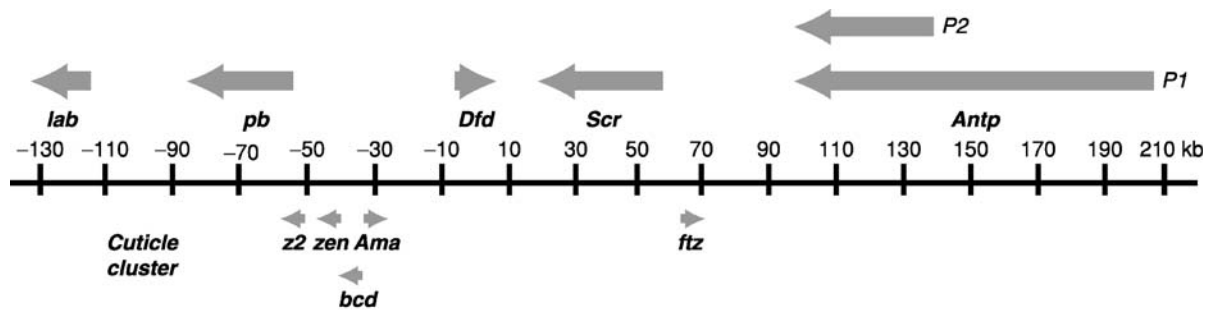


Figure 3 The molecular organization of the *Drosophila* Antennapedia Complex (ANT-C) locus. This portion of the *Drosophila melanogaster* Homeotic Complex (HOM-C) differs from the Bithorax Complex (BX-C) region by the presence of a number of genes not considered to have homeotic function, *per se*. A cluster of 16 cuticle genes lies between the labial (*lab*) and proboscipedia (*pb*) loci. The region between *pb* and Deformed (*Dfd*) is intervened by *zen 2* (*z2*), *zen* (*zen*), *bicoid* (*bcd*), and *amalgam* (*ama*). Finally, *fushi tarazu* (*ftz*), lies within the *Sex combs reduced* (*Scr*) regulatory region. The horizontal line represents approximately 340 kb of the ANT-C region marked in kilobases. The divisions derive from the extension of a chromosomal walk initiated by Scott *et al.* (1983) with the *Dfd* locus at the zero point. Arrows above and below the horizontal lines designate the genomic regions contributing to each gene's transcript(s) and the relative direction of transcription. The regions under the control of the P1 and P2 promoters of *Antennapedia* (*Antp*) are indicated separately.

and characterized in the fruit fly, and much of what is known of their regulation and function has come from this work. Characterization of HOM-C gene expression, function, and molecular organization also extends to a number of other animals, including nondrosophilid insect species and noninsect arthropods. Better-characterized insects include the apterygote insect *Thermobia domestica* (firebrat), *Schistocerca americana* (grasshopper), *Oncopeltus fasciatus* (milkweed bug), *Tribolium castaneum* (red flour beetle), *Bombyx mori* (silkworm), *Precis coenia* (butterfly), and *Apis mellifera* (honey bee). Less studied insects include *Ctenocephalides felis* (flea), *Aedes albopictus* and *Anopheles gambiae* (two mosquito species), *Schistocerca gregaria* (locust), *Acheta domesticus* (cricket), *Folsomia candida* (basal terrestrial apterygote), and *Neodiprion abietis* (sawfly).

In most animal species other than *Drosophila*, the HOM-C genes are clustered together in a single complex, differing from the split complexes observed in several *Drosophila* species (Beeman, 1987; Ferrier and Akam, 1996; Davenport *et al.*, 2000; Powers *et al.*, 2000; Cook, 2001). This split appears to have occurred within the *Drosophila* lineage, as different *Drosophila* species have split the complex in different locations (Von Allmen *et al.*, 1996; Negre *et al.*, 2003). This implies that the *Drosophila* ancestor likely had a single complex and the single insect HOM-C is the ancestral state. The size of the characterized clusters varies among insect species. For example, the portion of the *Tribolium* complex corresponding to the *Drosophila* ANT-C is approximately 279 kb, while the same region in *Drosophila* includes approximately 439 kb (Brown *et al.*, 2002a).

The HOM-C genes of the *Drosophila* ANT-C are labial (*lab*), proboscipedia (*pb*), Deformed (*Dfd*), Sex combs reduced (*Scr*), and Antennapedia (*Antp*). The HOM-C genes of the *Drosophila* BX-C are Ultrabithorax (*Ubx*), abdominal-A (*abd-A*), and Abdominal-B (*Abd-B*). Each HOM-C gene is expressed in a regional manner, and Figure 2 illustrates the generalized expression patterns of the HOM-C genes in a mid-staged *Drosophila* embryo. The maximum morphological and molecular similarity between insects is observed during the embryonic germ band stage, which may represent an evolutionarily constrained (phylotypic) developmental stage (Patel, 1994).

The characterization of each HOM-C gene in *D. melanogaster* is first reviewed in detail, followed by a discussion of studies in other insects with emphasis on their differences from flies. We will begin with the gene governing the anteriormost development and then move posteriorly, demonstrating the collinearity of gene order and anterior–posterior body axis.

Though often depicted with rather simple expression patterns, the HOM-C genes can be quite dynamic, varying in both the level and the extent of expression within each respective domain. Expression boundaries can be segmental, parasegmental, or a combination of the two, and, in some cases, the expression boundaries do not coincide with segmental or parasegmental boundaries.

Each segment can be thought of as being composed of an anterior and a posterior compartment (Figure 4). A parasegment has the same width as a segment, but the anterior of a parasegment is the posterior compartment of a segment, and the

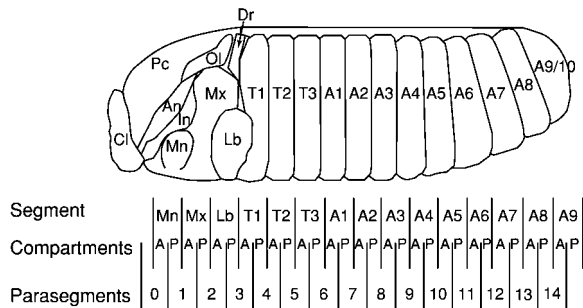


Figure 4 Segments, parasegments, and compartments. Gene expression boundaries in the developing *Drosophila* embryo can be described as segmental, parasegmental, a combination of the two, or sometimes expression boundaries coincide with neither. Parasegment (0–14) and segment boundaries are shown in relation to one another and to a germ band contracted *Drosophila* embryo. The anterior (A) and posterior (P) compartments of each segment are indicated. Parasegment boundaries are shifted anteriorly one compartment from the segmental boundaries. The anteriormost head segments are not labeled in this diagram. Cl, clypeolabrum; Ol, optic lobe; Dr, dorsal ridge; An, antennal; In, intercalary; Mn, mandibular; Mx, maxillary; Lb, labial; T#, thoracic segments; A#, abdominal segments.

posterior portion of a parasegment is the anterior compartment of the following segment. Therefore, a parasegment is similar to a segment, but it is shifted in register by one segmental compartment (Martinez-Arias and Lawrence, 1985).

The distinction between segments and parasegments is important with regard to *HOM-C* expression in the visceral mesoderm and the central nervous system (CNS). In the visceral mesoderm, the expression of most *HOM-C* genes is shifted posteriorly approximately the width of one parasegment, as compared to the ectoderm. The domains of *HOM-C* gene expression can overlap in the ectoderm, but their expression domains in the visceral mesoderm are mutually exclusive (Tremml and Bienz, 1989). As noted in the discussion below, *HOM-C* gene expression within the CNS is also frequently shifted, although by only half of a segment, such that the expression boundaries are parasegmental. The *Dfd* gene is an exception, with its expression boundaries shifting anteriorly in both the visceral mesoderm and the CNS.

1.2.2. The *HOM-C* Genes of the *ANT-C*

The genes of the *Drosophila* *ANT-C* (Figures 2 and 3) are expressed primarily in the head and thorax of the developing embryo and effect the development of corresponding regions in the adult.

1.2.2.1. labial (*lab*) As the most anteriorly expressed and most proximal (with regards to its chromosomal position) member of the *ANT-C*, *lab*

is required for proper development of the intercalary head segment (Mlodzik *et al.*, 1988; Diederich *et al.*, 1989). *lab* is also required for cell fate specification in the tritocerebral neuromere (Hirth *et al.*, 1998) and for copper cell differentiation in the gut endothelium (Hoppler and Bienz, 1995). The *lab* gene spans approximately 17 kb and contains three exons, with the homeobox divided between the second and third exon. The *lab* transcript is ~3.0 kb and encodes a 67.5 kDa protein of 629 amino acids.

Transcripts of *lab* are first detected at 2 h after egg laying (AEL), with the peak of embryonic expression occurring at 6–8 h AEL (Merrill *et al.*, 1989). Expression remains constant through 16–20 h AEL, but decreases before hatching. A low level of expression can be detected throughout pupal development, and no expression is detectable in adult flies.

Temperature sensitive mutants were used to ascertain the temporal requirement for *lab* expression. These experiments have shown that the requirement for *lab* extends from 3–4 h to 16–18 h of embryogenesis, with the period from 6 to 14 h being the most critical for viability to the adult stage.

The distribution of the Lab protein has been examined in embryos and in larval imaginal discs (Diederich *et al.*, 1989; Mahaffey *et al.*, 1989). In the embryo, Lab initially accumulates during germ band extension, with two regions of expression observed. An anterior, ectodermal stripe of expression is present along the ventrolateral border of the procephalon, extending to the anterior border of the postoral segments. This region corresponds to the intercalary segment, which contains the stomadium. A more posterior domain of expression is found in the midgut, where the anterior and posterior midgut primordia fuse. Despite the gene's name, there is no detectable accumulation of Lab in the gnathal lobes (mandibular, maxillary, labial), but expression is evident in the dorsal ridge and in the CNS in the supra/subesophageal ganglia at the base of both brain hemispheres. Lab protein also accumulates lateral to the ventral nerve cord (VNC) and in individual epidermal cells that are precursors to certain sensory organs. These include cells that correspond to the labral sense organ, the black dot organs, and the sense organs of the larval tail. The Lab protein also accumulates in the eye–antennal disc in third instar larvae.

Like other homeotic genes that specify head segment identities, *lab* mutants have no obvious homeosis in the larval head. Embryos homozygous for null mutations in *lab* have grossly disrupted mouthparts and die before hatching, after failing to undergo proper head involution (Merrill *et al.*, 1989). The mandibular and maxillary lobes, which

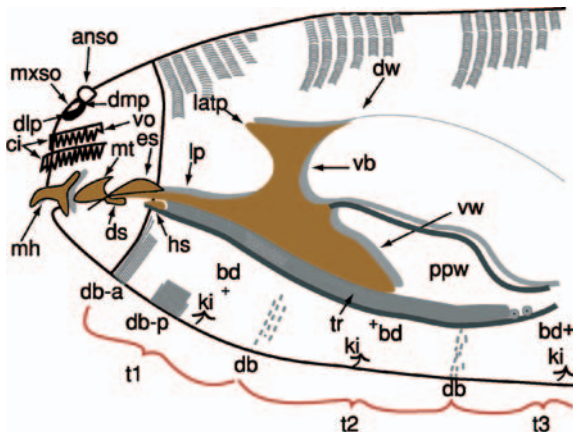


Figure 5 Structures of the *Drosophila* larval head. The cuticular and cephalopharyngeal structures of the larval head and first thoracic segments. Abbreviations, followed by the segmental origin: dw, dorsal wing, acron; latp, latticed piece (dorsal bridge), acron; vb, vertical bridge, acron; es, epipharyngeal sclerite, labral; mt, median tooth, labral; anso, antennal sense organ, antennal; ppw, posterior pharyngeal wall, intercalary; lp, lateral process, mandibular; vw, ventral wing, mandibular; tr, T-ribs, mandibular; mh, mouth hook, mandibular (tip)/maxillary (base); mxso, maxillary sense organ, maxillary; dmp, dorsal-medial papilla of the mxso, antennal; dlp, dorsal-lateral papilla of the mxso, mandibular; ci, maxillary cirri, maxillary; vo, ventral organ, maxillary; ds, dental sclerite, maxillary; hs, hypostomal sclerite (H-piece), labial; db, denticle belt, thoracic; db-a, first thoracic anterior belt, thoracic; db-p, first thoracic posterior belt, thoracic; ki, Keilin's organs, thoracic; bd, ventral kolbchen (black dot organs), thoracic. (Data from Jürgens, G., Lehman, R., Scharding, M., Nüsslein-Volhard, C., 1986. Segmental organization of the head in the embryo of *Drosophila melanogaster*. *Roux's Arch. Devel. Biol.* 195, 359–377 and FlyBase Consortium 2003. The FlyBase database of the *Drosophila* genome projects and community literature. *Nucl. Acids Res.* 31, 172–175. <http://flybase.org/>.)

normally fuse, fail to do so (see Figure 5 for a diagram of the *Drosophila* larval head structures). Further, the dorsal pouch, which gives rise to the dorsal portion of the larval head, develops abnormally. This disruption of head involution leaves the clypeolabrum, maxillary and mandibular lobes, and pharynx protruding from the anterior of the embryo, so that the larval cephalopharyngeal structures are not assembled properly. The lateral processes, the H-piece bridge, and the ventral arms of the cephalopharyngeal skeleton are absent. The median tooth is broadened, and amorphous sclerotized material is present in the head. In severe mutants, the T-ribs and pharynx are affected.

Interestingly, many of the affected tissues do not express *labial* during embryonic development, and thus may suffer effects secondary to *labial* loss. Also characteristic of *lab* mutants is the appearance of sclerotized material between the anal pads in the tail region. Despite the expression of *lab* in the midgut and peripheral nervous system during larval

development, these tissues appear to develop normally in the absence of *lab* (Merrill *et al.*, 1989).

Analysis of adult phenotypes demonstrates the homeotic nature of *lab* mutants (Merrill *et al.*, 1989). Though there are variable effects on the head (one or both of the maxillary palps are missing, the ventral margin of the eye is disrupted, and lumps of tissue with bristles protrude from the surrounding cuticle), the homeosis is obvious because thoracic-like bristles appear in posterior regions of the head, and a thoracic spiracle develops near the center of the postgena. (Labeled diagrams of the adult *Drosophila* head are available at <http://www.flybase.org/anatomy/image-browser.html>)

The expression of *lab* has been also examined in *Thermobia* and *Tribolium*. While expression in the intercalary segment mirrors that of the fly, *lab* is not detected in the midgut or dorsal ridge of the basal insect *Thermobia* (Peterson *et al.*, 1999; Nie *et al.*, 2001). Unfortunately, it is not clear whether this is due to differences in expression or to technical difficulties in the mRNA localization at later stages of development. Further, the exact role of *lab* in the intercalary segment is not well defined, as there are no common appendages arising from the intercalary segment, and it is only in the higher dipterans that *lab* would have a role in head involution (Hughes and Kaufman, 2002).

1.2.2.2. *proboscipedia* (*pb*) The *pb* gene is required for proper development of the adult maxillary and labial appendages but, interestingly, has no role in the development of the larva, even though it is expressed in the embryonic maxillary and labial segments (Pultz *et al.*, 1988). Perhaps this is reflection of a more ancient role, where appendages are found in the maxillary and labial segments of the larva, or perhaps this expression is necessary to prepare the tissues for the adult developmental program. The *pb* gene is approximately 34 kb and contains nine exons. Alternative splicing yields four different *pb* mRNA forms, each differing immediately upstream of the homeobox and encoding a different protein isoform (Cribbs *et al.*, 1992). Like *lab* and *Abd-B*, the homeobox of *pb* is split between two exons, a characteristic also observed in homologs of these genes in other animals.

Embryonic expression is first evident when the germ band is extended, immediately prior to segmentation, 5.5–6 h AEL. The Pb protein is first detected in the presumptive mesoderm behind the stomodeum. As the germ band contracts, Pb protein is detected under the mandibular lobe and in the ectoderm of the maxillary and labial lobes, gradually increasing as the gnathal lobes develop. Protein

accumulation is later evident in the ventral mandibular segment, though not in the ventral regions of the maxillary or labial segments. It overlaps partially with *Dfd* and *Scr* in these regions (Mahaffey *et al.*, 1989). *Pb* accumulates within several cells of each neuromere in the CNS (Pultz *et al.*, 1988; Mahaffey *et al.*, 1989). Expression of *pb* is also evident during the late larval and pupal stages in the labial disc and a small region of the eye–antennal disc.

In complete loss of function *pb* mutants, there is no observed embryonic phenotype, which makes *pb* unique among the *ANT-C* genes of *Drosophila*. Its function is required only for proper development of the adult labial and maxillary structures (Bridges and Dobzhansky, 1933; Pultz *et al.*, 1988). In strong loss-of-function alleles, the adult labial palps are transformed toward prothoracic legs, though weaker alleles transform the labial palps towards antennae. The fate of the maxillary palps in *pb* mutants is unclear; they are reduced and may be transformed toward antennae (Villem, 1944; Pultz *et al.*, 1988).

The expression of the *pb* gene has been characterized in several other insects, and its function in milkweed bugs and beetles has been studied using RNA interference (RNAi) and traditional genetics, respectively. In general, the expression of *pb* is similar to that of the fly, but there is a potentially significant difference in *Oncopeltus* (Rogers and Kaufman, 1997; Rogers *et al.*, 2002). Here, there is no expression of *pb* in the limb buds of the maxillary segment, but *pb* is expressed in the primordia of the maxillary plate and in the labial appendage. Rogers *et al.* (2002) suggest that this difference in *pb* expression may be correlated with, or even be the cause of, a change in the mouthpart structures from a mandibulate precursor to a stylate–haustellate type. They suggest that a change in *HOM-C* expression may cause a homeotic alteration of the maxillary appendage away from an appendage similar to that of the labial segment. If true, this would be quite interesting, as it would demonstrate that body plans could tolerate changes in *HOM-C* gene expression to yield differing developmental outcomes. RNAi reduction of *Oncopeltus pb* transforms the distal labium to first thoracic legs, similar to what occurs in the fly.

Unlike *Drosophila*, mutations in *maxillopedia* (*mxp*) (the *Tribolium* homolog of *pb*) have an embryonic effect (Beeman *et al.*, 1989; Shippy *et al.*, 2000). Null alleles are recessive larval lethals and cause the transformation of maxillary and labial palps toward legs. Only the distal portion of the each palp (telopodite) is transformed to leg. The proximal portion of the appendage (the coxopodite

and a ventral projection called the endite lobe) remains untransformed. Normally, the labial coxopodites fuse at the ventral midline; *mxp* mutants retain the fused coxopodites, but the telopodites are transformed to legs. The degree of transformation is variable – the homeotic legs may be warped, shortened, or may resemble normal palps with only a tarsal-like claw.

RNAi has been used to investigate the role of several *Tribolium HOM-C* genes, and with *mxp*, RNAi generates phenotypes similar to null larvae. Dominant gain-of-function *mxp* mutations also exist in *Tribolium*, with phenotypes that are interpreted to be a transformation of the legs and antennae toward maxillary or labial palps. In addition, the mutant larvae have altered head capsules. Thus, the phenotype of *mxp* mutants supports the theory that an ancestral *pb*-like *HOM-C/HOX* gene had embryonic functions, which were later lost in *Drosophila*. Similar phenotypic effects are observed in *A. albopictus pb*-like mutants (Quinn and Craig, 1971), but the mutation has not been localized to the mosquito *pb* locus.

1.2.2.3. Deformed (*Dfd*) *Deformed* gene expression is required for proper development of the *Drosophila* mandibular and maxillary segments (Wakimoto and Kaufman, 1984), as well as proper cell fate specification within the embryonic brain (Hirth *et al.*, 2001). The first allele was recovered in 1923 as a homozygous viable, dominant mutation reducing the anterior and ventral eye margins, and also disrupting the vibrissae (head bristles on the lower sides of the adult head capsule near the mouthparts). Later work by Wakimoto and Kaufman (1984) identified the requirement for *Dfd* in the specification of the embryonic head. The *Dfd* gene encompasses about 11 kb, having five exons, which produce a single transcript of 2.8 kb and a protein of 586 amino acids (Regulski *et al.*, 1987).

Deformed is the first *HOM-C* gene to be expressed, with its protein first detectable at the cellular blastoderm stage (Mahaffey *et al.*, 1989). Expression continues throughout the embryonic, larval, and pupal stages of development and is also detected in adult flies. Early embryonic expression of *Dfd* encircles the embryo, encompassing the cephalic furrow. Later accumulation is detected, transiently, in the primordia of the hypopharyngeal lobes, and in the developing mandibular and maxillary segments. Expression in the CNS and mesoderm is offset anteriorly, so that mesodermal and neural expression is parasegmental. *Deformed* (*Dfd*) protein expression is also present in cells between

the developing dorsal ridge and the optic lobe. By late germ band contraction, *Dfd* is strongly expressed in cells within the frontal sac that will contribute to the larval eye–antennal discs. Larval expression is localized to the eye–antennal disc in the peripodial membrane and the regions destined to become the maxillary palps. The *Dfd* protein is also detected in the basalmost portion of the labial discs (Martinez-Arias *et al.*, 1987; Diederich *et al.*, 1991).

Deformed gene activity is required at two critical developmental stages: from 3 to 10 h of embryonic development and later, during larval–pupal development (Merrill *et al.*, 1987). Like the other head *HOM-C* genes, *Dfd* mutants exhibit no obvious larval homeotic transformations. Null mutations in *Dfd* disrupt larval head development, causing defects in the cephalopharyngeal skeleton and in sensory structures deriving from the maxillary and mandibular segments (Merrill *et al.*, 1987; Regulski *et al.*, 1987). The mandibular and maxillary lobes fail to fuse and fail to internalize during head involution (a movie depicting *Drosophila* head involution is available at <http://flybase.bio.indiana.edu/images/lk/Animation>). Lost or reduced cephalopharyngeal structures include the mouth hooks, H-piece, maxillary cirri, maxillary sense organs, and portions of the antennal–maxillary sensory complex. In the adult, lack of *Dfd* causes head defects, including disruption of the dorsal and ventral postorbital bristles, changes in morphology of the postgena (including ectopic bristles), loss or reduction of the maxillary palps, the inability to appropriately extend the proboscis, and inclusion of thoracic-like bristles on the posterior head, thus suggesting a transformation to a thoracic-like identity (Merrill *et al.*, 1987; Regulski *et al.*, 1987).

The *Dfd* gene has been cloned from, and its expression examined in, several insects. In general, expression is similar to that in flies, being present in the mandibular and maxillary segments. Loss-of-function analysis using both genetic and RNAi techniques was undertaken in *Tribolium* and *Oncopeltus*, respectively (Brown *et al.*, 1999; Hughes and Kaufman, 2000). Here, a difference from *Drosophila* may occur. Loss of *Dfd* in *Tribolium* transforms the maxillary appendages to antennae and eliminates the endites from the mandibular coxopodites (Figure 6b) (Brown *et al.*, 2000). In *Oncopeltus*, depletion of *Dfd* results in the transformation of both the maxillary and the mandibular segments towards antenna (Hughes and Kaufman, 2000), and it has been suggested that this may represent a “ground state” for gnathal segment identity (Stuart *et al.*, 1991). The differences observed between *Drosophila* *Dfd* mutants and loss of *Dfd* in other insects may, however,

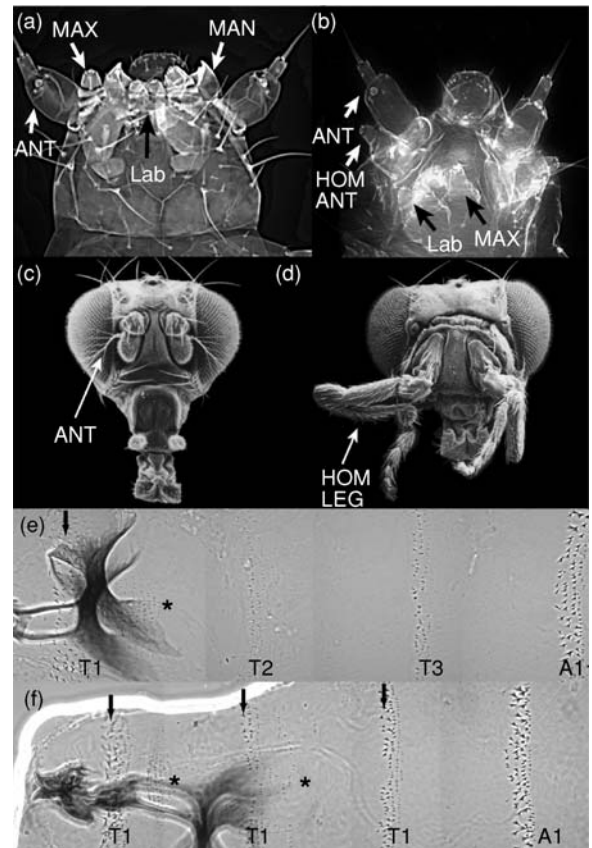


Figure 6 Phenotypic consequences of insect *HOM-C* mutations. Both loss-of-function mutations (b, f) and gain-of-function mutations (d) in the *HOM-C* genes can have dramatic phenotypic effects in *Tribolium* and *Drosophila*. Fluorescent, deconvoluted images of a wild-type (a) and a *TcDfd¹/TcDfd¹* mutant *Tribolium* larva (b). Normal antennae (ANT), mandibles (MN), maxillary palps (MAX), and labial palps (Lab) are visible in the wild-type larva. In a larva lacking *TcDfd* function (b), the mandibles are transformed to homeotic antennae (HOM ANT). A scanning electron micrograph of a wild-type (c) and a mutant (ectopic *Antp* expression) adult *Drosophila* head (d). The ectopic activation of *Antp* in the antennae (ANT) causes transformation to a second thoracic leg (HOM LEG). In a wild-type first instar *Drosophila* larva (e), the three thoracic segments differentiate characteristic denticle patterns. The first thoracic denticles include a main belt (marked with an arrow), and a second, small patch of denticles called the “beard” (marked by an asterisk). The morphology of the first thoracic denticles differs from those of the second and third segments, with the latter being smaller and finer. In the cuticle of an end-stage larva lacking *Antp* (f), the second and third thoracic segments are transformed towards a first thoracic identity. Note the appearance of a beard in the second thoracic denticle belt (marked by an asterisk), and the morphological change of the second and third thoracic denticles to a first thoracic type (arrows). (*Tribolium* images courtesy of S.J. Brown; *Drosophila* head images courtesy of FlyBase (FlyBase Consortium, 2003) <http://www.flybase.org>)

simply reflect the fact that there are no larval appendages in *Drosophila*. Accordingly, the phenotypes of flies and other insects may still represent equivalent developmental alterations, though one must be

careful in interpreting any phenotype as the ground state, as it is likely that some patterning genes are still present to influence appendage development.

1.2.2.4. *Sex combs reduced (Scr)* Spanning about 100 kb, *Scr* encodes at least four separate transcripts derived from alternative splicing of four exons (Andrew, 1995). A large portion of the *Scr* regulatory region is separated from the coding region by the *ftz* segmentation gene (Figure 3). Mutations effecting embryonic function are 5' to the *ftz* gene, closer to the *Scr* transcription unit, while semilethal lesions map 3' of the *ftz* gene, towards *Antp* (Pattatucci *et al.*, 1991; Pattatucci and Kaufman, 1991). The *Scr* protein product is 417 amino acids with a molecular weight of 44 kDa (Andrew, 1995).

The *Scr* gene is particularly interesting in that it controls segment identity in two different tagma of the embryo: the labial gnathal segment and the adjoining first thoracic trunk segment. Its transcripts are first detected as gastrulation begins, 2:50 to 3:10 h AEL, and continue to be present throughout the remainder of embryogenesis (Martinez-Arias *et al.*, 1987). Although lagging by 2–3 h behind the expression of RNA, protein accumulation follows the spatial pattern of accumulation of RNA transcripts, being found throughout the labial lobes and the anterior half of the first thoracic segment (Mahaffey and Kaufman, 1987). In addition, a few cells in the dorsal ridge also accumulate the *Scr* protein. As the germ band begins to contract, *Scr* protein accumulates in the midgut visceral mesoderm (Tremml and Bienz, 1989; Reuter and Scott, 1990). The *Scr* protein is also required for development of the salivary glands, and accumulates in the salivary gland primordia prior to invagination. However, *Scr* is entirely absent after invagination of the developing glands. The *Scr* transcripts and protein accumulate in third instar larval first thoracic discs (leg and humeral) and in the labial discs (Mahaffey and Kaufman, 1987). In larvae, the *Scr* protein is localized to the VNC and in the majority of the labial discs, the first thoracic leg discs, and the dorsal region of the first thoracic humeral discs (Mahaffey and Kaufman, 1987; Glicksman and Brower, 1988). Protein accumulation is also weakly detectable in the second and third thoracic leg discs, but not in the dorsal counterparts of these (wing and haltere). In the adult, the protein is detected in the CNS, in the subesophageal region only.

In the embryo, the homeotic nature of *Scr* appears only in the first thoracic segment, which, in null *Scr* mutants, is transformed to a second thoracic identity. The posterior denticle belt

(beard) is lost and the remaining anterior denticles assume a second thoracic morphology. Also, in embryos lacking *Scr*, the labial lobes fail to undergo head involution, leaving the normally internalized labial sense organs external. Although it was initially reported that the labial segment was transformed to a maxillary identity, closer examination indicated that this was not the case (Pederson *et al.*, 1996). Thus, as with other head homeotic genes, there is no larval homeotic transformation of the labial segment corresponding to the absence of *Scr*. Loss of *Scr* eliminates structures generated by the labial segment: the salivary glands and the bridge of the H-piece (Panzer *et al.*, 1992). Expression of *Scr* is further required in the visceral mesoderm for the formation of the gastric caeca (Reuter and Scott, 1990). In the adult, loss of *Scr* disrupts patterning of the first thoracic discs (leg and humeral) (Pattatucci *et al.*, 1991). In the transformed first thoracic segment of adults, the leg develops with a second thoracic identity, as evidenced by the bristle pattern and the reduction of sex comb teeth in males. Occasionally, a bit of wing-like appendage appears in the dorsal first thoracic segment (Struhl, 1982; Kaufman, 1990).

Along with *pb*, *Scr* is also required for adult proboscis development (Percival-Smith *et al.*, 1997; Rogers *et al.*, 1997; Abzhanov *et al.*, 2001). In null clones of *Scr*, the labial palps transform toward a maxillary palp identity. Dominant gain-of-function mutants also exist that affect adult structures. These cause a partial transformation of the second and/or third thoracic legs toward a first thoracic identity (Pattatucci and Kaufman, 1991).

Although labial expression of *Scr* appears conserved in other insects, indicating that this gene has a conserved role in development of this segment, its expression and role in the first thoracic region is somewhat variable. In *Oncopeltus*, *Acheta*, and *Thermobia*, *Scr* expression is limited to the leg spot domains in the first thoracic segment, in contrast to its expression in the entire anterior first thoracic compartment in *Drosophila*, *Tribolium*, and *Apis* (Rogers *et al.*, 1997; Walldorf *et al.*, 2000; Curtis *et al.*, 2001). It appears, therefore, that the role of *Scr* in the first thoracic segment has expanded in higher insects.

More controversial, however, is the dorsal patch of *Scr* expression in “lower” insects. In flies, expression of *Scr* in dorsal first thoracic tissue prevents development of ectopic wing structures. This has also been proposed to be the role of the dorsal expression in *Oncopeltus* and *Acheta*. However, a similar dorsal region of expression is found in *Thermobia*, and, as a more basal apterygote, *Thermobia*

is not likely to require wing suppression (Rogers *et al.*, 1997).

RNAi has been used to remove *Scr* function from *Oncopeltus*, and genetic analysis has been used to study the role of the *Scr* homolog *Cephalothorax* (*Cx*), in *Tribolium*. In *Oncopeltus*, the depletion of *Scr* results in transformation of the normally fused labium to two small leglike structures (Hughes and Kaufman, 2000). In the first thoracic leg, the bristles of the comb are altered to resemble those found on more posterior legs. In *Tribolium*, there are two classes of mutations in the *Cx* gene (Beeman *et al.*, 1989; Curtis *et al.*, 2001). RNAi was used to determine which is likely to be the null phenotype, and this is a transformation of the labium to antennae and a fusion of the first thoracic segment to the head. Interestingly, the labium to antennae transformation is observed in other insects only when both *pb* and *Scr* are removed. The second phenotypic class of *Cx* is more complicated, causing the labium to acquire a maxillary identity, and a duplication of the first thoracic segment. The reason for this transformation is unclear.

1.2.2.5. Antennapedia (*Antp*) The namesake of the *Drosophila* ANT-C (Kaufman *et al.*, 1980), *Antp*, is required for second thoracic identity and leg development. The *Antp* gene includes a total of eight exons, with two promoters (P1 and P2) producing four different sized transcripts (Bermingham and Scott, 1988). The genomic region controlled by P1 is 103 kb while that of P2 is 36 kb. The *Antp* open reading frame, approximately 1.1 kb in length, is found within the last four exons, which encode a protein of about 43 kDa (Schneuwly *et al.*, 1986). The homeodomain is encoded within the last exon. Multiple translation start sites and alternative splicing lead to at least four protein isoforms, but these vary only slightly, by up to 17 codons (Carroll *et al.*, 1986; Bermingham and Scott, 1988).

In the embryo, *Antp* expression overlaps with *Scr* in the first thoracic epidermis. Though transcripts are detected earlier (Levine *et al.*, 1983), the *Antp* protein is not detectable until germ band extension (Carroll *et al.*, 1986). At this time, the protein is weakly detected in the anterior ventrolateral portion of the presumptive thoracic region. Initial expression is parasegmental, extending from parasegment 3 to parasegment 6. Expression is dynamic and quickly resolves to include the posterior first thoracic segment and all of the second and third thoracic ectoderm. Expression in the visceral mesoderm begins during late germ band contraction, accumulating posterior to *Scr*, although not directly abutting it, and coinciding with parasegments 5

and 6. CNS expression initiates during early neurogenesis, with protein accumulating in the VNC from parasegments 4 through 12 (Carroll *et al.*, 1986; Wirz *et al.*, 1986). As the VNC contracts, *Antp* expression intensifies in the thoracic region, while the abdominal segments maintain only weak expression (Carroll *et al.*, 1986). Bermingham *et al.* (1990) constructed transcript-specific probes and demonstrated that the two promoters are differentially expressed during embryogenesis and have complex (both spatial and temporal) and differing expression patterns.

Transcript and protein accumulation have also been examined at the larval stages, and *Antp* transcripts persist in the ventral neuromere of the second thoracic segment and in the anterior spiracle (Levine *et al.*, 1983; Wirz *et al.*, 1986). Later, in third instar larvae, *Antp* accumulates in the humeral imaginal discs, in the wing blade portion of the wing imaginal discs, in portions of the haltere discs and, to some extent, in all of the leg discs (posterior compartment of the first thoracic leg discs and anterior compartment of the second and third thoracic leg discs).

Interestingly, there is differential expression of the P1 and P2 transcripts in the imaginal discs. The P1 transcripts localize to the anterior margins of the second thoracic wing and leg discs, while the P2 transcripts are evenly distributed in these tissues. Both transcripts are detected in the first and third thoracic leg discs (Jorgenson and Garber, 1987). Somatic clonal analysis reveals different developmental requirements for each *Antp* transcript (Abbott and Kaufman, 1986). Proper development of the adult dorsal thorax requires P1 promoter function, while the P2 transcript is required for appropriate leg and embryonic patterning.

There are three classes of dominant gain-of-function mutations within *Antp*, which often result from chromosomal rearrangements causing *Antp* expression outside its normal expression domain. Best known is a mutation responsible for the transformation of the adult antennae toward a second thoracic leg (Figure 6d). However, other mutations also transform dorsal head structures toward dorsal thoracic structures (Scott *et al.*, 1983), and the *Scr* class transforms the second and third thoracic legs toward a first thoracic identity.

Most dominant *Antp* alleles are also recessive embryonic lethals when hemizygous or heterozygous with an *Antp* null allele. The thoracic cuticle of embryos lacking all *Antp* function is transformed to a more anterior identity (Wakimoto *et al.*, 1984). Sclerotic plates resembling mouthpart material are occasionally present in the posterior of the

first thoracic segment, and the denticle belts of the second and third thoracic segments are transformed to resemble those of the first thoracic segment (Figure 6f). The fact that sclerotized structures form in the posterior of the first thoracic segment indicates a transformation towards a gnathal identity, while the more posterior thoracic segments adopt a first thoracic identity.

Clonal analysis was used to assess the adult requirement of *Antp* (Struhl, 1981). When clones are induced in the second thoracic leg discs, proximal and medial portions of the second thoracic legs are transformed into the corresponding portions of the antenna. The distal tarsus develops normally, however.

Hayward *et al.* (1995) compared the expression of *Drosophila Antp* to that of the grasshopper and found that, while the pattern of expression is conserved, the initial accumulation is quite different. The anterior limit in the grasshopper is segmental, corresponding to the labial/first thoracic border and, therefore, potentially would be overlapping with *Scr*, if *Scr* expression in the grasshopper occurs as in *Drosophila*. Additionally, in the grasshopper, early epithelial thoracic and CNS expression is not as widely modulated as in *Drosophila*, although the expression patterns become more similar later in development with the exception of the modulation that is observed in *Drosophila*.

Expression at the germ band stage is quite similar in all insects examined. The *Tribolium* homolog of *Antp* is called *prothoraxless (ptl)*; Beeman *et al.*, 1989, 1993), and larvae homozygous for this null mutation bear legs that are transformed to antennae. The dorsal thorax is also significantly reduced. This differs from the effects observed in *Antp* mutants in *Drosophila*, which have a more

limited leg transformation. An *Antp* mutant (*Nc*) has been also recovered in *Bombyx*, which results in the transformation of the first thoracic legs to antennae (Nagata *et al.*, 1996). Less extensive changes are also observed in the second thoracic legs, and the silk glands fail to develop properly.

1.2.3. The *HOM-C* Genes of the *BX-C*

The genes of the *Drosophila BX-C* govern pattern formation in the posterior thoracic and abdominal regions (Figure 2). The *BX-C* spans approximately 350 kb (Martin *et al.*, 1995) and is separated from the *ANT-C* by approximately 7.5 Mb (Martinez and Amemiya, 2002) (Figure 7).

1.2.3.1. *Ultrabithorax (Ubx)* The most proximal gene of the *Drosophila BX-C* is *Ultrabithorax (Ubx)*, which functions as a major determinant of segment identity in the thoracic and abdominal region by initiating abdominal development and repressing appendage development (Castelli-Gair and Akam, 1995). The *Ubx* gene spans a genomic region of approximately 76 kb (O'Conner *et al.*, 1988) and produces two transcripts, 3.2 and 4.6 kb in size, which vary at the 3' end due to alternate transcription termination signals. There are four exons including two microexons. The *Ubx* gene encodes at least five different protein variants, but the largest and smallest differ by only 43 amino acids.

Ultrabithorax transcripts are first detected in the syncytial blastoderm (Akam and Martinez-Arias, 1985) and their abundance increases as cellularization proceeds. By gastrulation, a region of strong accumulation includes the primordia of the third thoracic and first abdominal segments, or parasegment 6, with weaker pair-rule-like expression in parasegments 8, 10, and 12. After germ band extension, *Ubx*

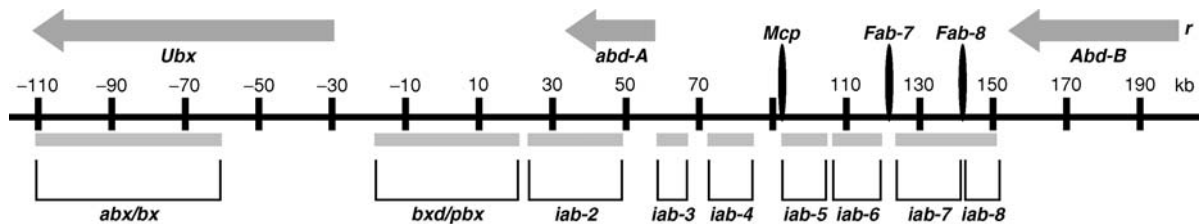


Figure 7 The organization and *cis*-regulatory control regions of the *Bithorax Complex*. The horizontal line, represents approximately 320 kb of the *BX-C* region. The transcription units are marked above the horizontal line for each *BX-C* gene product: *Ultrabithorax (Ubx)*, *abdominal-A (abd-A)*, and *Abdominal-B (Abd-B)*. The *BX-C cis*-regulatory regions are indicated below the horizontal line. The *abx/bx* (*anterobithorax/bithorax*) and *bxd/pbx* (*bithoraxoid/postbithorax*) regions are involved in *Ubx* regulation. The *infra abdominal control (iab)* regions regulate the expression of *abd-A* and *Abd-B*. Three chromatin insulator regions are also shown – *Mcp* (*Miscadastral pigmentation*), *Fab-7* (*Frontoabdominal*), and *Fab-8* – which prevent interactions between neighboring *cis*-regulatory regions. The zero point represents the point of initiation of a chromosome walk by Bender *et al.* (1983). The genomic regions Contributing to the *m* and *r* transcripts of *Abd-B* are also shown.

transcripts are present in parasegments 6 through 12; however, there is greater accumulation in the posterior portion of each parasegment. This is complementary to the expression of *abd-A* in these parasegments (see Section 1.2.3.2). Expression in the somatic and visceral mesoderm (parasegment 7, immediately adjacent to *Antp*) (Tremml and Bienz, 1989) begins as the germ band contracts. In the CNS, *Ubx* transcripts are present in the neuromeres, from parasegments 5 through 12 (Akam and Marinez-Arias, 1985). Although lagging transcription by 1–2 h, protein distribution generally follows the patterns of transcript accumulation (White and Wilcox, 1984; Beachy *et al.*, 1985).

In larvae, *Ubx* protein accumulates weakly in the wing and second leg discs, but more strongly in the haltere and third leg imaginal discs (Beachy *et al.*, 1985). Pupal expression is limited to the anterior compartment of the first abdominal segment and does not overlap with expression of other *BX-C* genes (Kopp and Duncan, 2002).

Homozygous null *Ubx* individuals die as larvae, with transformation of the third thoracic and first abdominal segments to a second thoracic identity (Lewis, 1978). Individuals hemizygous for viable combinations of *Ubx* alleles give rise to the famous “four-winged fly,” as the haltere is transformed to wing (Figure 1). In addition to ectopic wings, a wide band of notal tissue forms in the third thoracic segment, and third thoracic legs and thoracic pits can appear on the first abdominal segment. Gain-of-function alleles ectopically expressing *Ubx* exhibit a transformation of the adult second thoracic segment toward a third thoracic identity, causing a partial wing to haltere transformation (Bender *et al.*, 1983). Interestingly, there appears to be a temporal component to *Ubx* function, as null clones induced at different times in development have different effects. Clones induced earlier than 8 h AEL transform the posterior compartments of the second and third legs towards a first thoracic leg identity, while clones induced later than 8 h AEL have normal second legs, but transform dorsal and ventral appendages of the third thoracic segment (haltere and third leg) towards the corresponding appendage of the second thoracic segment (wing and second leg) (Morata and Kerridge, 1981; Struhl, 1982).

Ubx expression and function have been examined in other insects, and there are some differences when compared with *Drosophila*. In the lepidopterans *P. coenia* and *Manduca sexta*, there are circular clearings within the *Ubx* and *Abd-A* domains, where the larval prolegs will form (Suzuki and Palopoli, 2001),

supporting a role for *Ubx* in limb suppression. Interestingly, even though sawfly (*Neodiprion*) larvae also develop prolegs, *Ubx* and *Abd-A* proteins are detected throughout the developing prolegs, perhaps implying a convergent mechanism for proleg development. This is discussed further, when we consider *HOM-C* target genes. In *Tribolium*, *Ultra-thorax* (*Utx*; the *Tribolium* homolog of *Ubx*) expression is initially detected in parasegments 4 and 5, but this expression later resolves to parasegment 5, more similar to the fly (Bennett *et al.*, 1999). Genetic analysis uncovered a haploinsufficient *Utx* phenotype, demonstrating that the gene is required for proper development of the adult elytra. Heterozygous adults have warped, blistered elytra that fail to meet at the dorsal midline. Homozygosity is lethal and, similar to flies, the first abdominal and third thoracic segments are transformed to a second thoracic identity.

1.2.3.2. abdominal-A (*abd-A*) The *abd-A* gene spans a genomic region of approximately 60 kb, with the transcribed region consisting of at least eight exons (Karch *et al.*, 1990). This gene is required for the promotion of abdominal development and the suppression of thoracic development in parasegments 7 through 9, for the patterning of parasegments 10 through 13, and the specification of the heart cell fate (Busturia *et al.*, 1989; Lovato *et al.*, 2002). The gene encodes a single protein, which is 330 amino acids in length (Cumberledge *et al.*, 1990).

Abd-A protein accumulates simultaneously in the first through eighth abdominal segments and in the amnioserosa at about 4 h AEL, during germ band extension, although the mRNA can be detected earlier (Macías *et al.*, 1990). The anterior expression border coincides with parasegment 7, but the posterior border of expression appears to be segmental, laterally, extending through the seventh abdominal segment. Ventrally, the posterior expression boundary does not coincide with a compartment border but ends within the posterior region of parasegment 13. There is a gradient of *Abd-A* accumulation within each parasegment, such that the highest amount of protein occurs at the anterior border of each parasegment fading to nearly complete absence at the posterior border. This pattern is complementary to *Ubx* in parasegments 7–12, with *Ubx* being strongest where *Abd-A* is absent. Maximum *Abd-A* accumulation occurs in the segmental grooves, but is restricted to only the anterior side of the seventh and eighth segmental grooves. Accumulation of *Abd-A* is also strong around the tracheal pits.

In the visceral mesoderm, Abd-A is evident in cells abutting those cells expressing Ubx, and extends posteriorly to the end of the visceral midgut mesoderm (Tremml and Bienz, 1989; Karch *et al.*, 1990). In the CNS, Abd-A accumulation reflects the pattern in the epidermis (Karch *et al.*, 1990; Macías *et al.*, 1990). Strong expression is evident in the dorsal vessel, in the cells that will form the posterior portion of the heart (Lovato *et al.*, 2002). Somatic cells of the developing gonad also stain weakly in late embryos (Karch *et al.*, 1990). In pupae, Abd-A accumulates in parasegments 7–12, gradually increasing in amount from anterior to posterior. Its domain is adjacent and nonoverlapping to that of Ubx, in contrast to the overlapping domains observed during embryonic development (Kopp and Duncan, 2002).

Defining homeosis within the abdominal segments is difficult, due to the morphological similarities between these segments, in both the larva and the adult (Mathog, 1991). Null alleles of *abd-A* are embryonic lethals, with abdominal parasegments 7 through 9 completely transformed and parasegments 10 through 13 weakly transformed to a parasegment 6 identity. Hemizygous viable alleles allow the characterization of *abd-A* reduction in adult flies, where the second, third, and fourth abdominal segments are weakly transformed toward the first abdominal segment, with the second abdominal segment being most affected. In addition to mutations disrupting the coding region of the gene or affecting the entire *abd-A* locus, some mutations disrupt *cis*-regulatory elements and affect expression of *abd-A* in specific parasegments (Busturia *et al.*, 1989; Karch *et al.*, 1990). Dominant gain-of-function mutations often result from changes in the *infra abdominal control* (*iab*) regulatory regions, leading to ectopic expression of *abd-A* (Busturia *et al.*, 1989) and affecting adult development (Figure 7). For example, one allele, *abd-A^{iab2-S3}*, causes a partial transformation of the first abdominal segment towards a more posterior, second abdominal identity. Other regulatory mutations cause reciprocal transformations of the first abdominal segment towards the second, and the second abdominal segment towards the first (e.g., *abd-A^{iab2-uab1}*). These mutations allow ectopic expression of Abd-A in the developing first abdominal segment, while at the same time reducing Abd-A expression in the developing second abdominal segment. Both of these later mutations only affect imaginally derived tissues. A third gain-of-function allele, *abd-A^{iab3-277}*, causes a divergent transformation, with the first abdominal and third through fifth abdominal segments being all transformed to a second abdominal identity.

While the ninth and tenth abdominal segments in *Drosophila* are quite reduced, many lower insects have ten full abdominal segments. Expression of *abd-A* homologs reflects this anatomical difference. In *Tribolium*, *Abdominal (A)* (the homolog of *abd-A*) has a broader pattern of posterior abdominal expression, reflecting the increased number of abdominal segments (Stuart *et al.*, 1993; Shippy *et al.*, 1998). In *A* mutants, all abdominal segments develop pleuropodia in their anterior compartment (normally only found in the first abdominal segment) and protrusions with a posterior third thoracic leg identity in their posterior compartment. In *Bombyx*, loss of the *abd-A* homologue (*E^{Ca}*) is thought to cause a transformation of the second through seventh abdominal segments to a more anterior abdominal identity (Ueno *et al.*, 1992). Larval prolegs are normally present on the third through sixth abdominal segments, but not on the first, second, and seventh segments. In *E^{Ca}* mutants, the prolegs are lost from the third through the sixth abdominal segments. Although the exact identity of the transformed segments is not clear, one expects an anterior transformation to a first abdominal identity, by analogy to the role of *abd-A* in *Drosophila*. Another mutation in *Bombyx*, *E^N*, removes both the *Ubx* and *abd-A* homologs. Larvae homozygous for this allele develop thoracic-type legs in the first through the seventh abdominal segments with a mixed leg/proleg identity in the eighth abdominal segment. This transformation to a more anterior identity supports the proposed anterior transformation in the *E^{Ca}* mutants.

1.2.3.3. Abdominal B (*Abd-B*) *Abd-B* governs the development of the most posterior abdominal segments of *Drosophila*, and limits the size of the *Drosophila* abdomen by repressing *abd-A* function. The *Abd-B* gene spans approximately 100 kb, containing at least eight exons with multiple promoters. The homeobox is encoded by exons seven and eight, and the position of the intronic interruption is the same as in *lab* and *pb*. Five transcripts are produced, ranging in length from 3.3 to 7.8 kb, with each transcript sharing four common exons (Kuziora and McGinnis, 1988a). These transcripts are expressed in specific spatial and temporal patterns.

The *Abd-B* gene encodes two distinct homeoproteins of 54 and 36 kDa (Celniker *et al.*, 1989), referred to as the “m” and “r” forms, respectively (Figure 7). The m form has a morphogenetic role in parasegments 10–13, specifying identity in the posterior abdominal segments. The r form has a regulatory function. It suppresses the m protein’s morphogenetic role in parasegment 14 and possibly

limits abdominal segmentation (Casanova *et al.*, 1986; Boulet *et al.*, 1991; Kuhn *et al.*, 1995). The r protein form lacks a glutamine-rich M- or opa-repeat (Wharton *et al.*, 1985), which is a transcription activation domain that is found in the m form. The 7.8, 3.7, and 3.3 kb transcripts encode the r protein and accumulate in parasegments 14 and 15, while the 4.3 and 4.7 kb transcripts encode the m protein and accumulate in parasegments 10 through 15 (Celniker *et al.*, 1989; Zavortink and Sakonju, 1989).

The Abd-B proteins are first detectable in the posterior ectoderm of parasegments 13 through 15 of early germ band extended embryos. Relative to the detection of the transcripts, the protein is delayed by approximately 1.5–2 h (Celniker *et al.*, 1989; Delorenzi and Bienz, 1990). As embryogenesis continues, Abd-B expression spreads anteriorly into portions of parasegments 11 and 12. There is a stepwise increase in protein concentration towards the posterior region of the embryo. During early germ band retraction, visceral mesodermal expression is first detected laterally in parasegments 12 through 14 of the mesoderm (Tremml and Bienz, 1989). Expression in the VNC is also evident during this period, and follows the pattern of the epidermis (Celniker *et al.*, 1989; Delorenzi and Bienz, 1990). Following head involution, protein accumulation fades and is not detectable again until the third instar larval stage (in the genital discs). Expression returns to the abdominal segments in pupae, and is found at progressively higher levels in the fifth through seventh abdominal segments (Kopp and Duncan, 2002).

Although the antibody used to detect Abd-B protein recognizes both the m and r forms, *iab-8* mutants are m^- , so protein accumulation in these embryos allows inference of the r form's expression pattern in parasegments 14 and 15 and in the mesoderm of parasegment 14. The anterior margin of the r protein is unchanging during development, always coinciding with the border of parasegments 13 and 14. This differs from the shifting anterior margin observed with this antibody in wild-type embryos, when both forms of the protein are present and detected. It is likely that all of the Abd-B protein detected anteriorly to parasegment 14 corresponds to the m protein. Embryos lacking the r form have a wild-type protein accumulation pattern, reflecting normal m protein in parasegments 10 through 13, and ectopic m protein in parasegment 14 that is due to its derepression by the lack of the r function (Delorenzi and Bienz, 1990).

Loss of *Abd-B* function is lethal and causes the transformation of all abdominal segments posterior

to the fifth abdominal segment to a fourth or fifth abdominal cuticle pattern (Sánchez-Herrero *et al.*, 1985). Exact identification is difficult, again, due to similarity between these segments. Also, the larval posterior spiracles are reduced or eliminated. Some homozygous lethal alleles produce a few escapers, allowing the adult effects of *Abd-B* loss to be directly assessed. In adults, the fifth through eighth abdominal segments are transformed to a fourth abdominal identity. The dominant haploinsufficient effects of *Abd-B* are weak transformations of the sixth and seventh abdominal tergites which resemble the fourth or fifth abdominal type, and adult infertility.

Absence of Abd-B in the genital disc results in a transformation of the male and female genitalia to leg, with claws, tarsi, and/or tibia (Estrada and Sánchez-Herrero, 2001). Less frequently, there was a transformation towards antennae, producing second and/or third antennal segments and arista.

In *Drosophila Abd-B* mutants, a full ninth abdominal segment develops concomitant with posterior expansion of *abd-A* expression (Casanova *et al.*, 1986; Karch *et al.*, 1990). This, and comparisons with other insects, has led to the proposal that *Abd-B* sets the limit on the number of abdominal segments to be formed by repressing *abd-A* activity (Hughes and Kaufman, 2002). The *Tribolium Abd-B* homolog, *extra urogomphi (eu)*, has a more limited expression than *Abd-B*, corresponding only to the posteriormost segments (the tenth and eleventh abdominal segments), whereas *Drosophila Abd-B* is found in approximately five abdominal segments. However, the results from *Thermobia* and *Schistocerca* may contradict this model. *Schistocerca* has a limited *Abd-B* expression domain initially (only the eleventh abdominal segment), but later expression expands anteriorly through the posterior eighth abdominal segment (Kelsh *et al.*, 1993). In *Thermobia*, the *Abd-B* homolog (*TdAbd-B*) is initially expressed posterior to the growth zone, behind the forming posterior abdominal segments (Peterson *et al.*, 1999), but extends anteriorly into the eighth abdominal segment after germ band extension.

The anterior expression in *Thermobia* and *Schistocerca* is perhaps contrary to the model predicting that extended *Abd-B* expression limits the number of abdominal segments, except that this expansion occurs after *abd-A* has been expressed in these segments. Interestingly, the domains of *Abd-B* homolog expression in other insects is similar to the expression pattern of the r-type *Drosophila* protein, while the expression domains coinciding with the *Drosophila* m-type protein appear to be absent.

Also of interest is the difference between *TdAbd-B* expression from that of other *Thermobia* HOX genes, in the limbs that develop in each gene's expression domain. The *Thermobia* homologs of *pb*, *Dfd*, *Scr*, *Antp*, and *Ubx* are expressed throughout the limbs developing in their segments, while *TdAbd-B* is only expressed in the cercal appendage primordia, and not in the appendages themselves as they develop (Peterson *et al.*, 1999). A similar lack of expression is noted in the cercal appendages of *Schistocerca* (Kelsh *et al.*, 1993). Peterson *et al.* (1999) propose that this may indicate a fundamental difference in the nature of terminal appendages versus anterior appendages.

A final interesting note is the association of *Abd-B* with genital development, a role conserved in other insects, spiders (Damen and Tautz, 1999), and even vertebrates, suggesting a general role in establishing the genitalia (Akam *et al.*, 1988).

1.2.4. HOX Expression in Related Arthropods

HOM-C/HOX genes expression has been examined in several noninsect arthropods. Expression has been examined in crustaceans, chelicerates (spiders and mites), and myriapods (centipedes) and significant variations were found from the expression patterns observed in insects. These have been reviewed by Hughes and Kaufman (2002), who also discuss the evolutionary implications of the variability of these genes in arthropod body patterning.

1.3. Regulation of *HOM-C* Gene Function

1.3.1. Introduction

The *HOM-C* genes' clustered genomic structure and collinear expression are rather uniquely conserved across phyla. This raises the question: why are the *HOM-C/HOX* genes found in such highly conserved arrangements, and is this a requirement for their expression and function? Studies in vertebrates reveal shared *HOX* regulatory regions and indicate that changes in the arrangement of the complex can impact the expression of multiple *HOX* genes (Gould *et al.*, 1997; Sharpe *et al.*, 1998). The fact that splits in the *HOM-C/HOX* cluster have only been observed (thus far) in *Drosophila* and in the nematode *Caenorhabditis elegans* (Ruvkun and Hobert, 1998), suggests a strong evolutionary constraint for the ordered *HOM-C/HOX* cluster, and perhaps a loosening of this constraint in these two organisms, in which shared *HOM-C* gene regulatory regions have not been identified. The conservation of ordered, clustered genomic organization may

be required for the temporal and spatial collinearity observed in *HOM-C* expression (van der Hoeven *et al.*, 1996; Kondo and Duboule, 1999), but the mechanism underlying this requirement has yet to be determined (review: Kmita and Duboule, 2003). In spite of the existing uncertainties, however, some of the mechanisms of *HOM-C* gene regulation in *Drosophila* are understood.

As seen by their mutant phenotypes, the *HOM-C* proteins are powerful transcription factors, directing the downstream expression of other genes in specifying developmental pathways. Loss or misexpression of a *HOM-C* gene is sufficient to direct cells, and even whole body regions, towards dramatically different developmental fates. Frequently, these changes in expression are lethal and accompanied by gross morphological defects, and even when viable adults are produced, fitness may be greatly effected. Therefore, strict spatial and temporal control of the *HOM-C* selector genes is critical to normal body patterning. Interestingly, and perhaps predictably, alterations in *HOM-C* gene expression appear to play a role in the differential body patterning between some insect species.

The transcriptional control of the *Drosophila* *HOM-C* genes can be exercised at two levels, related to (1) the initiation or establishment of the active state, and (2) the maintenance of this state. This is true whether we are discussing activation or repression. Initiation or establishment of *HOM-C* gene activity is generally a short term phenomenon and, where mapped, involves local regulatory elements within close proximity to the promoters (Bienz and Müller, 1995). *Trans*-acting factors that initiate *HOM-C* expression include gap and pair rule segmentation gene products. The maintenance phase is long term and may involve DNA elements that are a large distance away from the promoter. Two protein groups, the Polycomb and the Trithorax groups, have opposing effects on the long-term regulation of *HOM-C* transcription. Self-regulation by the *HOM-C* genes and cross-regulation between *HOM-C* genes are also important to the regulation of *HOM-C* gene activity.

A summary of the regulatory factors discussed in this chapter is shown in Table 1.

1.3.2. Initiation and Establishment of *HOM-C* Gene Expression

In the *Drosophila* embryo, the establishment of early *HOM-C* expression occurs during the syncytial or early cellular blastoderm stage and is controlled by the early upstream gap and segmentation gene products. Several studies have demonstrated regulation of *HOM-C* gene expression by the gap

Table 1 Summary of the HOM-C gene regulators discussed in the text^a

Regulator	HOM-C gene(s)	Reference
Involved in activation		
<i>bicoid</i> (<i>bcd</i>)	<i>Dfd</i>	McGinnis <i>et al.</i> (1990)
<i>decapentaplegic</i> (<i>dpp</i>)	<i>lab, Ubx</i>	Staepling-Hampton <i>et al.</i> (1994), Eresh <i>et al.</i> (1997)
<i>engrailed</i>	<i>Dfd, Scr</i>	Jack <i>et al.</i> (1988), Pelaz <i>et al.</i> (1993)
<i>even-skipped</i> (<i>eve</i>)	<i>Dfd, Ubx</i>	Ingham <i>et al.</i> (1986), Jack <i>et al.</i> (1988)
<i>fushi tarazu</i> (<i>ftz</i>)	<i>Scr, Antp, Ubx</i>	Riley <i>et al.</i> (1987), Martinez-Arias and White (1988), Qian <i>et al.</i> (1993)
<i>giant</i>	<i>Scr, Antp, Abd-B</i>	Reinitz and Levine (1990), Wu <i>et al.</i> (2001)
<i>hunchback</i> (<i>hb</i>)	<i>Scr, Antp</i>	White and Lehmann (1986), Riley <i>et al.</i> (1987), Harding and Levine (1988), Shimell <i>et al.</i> (1994), Casares and Sanchez-Herrero (1995), Wu <i>et al.</i> (2001)
<i>Krüppel</i> (<i>Kr</i>)	<i>Antp, Ubx</i>	White and Lehmann (1986), Riley <i>et al.</i> (1987), Harding and Levine (1988), Wu <i>et al.</i> (2001)
<i>odd-paired</i> (<i>opa</i>)	<i>Dfd</i>	Jack <i>et al.</i> (1988)
<i>paired</i> (<i>prd</i>)	<i>Dfd</i>	Jack <i>et al.</i> (1988)
<i>phosphatase 2A</i> (<i>dPPP2A, B'</i>)	<i>Scr</i>	Berry and Gehring (2000)
<i>runt</i>	<i>Dfd</i>	Jack <i>et al.</i> (1988)
<i>serine/threonine kinase casein kinase II</i> (<i>CKII</i>)	<i>Antp</i>	Jaffe <i>et al.</i> (1997)
<i>tailless</i> (<i>ttl</i>)	<i>Dfd, Ubx, Abd-B</i>	Reinitz and Levine (1990), Casares and Sanchez-Herrero (1995)
<i>wingless</i> (<i>wg</i>) (low levels)	<i>lab</i>	Hoppler and Bienz (1995)
Involved in repression		
<i>engrailed</i> (<i>en</i>)	<i>Ubx</i>	Qian <i>et al.</i> (1993)
<i>fushi tarazu</i> (<i>ftz</i>)	<i>Dfd</i>	Jack and McGinnis (1990)
<i>hairy</i>	<i>Dfd</i>	Jack and McGinnis (1990)
<i>hunchback</i> (<i>hb</i>)	<i>Ubx, abd-A</i>	White and Lehmann (1986), Qian <i>et al.</i> (1993), Casares and Sanchez-Herrero (1995), Shimell <i>et al.</i> (2000)
<i>knirps</i> (<i>kni</i>)	<i>Antp, Ubx, Abd-B</i>	White and Lehmann (1986), Harding and Levine (1988), Casares and Sanchez-Herrero (1995)
<i>Krüppel</i> (<i>Kr</i>)	<i>Ubx, abd-A, Abd-B</i>	White and Lehmann (1986), Harding and Levine (1988), Shimell <i>et al.</i> (1994), Casares and Sanchez-Herrero (1995), Shimell <i>et al.</i> (2000)
<i>odd-skipped</i> (<i>odd</i>)	<i>Dfd</i>	Jack and McGinnis (1990)
<i>tailless</i> (<i>ttl</i>)	<i>Ubx, abd-A</i>	Qian <i>et al.</i> (1993), Casares and Sanchez-Herrero (1995)
<i>wingless</i> (<i>wg</i>) (high levels)	<i>lab</i>	Hoppler and Bienz (1995)
Cross-regulation and Autoregulation		
Repression		
<i>abd-A</i>	<i>lab, pb, Dfd, Scr, Antp, Ubx</i>	Karch <i>et al.</i> (1990); Appel and Sakonju (1993), Miller <i>et al.</i> (2001b)
<i>Abd-B</i>	<i>lab, pb, Dfd, Scr, Antp, Ubx, abd-A</i>	Karch <i>et al.</i> (1990), Pelaz <i>et al.</i> (1993), Miller <i>et al.</i> (2001b)
<i>Antp</i>	<i>lab, pb, Dfd, Scr</i>	Riley <i>et al.</i> (1987); Gonzalez-Reyes <i>et al.</i> (1992), Pelaz <i>et al.</i> (1993), Miller <i>et al.</i> (2001b)
<i>Scr</i>	<i>lab, Dfd, pb</i> (in the embryonic mesoderm)	Gonzalez-Reyes <i>et al.</i> (1992), Miller <i>et al.</i> (2001b)
<i>Ubx</i>	<i>lab, pb, Dfd, Scr, Antp</i>	Struhl (1982), Appel and Sakonju (1993), Miller <i>et al.</i> (2001b)
Activation		
<i>Antp</i>	<i>Scr</i> (in the embryonic mesoderm)	Reuter and Scott (1990)
<i>Dfd</i>	<i>pb</i>	Rusch and Kaufman (2000)
<i>pb</i>	<i>Scr</i> (larval labial disc)	Abzhanov <i>et al.</i> (2001)
<i>Scr</i>	<i>pb</i>	Rusch and Kaufman (2000)
Autoregulation		
<i>Dfd</i>	<i>Dfd</i>	Kuziora and McGinnis (1988a), Bergson and McGinnis (1990), Regulski <i>et al.</i> (1991), Gonzalez-Reyes <i>et al.</i> (1992), Zeng <i>et al.</i> (1994), Lou <i>et al.</i> (1995)
<i>Lab</i>	<i>lab</i>	Lou <i>et al.</i> (1995)
<i>Ubx</i>	<i>Ubx</i>	Bienz and Tremml (1988)

Continued

Table 1 Continued

Regulator	HOM-C gene(s)	Reference
Involved in maintenance		
Polycomb complexes		Simon (1995), Simon and Tamkun (2002)
PRC1 (inhibits nucleosome remodeling) includes:		
<i>Polycomb (Pc)</i>		
<i>polyhomeotic (ph)</i>		
<i>Posterior sexcombs (Psc)</i>		
ESC-E(Z) (histone deacetylase activity) includes:		
<i>extra sex combs (esc)</i>		
<i>enhancer of zeste (e(z))</i>		
Trithorax complexes		Ingham (1998), Simon and Tamkun (2002)
TAC1 (histone modification) includes:		
<i>Trithorax (trx)</i>		
<i>Calbindin 53E (Cbp53E)</i>		
BRM (ATP dependent nucleosome alterations) includes:		
<i>brahma (brm)</i>		
<i>moria (mor)</i>		
<i>Snf5-related 1 (Snr1)</i>		
<i>eyelid/osa (osa)</i>		

^aNote that *Drosophila* gene names follow a standard nomenclature: genes are generally named for the mutant phenotype, and dominant mutations are capitalized whereas recessive mutations are lowercase and are shown in italics. Protein names are generally capitalized and not italicized.

gene products. For example, Gap proteins regulate the expression of the *abd-A* and *Abd-B* genes through the *infra-abdominal (iab)* regions (Figure 7), which represent *cis*-regulatory regions of the *abd-A* and *Abd-B* genes (Casares and Sánchez-Herrero, 1995). The number following the *iab* abbreviation reflects the abdominal segment in which each region has its most prominent regulatory effect. For example, the *iab-5*, *iab-6*, *iab-7*, and *iab-8* *cis*-regulatory regions control *Abd-B* expression in segments 5, 6, 7, and 8, respectively. The *iab* regions, which do not produce protein-coding mRNAs, are transcribed initially at the blastoderm stage, and the anterior limits of their expression are collinear with the chromosomal location of each region (Sánchez-Herrero and Akam, 1989; Cumberland *et al.*, 1990). When fused to *lac-Z* reporter genes, the *iab* regions direct expression in precise and distinct domains (Simon *et al.*, 1990; Galloni *et al.*, 1993; McCall *et al.*, 1994; Zhou and Levine, 1999; Barges *et al.*, 2000). Their function appears to be the integration of positional information from the Gap proteins and the transmission of this information into specific regional *abd-A* and *Abd-B* activation domains. It has been proposed that the transcription of these regions “opens” the *abd-A* and *Abd-B* regions to allow activation (Gyurkovics *et al.*, 1990).

The protein products of the gap genes *hunchback (hb)*, *Krüppel (Kr)*, *tailless (tll)*, and *knirps (kni)* control early *abd-A* and *Abd-B* expression through the selective regulation of the *iab* regions (Casares and Sánchez-Herrero, 1995; Shimell *et al.*, 2000).

Loss of either Hb or Kr allows anterior expansion of *iab-2* (via Kr), *iab 3–4* (via Kr and Hb) and *iab 5–6* (via Kr) transcription, and causes a concomitant anterior expansion of *abd-A* and *Abd-B* expression domains (Shimell *et al.*, 1994). In this manner, Kr and Hb define the anterior limits of initial *abd-A* and *Abd-B* expression. The Tll protein similarly limits the posterior expression of *abd-A*; loss of Tll allows *abd-A* expression to occur almost to the end of the germ band (Casares and Sánchez-Herrero, 1995). Further, Tll and Kni regulate transcription in the *iab-8* domain and have opposing effects upon *Abd-B* activity. The loss of Kni causes anterior expansion of *Abd-B* expression through an expansion of *iab-8* transcription, while the loss of Tll eliminates *Abd-B* expression. Differing gradients of gap gene products along the anterior–posterior axis may drive the differing spatial regulation of the *iab* regions. The transcription of the *iab* regions is further regulated by chromatin insulator regions (review: Müller, 2000), which intervene between the *iab* elements (Figure 7). The loss of a chromatin insulator region allows transcription to occur across *iab* regions, and can result in homeotic transformation of one abdominal segment to another (Mihaly *et al.*, 1997, 1998; Drewell *et al.*, 2002).

Gap gene products also affect the expression of other HOM-C genes. The *cis*-regulatory regions of *Ubx – abx/bx (anterobithorax/bithorax)* and *bxdl/pbx (bithoraxoid/postbithorax)* (Figure 7) – also require gap gene products for early regulation, although it does not appear that this involves the

transcription of noncoding RNAs (Qian *et al.*, 1993). Additionally, the loss of Hb affects the expression of other *HOM-C* genes functioning within its domain (*Scr*, *Antp*, and *Ubx*) (White and Lehmann, 1986; Riley *et al.*, 1987; Harding and Levine, 1988).

The control of the *ANT-C* gene *Dfd* by early patterning genes has also been investigated (Jack *et al.*, 1988). Early *Dfd* activity is not significantly affected by changes in the zygotic gap genes, but mutations in the pair-rule genes do affect *Dfd* expression. Mutations in *hairy* (*h*), *runt* (*run*), *even-skipped* (*eve*), *fushi tarazu* (*ftz*), *paired* (*prd*), *odd-paired* (*opa*), *odd-skipped* (*odd*), and *engrailed* (*en*), all affect *Dfd* expression. Mutations in *odd* and *ftz* cause a spatial expansion of *Dfd* expression, indicating a negative regulation of *Dfd* by the respective gene products. The remaining mutants result in contracted *Dfd* expression by varying degrees, indicating a role in the early activation of *Dfd*.

These results support a hierarchical mode of regulation, where Gap proteins regulate pair-rule genes, and pair-rule products regulate *HOM-C* genes. Other work, however, suggests a combinatorial role for early patterning genes in *HOM-C* gene regulation. In separate studies investigating the expression patterns of *Scr*, *Antp* P2, and *Ubx*, Ftz was shown to be required for the initial activation of these *HOM-C* genes (temporal control), while the gap gene products control their spatial domains of expression (Ingham and Martinez-Arías, 1986; Riley *et al.*, 1987; Martinez-Arías and White, 1988). More recent work demonstrates a more direct role for the gap protein Hb in the activation of *Antp*. The authors proposed that this may also be true for the Gap proteins Giant (Gt) and Kr, in terms of the activation of *Scr* and *Ubx*, respectively (Wu *et al.*, 2001). It must be noted, however, that the large-scale morphological defects in Gap gene mutants can confound the assessment of their effects on *HOM-C* expression.

1.3.3. Maintenance of *HOM-C* Gene Regulation

The maintenance of *Drosophila HOM-C* gene expression or repression is required beyond the early stages of embryogenesis, after the early patterning gene products are no longer available. Because cells continue to express the *HOM-C* genes in spatially restricted patterns throughout development, the cells must “remember” their *HOM-C* transcription state to avoid inappropriate activation or loss of selector gene function. This long-term retention of *HOM-C* transcription state is controlled by two large groups of *trans*-acting proteins, the Polycomb Group (PcG) (review: Simon, 1995) which maintains the *HOM-C* genes in the repressed state, and

the Trithorax (Trx) Group (review: Ingham, 1998) which maintains the genes in the active state. Both the Trx group and PcG group proteins are widely conserved between *Drosophila* and mammals (Müller *et al.*, 1995; Simon, 1995).

The PcG proteins function as long-term repressors of *HOM-C* function (Bienz and Müller, 1995; Jacobs and van Lohuizen, 1999). These proteins form multimeric complexes, which are vital for the maintenance of the spatial boundaries of *HOM-C* gene expression (Franke *et al.*, 1991; Simon *et al.*, 1992). They are expressed throughout the embryo (e.g., Franke *et al.*, 1991; Paro and Zink, 1992; Lonie *et al.*, 1994; Martin and Adler, 1993) and, therefore, cannot on their own, define spatial expression of the *HOM-C* genes (Simon *et al.*, 1995). Rather, these protein complexes maintain the expression state initiated by the earlier segmentation proteins by binding PcG response elements (PREs) found within the *HOM-C* gene regions (Zink and Paro, 1989; Castelli-Gair and Garcia-Bellido, 1990; Simon *et al.*, 1995; Gindhart and Kaufman, 1995; Simon, 1995). In PcG mutants, the initial expression of the *HOM-C* genes is normal; however, as early segmentation gene products begin to decay, ectopic expression of the *HOM-C* genes becomes apparent (Wedeen *et al.*, 1986). This leads to homeotic transformations similar to those observed in *ANT-C* and *BX-C* gain of function mutants (e.g., Lewis, 1978; Duncan, 1982; Dura *et al.*, 1985; Choi *et al.*, 2000).

The PcG proteins are thought to mediate repression through the formation of higher-order chromatin structures associated with histone methylation (Cao *et al.*, 2002). The PREs do not have a specific recognizable consensus DNA sequence (Jacobs and van Lohuizen, 1999), and the PcG proteins are likely to be recruited to the PREs by either the repressive Gap or pair-rule proteins, or another DNA binding intermediary.

The Trithorax (Trx) group proteins serve a reciprocal role to the PcG proteins and maintain the active transcriptional state of the *ANT-C* and the *BX-C* genes (Breen and Harte, 1993; Kennison, 1993; Gindhart and Kaufman, 1995). Trithorax (Trx), the namesake and best-characterized member of the group (Ingham and Whittle, 1980), accumulates in a spatially modulated pattern, beginning with pair-rule-like stripes in the posterior of the embryo. This may regulate early *BX-C* transcription (Sedkov *et al.*, 1994), as *trx* mutants have altered *BX-C* gene expression during germ band extension. However, *ANT-C* gene expression is unaltered until the late stages of development.

There are alleles of *trx* that affect late *ANT-C* gene expression with no effect upon *BX-C* expression,

indicating the generation of multiple products, with multiple roles, from the *trx* locus. Null mutants of *trx* mimic loss-of-function mutations in the *ANT-C* and *BX-C* clusters, sometimes producing flies with six dorsal thoracic appendages of wing-like identity, the phenotype for which the gene and the group are named (Ingham, 1998).

Like the case of the PcG proteins, it is suspected that the Trx group proteins may not bind DNA directly, but act through other proteins with DNA binding activity. Interestingly, analysis of PcG and Trx protein localization on salivary gland polytene chromosomes demonstrates that these proteins bind many of the chromosomal regions that contain *ANT-C* and *BX-C*. Thus, interaction between these opposing regulatory proteins at the same chromosomal sites may be important to their function (Kuzin *et al.*, 1994; Chinwalla *et al.*, 1995). However, it is also important to note that although these protein complexes function to maintain the initial *HOM-C* gene activity state in a particular region, the *HOM-C* genes are by no means “locked in” to a particular activity state for the duration of development. This is evident by the dynamic patterns of expression observed for individual *HOM-C* genes during development (discussed earlier in this chapter).

1.3.4. Regulation between the HOM-C Proteins: Auto- and Cross-Regulation

The earliest recognized *Drosophila HOM-C* gene regulation was from proteins encoded within the complexes themselves. The *HOM-C* genes cross regulate one another’s activity, and the expression of several of the *HOM-C* genes is partially directed by autoregulatory loops. Several of the *HOM-C* proteins function to limit the expression of *HOM-C* genes expressed in adjoining regions. For example, Abd-B represses both *Ubx* and *abd-A* to exclude their expression in the posterior abdomen (Macías *et al.*, 1990). Abd-A limits the expression of *Ubx*, giving the two *BX-C* proteins complementary expression patterns in the trunk (Karch *et al.*, 1990). Similarly, a high level of Antp in the second thoracic segment, and *BX-C* proteins in the remaining trunk segments, are required to limit the posterior boundary of *Scr* expression (Riley *et al.*, 1987). In the absence of the *BX-C* genes, *Scr* expands its domain posteriorly to include epidermal expression in the posterior compartments of the thoracic and abdominal segments (Pelaz *et al.*, 1993). Interestingly, this expansion is dependent upon moderate levels of ectopic Antp (normally repressed by the *BX-C* proteins), which, in the absence of the *BX-C* proteins, activates *Scr* in the context of the posterior trunk segments.

In two recent studies, Miller *et al.* (2001a, 2001b) utilized the UAS-Gal4 system (an inducible expression system which makes use of the yeast Gal4 transcriptional activator and its binding site, the Upstream Activation Sequence; Brand and Perrimon, 1993) to investigate the embryonic cross-regulatory relationships of the *HOM-C* genes. They separately focused on cross-regulation in the epidermis, the CNS, the visceral mesoderm, and the somatic mesoderm. In the epidermis, cross-regulation generally follows a hierarchy, whereby the more posterior proteins can repress the activation of the anterior genes, but not vice versa. For example, ectopic expression of *Lab* is unable to repress the activity of more posteriorly expressed *HOM-C* genes, while ectopic *Abd-A* protein results in reduced expression of all *HOM-C* genes expressed more anteriorly.

The *HOM-C* genes demonstrate tissue-specific cross-regulatory activities. For example, ectopic *Scr* and ectopic *Dfd* activate *pb* in the ectoderm, which is consistent with the normal role for these proteins in activating *pb* expression in the embryo (Rusch and Kaufman, 2000). In the mesoderm, however, ectopic *Scr* represses the expression of *pb* (Miller *et al.*, 2001a). The hierarchy of cross-regulation in the CNS also differs from that in the epidermis. Ectopic *Ubx* and *Abd-A* are only able to repress two *HOM-C* genes (*Scr*, *lab*) in the CNS, whereas *Ubx* and *Abd-A* have greater cross-regulatory effects in the epidermis. Miller *et al.* (2001a) conclude that no simple model can describe comprehensively the regulatory relationships between the *HOM-C* genes. Their interactions in the CNS and mesoderm are more complex, and signaling cascades likely contribute to this complexity. Assessment of the relationship between cell autonomous regulation and signal transduction led these authors to propose a new hierarchical relationship in the mesoderm, called “autonomic dominance,” in which the extrinsic determination of cell fate via signaling can be overridden by the expression of *HOM-C* proteins.

The integration of signaling and *HOM-C* gene regulation is illustrated by the induction of *HOM-C* signaling in the endoderm via signals from the overlying visceral mesoderm. This pathway links the regulation of two *HOM-C* genes indirectly through signaling and feedback loops (Bienz, 1997) (Figure 8). Ultrabithorax and Extradenticle (*Exd*; expressed in the visceral mesoderm) direct this complex pathway by activating the signaling molecules Decapentaplegic (*Dpp*) and Wingless (*Wg*). These molecules signal to cells in the endoderm, eventually resulting in the activation of *lab* expression and specification of endodermal cell fate (Immerglück *et al.*, 1990; Reuter *et al.*, 1990; Staehling-Hampton *et al.*,

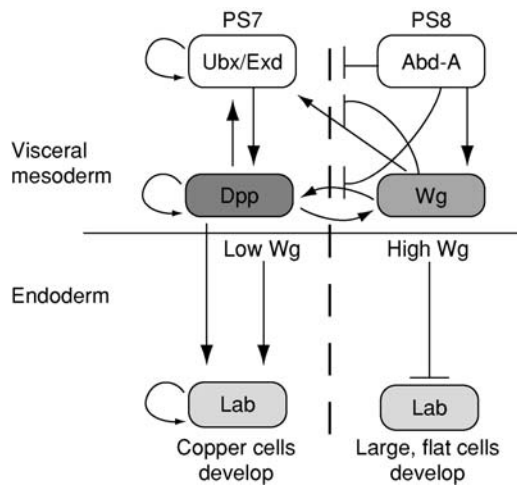


Figure 8 The integration of signaling and *HOM-C* gene regulation in endoderm induction and copper cell specification. Parasegments are approximately six to eight cells wide at the time of endoderm induction. Positive interactions are denoted with arrows, and repressive ones are denoted with bars. In parasegment 7, *Ubx/Exd* directly induces *dpp* expression in the visceral mesoderm overlying the presumptive endoderm. *Dpp* signaling results in a band of *Wg* expression in the visceral mesoderm of parasegment 8. The levels of *Wg* signaling modulate the position of *labial* expression in the endoderm. High levels of *Wg* in parasegment 8 repress the activation of *labial* and allow the development of large, flat endodermal cells. In parasegment 7, signaling input from *Dpp* and low levels of *Wg* activate *labial* and the subsequent specification of crescent-shaped endodermal copper cells. Both *Wg* and *Abd-A* repress *Ubx* transcription in parasegment 8, contributing to the spatial limitation of copper cell differentiation to the endoderm of parasegment 7. Numerous autoregulatory and feedback loops act in this pathway: both *Dpp* and *Wg* act synergistically to maintain *Ubx* expression; *Ubx*, *dpp* and *lab*, all demonstrate autoactivation; and *Ubx* feeds back on *Dpp* to maintain *dpp* expression in the midgut.

1994; Sun *et al.*, 1995; Eresh *et al.*, 1997; Grieder *et al.*, 1997). The concentration of *Wg* directs the activity state of *lab* in these cells. Low *Wg* levels promote *lab* activation and endodermal copper cell differentiation. High levels of *Wg*, on the other hand, repress *lab* activation and, thus, repress the differentiation of copper cells (Hoppler and Bienz, 1995). This regulation may be mediated through the *Wg* signal transducers, such as *disheveled* (*dsh*), *armadillo* (*arm*), and *shaggy* (*sgg*), and clearly illustrates a link between differential regulation of a *HOM-C* gene and a specific terminal cell fate.

Autoregulation can also direct the activity of the *HOM-C* genes. Some of the earliest characterized *HOM-C* protein binding sites were autoregulatory elements. Several regionally specific autoregulatory elements have been characterized in *Dfd*, *Ubx*, and *lab* (Bienz and Tremml, 1988; Kuziora and McGinnis, 1988b; Bergson and McGinnis, 1990;

Chouinard and Kaufman, 1991; Regulski *et al.*, 1991). These elements may be located upstream of the promoter or within intronic regions. For example, a CNS-specific autoregulatory element of *Dfd* maps within the large *Dfd* intron (Lou *et al.*, 1995), while an epidermal enhancer resides upstream of the *Dfd* promoter (Bergson and McGinnis, 1990; Zeng *et al.*, 1994).

1.3.5. Posterior Prevalence–Functional Hierarchies

Posterior prevalence, also called functional dominance and phenotypic suppression, refers to the phenomenon whereby a posteriorly expressed protein can override the function of a more anteriorly functioning protein, when these are simultaneously expressed in the same cells. For example, the ubiquitous ectopic expression of *Scr*, under the control of a heat shock promoter, is lethal and results in the transformation of the embryonic maxillary segment towards a labial identity, as evidence by the partial loss of mouthpart structures and the formation of ectopic salivary glands (Zeng *et al.*, 1993). The *Scr* protein overrides the normal patterning directed by *Dfd* in the mandibular and maxillary lobes. This could be due to a higher level of *Scr* than *Dfd* in these regions. However, there is no phenotypic effect in the trunk posterior to the second thoracic segment. Ectopic *Scr* appears unable to supersede the functions of endogenous *Ubx*, *Abd-A*, and *Abd-B*, even though these proteins are probably less abundant. Similarly, the ubiquitous ectopic expression of *Ubx* causes the transformation of segments anterior to the first abdominal, including the development of abdominal type denticles in the head and in the thorax (González-Reyes and Morata, 1990). However, segments posterior to the first abdominal are not altered. Therefore, like *Scr*, *Ubx* is unable to override *Abd-A* and *Abd-B* function in the epidermis.

There are, however, violations to the posterior prevalence model. For example, the ectopic expression of *Dfd* is able to induce ectopic mouth hooks in the labial segment, alter the denticle patterning of the second thoracic segment to resemble the first thoracic segment, and produce ectopic sensory cirri and sclerotized mouthpart-like material in the trunk segments (Kuziora and McGinnis, 1988a), thus apparently altering the patterns directed by the endogenous *HOM-C* proteins in these cells.

1.3.6. Regulation of *HOM-C* Protein Function by Phosphorylation

Not all *HOM-C* gene regulation occurs at the level of transcription; *HOM-C* protein function is also

modulated posttranslationally. The N-terminal portion of the homeodomain is important for the specificity of homeodomain function (see Section 1.4), and this region of the Scr protein is a target for a regulatory subunit of the serine–threonine specific protein phosphatase 2A (dPP2A, B') (Berry and Gehring, 2000). The phosphorylation state of two key residues in the N-terminal portion of the homeodomain (Thr6 and Ser7) is critical to Scr function. These residues can be phosphorylated by cAMP-dependent kinase A (PKA), and dephosphorylated by dPP2A in cultured cells. Ectopic expression of an Scr protein construct mimicking constitutive dephosphorylation (with Thr6 and Ser7 altered to alanine), reproduces the embryonic phenotype induced by ectopic expression of the wild-type protein (loss of some mouthpart structures, ectopic salivary gland formation, and transformation of the second and third thoracic segments towards a first thoracic identity). Conversely, an ectopically expressed Scr protein construct, in which Thr6 and Ser7 are altered to mimic constitutive phosphorylation (by substitution with aspartic acid) is unable to reproduce the embryonic ectopic Scr phenotype and has a reduced ability to bind a target DNA sequence *in vitro*. Further, dPP2A,B' appears to be required for endogenous Scr function, as embryos lacking dPP2A,B' fail to form salivary glands.

A second example of HOM-C protein functional regulation by phosphorylation involves Antp. Phosphorylation by the serine–threonine kinase casein kinase II (CKII) is implicated in preventing spatially inappropriate Antp function during *Drosophila* embryogenesis (Jaffe *et al.*, 1997). Together, these results suggest that posttranslational modifications may control the functional state of a HOM-C protein.

1.3.7. Regulation of HOM-C Genes in Other Insects

Because *Drosophila* is a long germ insect, patterning its trunk segments from a continuous existing group of cells, aspects of its HOM-C gene regulation might be expected to differ from intermediate or short germ insects, which form their trunk segments sequentially from a continuously dividing growth zone. Homologs to many of the *Drosophila* early segmentation genes have been identified, in other insects, and their expression patterns examined. In *Tribolium*, homologs to each class of the *Drosophila* segmentation genes have been identified, and the expression of most was shown to be similar to that observed in *Drosophila* (Sommer and Tautz, 1993; Brown *et al.*, 1994; Nagy and Carroll, 1994; Patel

et al., 1994; Wolff *et al.*, 1995; Brown *et al.*, 1997). Yet, their function may not always be the same. For example, in *Tribolium*, deletion of *Tcftz* has no effect on segmentation (Stuart *et al.*, 1991). Similarly, the homologs of the pair-rule genes *eve* and *ftz* appear to have no pair-rule function in *Schistocerca* (Akam and Dawes, 1992; Patel *et al.*, 1992). Differences in the roles of these genes may also reflect differences in their ability to regulate HOM-C gene expression, but this remains to be investigated.

One proposed regulator of HOM-C gene activity in other insects is the *Tribolium* gene, *jaws*. Mutants in this gene bear a phenotype similar to one resulting from the overexpression of *mxp*, the *Tribolium* homolog of *pb* (Sulston and Anderson, 1998). Embryos lacking *jaws* have a homeotic transformation of the thoracic and anterior abdominal segments into four alternating maxillary and labial segments, and also fail to form a number of posterior abdominal segments (Sulston and Anderson, 1996). The *mxp* gene is ectopically expressed in each transformed segment, with Ultrathorax (the homolog to Ubx) and Abdominal (the homolog to Abd-A) being virtually absent in the few remaining trunk segments. The *jaws* gene is not located within the *Tribolium* HOM-C, and thus is a candidate negative regulator of HOM-C gene activity. Sulston and Anderson postulate that *jaws* may represent a member of the *Tribolium* Polycomb group.

Cross-regulation of HOM-C genes also is observed in other insects. *Tribolium* shares regulatory relationships between *pb* and *Scr* similar to those present in *Drosophila*. In the *Drosophila* embryo, *Scr* and *Dfd* positively regulate *pb* expression in the gnathal segments (Rusch and Kaufman, 2000), while in the distal region of the larval labial disc, *pb* positively regulates the activity of *Scr* (Abzhanov *et al.*, 2001). In *Tribolium*, development of the larval labial appendage requires *Cx* (the *Scr* homolog) to properly activate early and midstage embryonic expression of *mxp* (the *pb* homolog) (DeCamillis *et al.*, 2001), and MXP positively regulates *Cx* expression in the distal gnathal appendage fields in later embryonic development (DeCamillis and French-Constant, 2003). However, this does not hold true in all insects. Similar patterns of *Scr* and *pb* expression are observed in the milkweed bug, but RNAi experimentation indicates no apparent regulation of *Scr* by *pb* in gnathal appendage buds, or cross-regulation of *pb* by *Scr* (Hughes and Kaufman, 2000). It is clear therefore, that there may be differences in HOM-C regulation, and this is an area where additional information from other insects is needed.

1.4. The Homeobox and the Homeodomain: Identification, Structure, and Function

1.4.1. Identification and Characterization of the Homeobox and Homeodomain

Following the cloning of the *ANT-C* and *BX-C*, two groups identified a strongly conserved DNA sequence in several genes within the two complexes, which was called the H-repeat (McGinnis *et al.* 1984b; Scott and Weiner, 1985). Further testing pinpointed their genomic locations to regions within the *BX-C* (between the *bxd/pxd* and *iab-2* mutations) and the *ANT-C* (in the region of *pb*, *Dfd*, and *zen*). The H-repeat was named the “homeobox,” and a second conserved repeated sequence, the M-repeat, was also identified. The M-repeat encodes a poly-glutamine repeat that had been previously identified in genes outside of the *HOM-C*, and is alternatively called the opa-repeat (Wharton *et al.*, 1985). Sequence analysis revealed strong conservation of predicted amino acid sequence between the gene regions. Using the homeobox sequences from *Antp* and *Ubx*, Regulski and collaborators mapped homeobox sequences to each *ANT-C* gene cloned at the time (*ftz*, *Antp*, *Dfd*, and *Scr*) and to three regions within the *BX-C* (*Ubx*, *abd-A*, and *Abd-B*) (Regulski *et al.*, 1985). They concluded, “all of the copies of homeobox homology that are well conserved with respect to both *Antp* and *Ubx* copies, map within genes of the *ANT-C* or *BX-C*.” Later work showed that both *pb* and *lab* are also

homeobox and M-repeat containing genes (Mlodzik *et al.*, 1988).

The homeobox is a nucleotide sequence of 180 well-conserved base pairs, encoding a 60 amino acid motif called the homeodomain (Figure 9) (McGinnis *et al.*, 1984c). Within the homeobox, nucleotide sequence similarities among the *HOM-C* genes are as high as 75–80%, and many of the nucleotide differences are silent. For example, *Antp* and *Ubx* share 79% nucleotide identity within the homeobox, but share 89% amino acid identity in the homeodomain. Outside of the 180 bp region, the sequence similarity among the *HOM-C* genes is limited to small regions, or is nonexistent.

Evolutionary conservation of the homeobox was initially reported in the genomes of three other segmented invertebrates (*D. hydei*, the beetle *Tenebrio molitor*, and the earthworm *Lumbricus* sp.) and three vertebrates (chicken, frog *Xenopus laevis*, and mouse) (Carrasco *et al.*, 1984; McGinnis *et al.*, 1984a, 1984b). The homeobox sequences from the *Drosophila Antp* and *Ubx* genes were used in cross-species genomic surveys to identify homeodomain-containing fragments in these animals, and the similarity within the homeodomains was found to be quite high. This conservation across phyla was reinforced by results from subsequent studies, with *HOM-C* homologs found in the genomes of every animal searched. In fact, remarkable conservation is even noted between the *Drosophila HOM-C* genes and their mammalian counterparts despite at least 600 million years of evolutionary separation. For

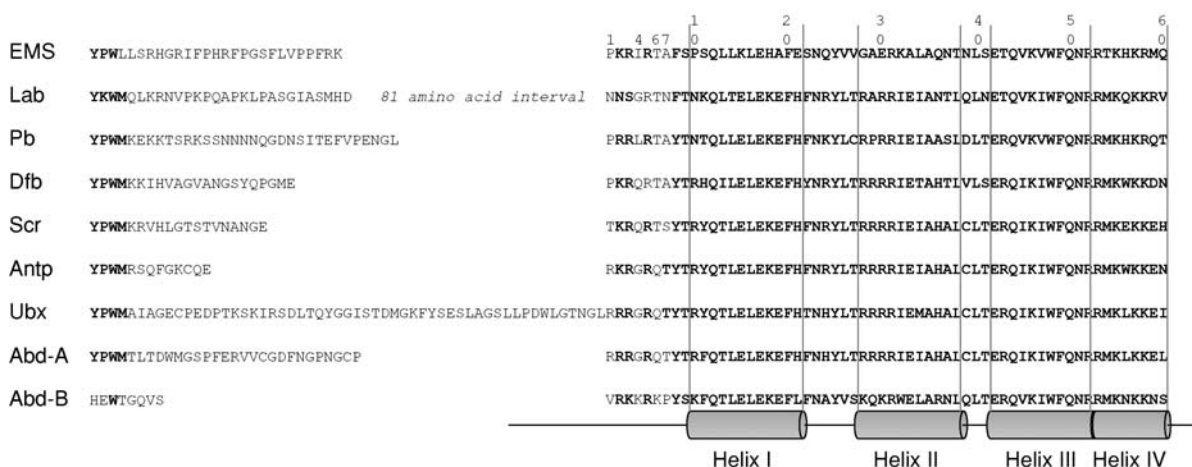


Figure 9 The *HOM-C* protein homeodomain and YPWM motif. The amino acid sequence of each *HOM-C* protein and EMS homeodomain, plus a number of residues N-terminal to the homeodomain are aligned to show sequence conservation. Some of the amino acid sequence between the LABIAL homeodomain and YPWM motif is omitted, as 81 amino acids intervene the two conserved domains. *Abd-B* does not exhibit the conserved YPWM motif, but a conserved tryptophan (W) residue just upstream of the homeodomain may represent the remainder of that motif, and is thus aligned as such. The secondary structure of the homeodomain is shown below the amino acid sequence. Note that α -helices III and IV abut and frequently the homeodomain is considered to have only three α -helices. N-terminal residues key for functional specificity are highlighted and discussed further in the text.

example, *Dfd*, and a mammalian homolog *HOXD-4* (*HOX-4.2*), are conserved within the homeodomain at 55 of 60 residues. *HOXD-4* expression in *Drosophila* is able to activate a *Dfd* responsive reporter in embryos, and produces adult phenotypes similar to those caused by a dominant gain-of-function *Dfd* allele (McGinnis *et al.*, 1990). This level of cross-phylum conservation within the homeobox sequence led Jonathan Slack (1984) to wonder if this conserved sequence could prove to be “the biological equivalent of the slab of basalt found in 1799 near the Egyptian town of Rosetta ... [which] provided the most important clue in the decipherment of the hieroglyphics,” and touched off a profusion of study into the homeobox, the homeodomain, and *HOM-C* gene function.

The homeodomain-containing genes of all organisms are classified based on amino acid sequence similarity in and around the homeodomain (Bürglin, 1994). Six of the eight *HOM-C* genes are grouped together as part of the “Antennapedia class” (*pb*, *Dfd*, *Scr*, *Antp*, *Ubx*, *abd-A*), and share at least 60% sequence similarity within the homeodomain. The *labial* gene is in a class bearing its name, and its homeodomain is 55–67% similar to members of the *Antp* class. Just upstream from the homeodomain, the *Antp* and *labial* classes share a short conserved motif (the hexapeptide, or YPWM motif), making these classes members of the Hexapeptide superclass of homeobox genes. The *Abd-B* homeodomain is no more than 55% similar to any other *HOM-C* homeodomain, diverging significantly in the first two helices of the homeodomain (Celniker *et al.* 1989). The third helix, however, is quite similar, differing from the corresponding *Ubx* region by only a single amino acid. The *Abd-B* homeodomain does not contain the hexapeptide motif, but does retain a conserved tryptophan residue (W) just upstream of the homeodomain. In the mouse, this residue appears capable of mediating protein interactions similar to those mediated by the hexapeptide motif (Shen *et al.*, 1997). Because its homeodomain sequence diverges significantly from the other *HOM-C* homeodomains, *Abd-B* is classified separately, in the *Abd-B* class (Bürglin, 1994).

In addition to the *HOM-C* genes, there are numerous other *Drosophila* homeodomain-containing genes. Several are well studied and have provided much of the basis for our understanding of homeodomain structure and function. Particularly well-characterized examples include the segmentation genes *engrailed* (*en*) (Desplan *et al.*, 1985, 1988; Ohkuma *et al.*, 1990; Kissinger *et al.*, 1990; Jaynes and O’Farrell, 1991; Ades and Sauer, 1994; Clarke *et al.*, 1994; Bourbon *et al.*, 1995) and *ftz* (Nelson

and Laughon, 1990; Percival-Smith *et al.*, 1990; Florence *et al.*, 1991; Walter *et al.*, 1994), and the maternal factor *bicoid* (*bcd*) (Hanes and Brent, 1989; Treisman *et al.*, 1989; Hanes *et al.*, 1994). All of the *HOM-C* proteins, and other homeodomain-containing proteins including Even-skipped (*Eve*), *Ftz*, and *En*, have a conserved glutamine (Q) residue at position 50 of the homeodomain and are referred to as the Q50 homeoproteins (Biggin and McGinnis, 1997). Thus, a generalized *HOM-C* protein can be described as having the following structure: a long N-terminal segment containing the conserved M-repeat/opa-repeat region, a central region, the homeodomain, a short C-terminal region, and a trailer sequence (Desplan *et al.*, 1988). Because the general properties of the homeodomains are quite similar, pertinent data from other homeodomain proteins is useful to fully characterize *HOM-C* homeodomain structure and function.

1.4.2. The *HOM-C* Homeodomains Bind DNA at a Core Consensus Sequence

The homeodomain sequence was recognized as having features similar to a known transcription factor, the yeast Mat α protein, and to prokaryotic helix–turn–helix repressor proteins (Laughon *et al.*, 1984). It was therefore predicted that the *HOM-C* proteins might function as DNA binding factors, with the homeodomain as the likely binding motif. Studies undertaken to characterize homeodomain function included genetic interactions, transcription assays, DNA–protein cross-linking assays, immunoprecipitation, and DNA footprinting experiments. The results of this work confirmed the predicted function of the homeodomain as a DNA binding motif and that of the *HOM-C* proteins as transcriptional regulators (e.g., see Desplan *et al.*, 1985; Müller *et al.*, 1988; Samson *et al.*, 1989; Krasnow *et al.*, 1989; Johnson and Krasnow, 1990, respectively). In fact, it is now known that the homeodomain is one of the most common DNA binding motifs found in eukaryotic transcriptional regulators, second only to the C2H2 zinc finger class (Tupler *et al.*, 2001).

Numerous *in vitro* studies with the *HOM-C* homeodomains resulted in the characterization of a core consensus DNA recognition sequence. An early study of *HOM-C* protein/DNA interactions used DNase protection to characterize the *Antp* homeodomain (ANTP-HD) recognition sequence. This peptide exhibited specific DNA binding affinity for the sequence 5'-TAATG-3' (Müller *et al.*, 1988; Affolter *et al.*, 1990; Ekker *et al.*, 1994). The ANTP-HD was also found to form a more stable complex with DNA than did the similarly structured prokaryotic HLH proteins. The extended recognition

helix, formed by helices three and four, may contribute to this increased stabilization. Other studies of HOM-C/DNA interactions involve partial or full-length Dfd, Antp, Ubx, and Abd-B proteins (Desplan *et al.*, 1988; Ekker *et al.*, 1991, 1992, 1994; Wilson *et al.*, 1996). Although Abd-B appears to bind preferentially to a slightly different core sequence of 5'-TTAT-3' (Ekker *et al.*, 1994), the Q50 homeodomains consistently recognize a consensus core sequence of 5'-TAAT-3' within characterized recognition sites of 5–9 bp (Figure 10).

Structural and genetic studies indicate that the nucleotides flanking the core DNA sequence are important for homeodomain recognition, with one upstream base and two downstream bases being of particular importance (Desplan *et al.*, 1988; Hanes and Brent, 1991). Genetic analysis also indicates the importance of bases several positions downstream of the 5'-TAAT-3' to homeodomain recognition specificity. Interestingly, *in vitro* studies revealed that a single homeodomain peptide can bind different DNA sequences with similar affinities, and that even divergent homeodomain proteins appear to recognize very similar DNA sequences (Desplan *et al.*, 1988; Hoey and Levine, 1988; Walter *et al.*, 1994; Walter and Biggin, 1996). Ubx and Antp have

been shown, *in vitro*, to recognize identical consensus DNA sequences (Ekker *et al.*, 1994). Thus, although the HOM-C proteins recognize and bind specific DNA sequences, it appears that they all recognize the same core sequence, suggesting that there must be additional *in vivo* mechanisms involved in directing their biological patterning specificities.

1.4.3. Functional Specificity of the HOM-C Homeodomain

Because of the strong amino acid conservation and apparent similarities in target site recognition, several studies have attempted to dissect HOM-C protein homeodomains to identify specific residues critical to their biological function. Several groups approached this problem by constructing chimeric HOM-C proteins (swapping various regions of one HOM-C protein for another), then expressing the chimeras in *Drosophila* embryos and adults to assay developmental outcomes and the effects on target gene expression. Although these types of studies have involved the majority of the HOM-C proteins, we summarize, below, the results of selected swapping studies involving the Ubx homeodomain inserted into the Dfd protein (Kuziora and McGinnis, 1989; Dessain *et al.*, 1992; Lin and McGinnis, 1992), and the Scr homeodomain inserted into the Antp protein (Gibson *et al.*, 1990; Furukubo-Tokunaga *et al.*, 1993).

Substitution of the Ubx homeodomain into an otherwise normal Dfd protein is sufficient to introduce Ubx target regulation, and override normal Dfd activity. Wild-type Dfd can autoregulate its own expression in some cells, binding to defined autoregulatory elements to maintain its transcription (Kuziora and McGinnis, 1988b; Lou *et al.*, 1995). The ectopic activation of wild-type Dfd results in the development of ectopic mouthpart material and maxillary cirri in the trunk segments, and the auto-activation of Dfd outside of its normal domain. Activation of a chimeric heat shock-inducible Dfd construct, encoding the Ubx homeodomain in place of the Dfd homeodomain, results in a transformation of the larval head segments toward a thoracic identity, as also results from ectopic Ubx expression (González-Reyes and Morata, 1990). The ectopic activation of *Antp* P1, a known *Ubx* target (Beachy *et al.*, 1988), and the concomitant loss of *Dfd* autoregulation are also observed (Kuziora and McGinnis, 1989). Thus, the Dfd/Ubx chimera, with only the homeodomain region changed, alters the protein function to resemble a wild-type Ubx protein construct.

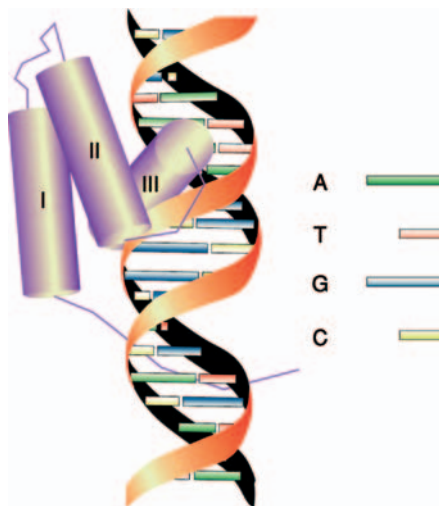


Figure 10 A schematic view of the homeodomain/DNA complex. The three helices of the homeodomain are shown as purple cylinders and numbered (I, II, III). The DNA helix is represented by ribbons and bars. The bar colors denote base identity: green represents adenine (A), red represents thymine (T), blue represents guanine (G), and yellow represents cytosine (C). The recognition helix (helix III) of the HOM-C homeodomain binds directly to DNA in the major groove, recognizing a core consensus sequence of 5'-TAAT-3'. The C-terminal region of this helix is less stable, and is sometimes referred to as helix IV (Qian *et al.*, 1989). The flexible N-terminal arm makes contact with DNA in the minor groove.

Further studies mapped the functional specificity of the Ubx homeodomain using a series of chimeric proteins, in which different portions of the Ubx homeodomain were substituted into an otherwise normal Dfd protein construct (Lin and McGinnis, 1992). Each construct was tested for its ability to activate the *Dfd* autoregulatory element and the *Antp* P1 promoter. Constructs retaining the Ubx N-terminal homeodomain region also retain the larval head to thoracic transformation produced by the original Dfd/Ubx construct, and activate ectopic *Antp* P1 transcription. Further, substituting only a few N-terminal Ubx homeodomain residues (numbers 0–9) (Figure 9) is sufficient to alter ectopic Dfd function and allow initiation of ectopic *Antp* P1 transcription. A chimera containing the Ubx homeodomain with the N-terminal Dfd homeodomain region fails to activate ectopic *Antp* P1 transcription and loses the ability to induce a head to thorax transformation. Both the N-terminal and C-terminal homeodomain regions are required for full Dfd autoactivation: no autoactivation is possible without the N-terminal Dfd region, and only weak autoactivation is possible with the C-terminal portion absent. In summary, these studies demonstrate that the homeodomain is capable of directing HOM-C protein function, and specifically, the N-terminal homeodomain region alone is sufficient to alter HOM-C protein function. The C-terminal flanking region also contributes to functional specificity.

Others took a similar approach in comparing two ANT-C proteins, Antp and Scr. Gibson *et al.* (1990) used the degree of transformation and target gene transcript accumulation to assess the functional specificity of a series of ectopically expressed, chimeric proteins. The Scr homeodomain was substituted into an otherwise normal Antp protein construct and the chimera was expressed in embryos and adults, utilizing an inducible heat shock promoter. This work determined that functional specificity lies in residues both within and adjacent to the homeodomain, and corroborated results obtained with the Dfd and Ubx studies.

Extending the Scr/Antp study, Furukubo-Tokunaga *et al.* (1993) used the chimeric construct to assess the functional requirement of regions outside of the homeodomain, and to more specifically investigate residues within the homeodomain that confer functional specificity. Interchange of the entire protein region N-terminal to the homeodomain seems only to affect the extent of transformation, or “potentiation,” but does not alter the overall function from that of the original chimeric protein. Positions 1, 4, 6, and 7 of the homeodomain (Figure 9) are the residues that distinguish the wild-type Antp

and Scr homeodomains and prove critical to the functional specificity of the chimeric proteins. By substituting the Antp-specific homeodomain residues into the chimeric Scr/Antp protein (wild-type Antp with the Scr homeodomain), a functional Scr protein is reverted to a functional Antp protein. Thus, substitution of these four amino acids alone is sufficient to determine the functional difference between Antp and Scr. Therefore, functional specificity between the homeodomains of two different ANT-C proteins appears to be determined by only a few critical amino acids within the N-terminal portion of the homeodomain, while residues within the large region N-terminal to the homeodomain direct only the extent of the protein’s functional effects. Similar studies involving Ubx and Antp, and Dfd and Abd-B produced similar results (Kuziora and McGinnis, 1991; Chan and Mann, 1993; Zhu and Kuziora, 1996).

As discussed above (see Section 1.3.6), the phosphorylation state of two N-terminal homeodomain residues (6 and 7) is critical to Scr function. At position 6 of the homeodomain, Scr has a threonine residue, while Antp has a nonphosphorylatable glutamine residue. The phosphorylation state of this N-terminal homeodomain residue may, at least in part, explain some of the differing functional specificities of the Scr and Antp homeodomains.

1.4.4. Homeodomain Structure and the Physical Interaction with DNA

The homeodomain structure is highly conserved, as evidenced by strong sequence conservation and three-dimensional structural analysis. The Antp homeodomain was the first HOM-C homeodomain to be structurally resolved, and is the best characterized with regard to the three-dimensional structure. The structure of the Antp protein, both alone and bound to DNA, was resolved by nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography, revealing detailed information concerning the *in vitro* physical contacts within the protein–DNA complex. These studies are consistent with the structural characterization of other homeodomain–DNA interactions (Fraenkel *et al.*, 1998; Hovde *et al.*, 2001). Because of strong sequence similarity between the HOM-C homeodomains, the data and conclusions from these studies can be reasonably applied to the remaining HOM-C genes and probably other Q50 homeodomain proteins, as well.

NMR resolution of the Antp homeodomain tertiary structure reveals that the homeodomain forms a globular domain, consisting of four helices (Figure 9), three well-defined alpha helices and one less organized fourth helix directly abutting the

third (Billeter *et al.*, 1990). The C-terminal end of the third helix, or recognition helix, is somewhat less stable and, therefore, occasionally characterized as a fourth helix, although the homeodomain is most commonly described as having only three helices. An extended N-terminal arm is evident, upstream of the first helix (Qian *et al.*, 1989).

A number of NMR and X-ray crystallography studies have provided the structural details of homeodomain–DNA interactions (Otting, *et al.*, 1990; Hirsch and Aggarwal, 1995; Wilson *et al.*, 1995; Tucker-Kellogg *et al.*, 1997; Fraenkel *et al.*, 1998; Fraenkel and Pabo, 1998; Hovde *et al.*, 2001). A schematic of the homeodomain–DNA interaction is shown in **Figure 10**. Contact between the homeodomain and DNA occurs at the recognition helix and at amino acid residues flanking helix one. The DNA is contacted within a 7 nucleotide region on both sides of the core binding sequence and all but one contact sites are within the major groove. Overall, contacts are made by the homeodomain across two major grooves of the DNA, with the N-terminal arm (containing four amino acids important for functional specificity, and discussed above) contacting the minor groove.

Further support for the importance of the N-terminal homeodomain residues is provided by the structural study of a truncated Antp peptide lacking the first six amino acids of the homeodomain (Qian *et al.*, 1994). Although its three-dimensional structure is virtually identical to that of the intact homeodomain, the K_d for the truncated peptide bound to DNA was found to be approximately 10-fold less than that of the intact Antp peptide. Thus, the N-terminal homeodomain residues contribute significantly to DNA binding affinity and stability, and perhaps contribute to the motif's ability to stably bind and activate target DNA sequences. Interestingly, inclusion of the conserved hexapeptide (YPWM) motif does not add to the stability of the homeodomain–DNA interaction, nor does it increase the DNA binding specificity (Qian *et al.*, 1992). These results support genetic and later structural studies demonstrating that this highly conserved motif functions in protein–protein interactions, rather than being involved in DNA sequence recognition. The YPWM motif and its function are discussed further in the section that addresses the cooperative interactions between HOM-C proteins and other factors.

In summary, the protein products of the HOM-C genes are DNA binding factors recognizing a consensus sequence with a canonical 5'-TAAT-3' at its core. It is difficult to reconcile how the HOM-C proteins direct developmental pathways leading to

distinct and varying cell morphologies when their target recognition sequences are so similar. For example, it is reasonable to assume that the genetic cascade initiated by one HOM-C protein to pattern a gnathal segment is quite different than the cascade initiated by a different HOM-C protein to pattern an abdominal segment. Yet, we see identical amino acid residues within the recognition (third) helix and the ability of multiple homeodomains to recognize identical sequences. Chimeric studies identify a few N-terminal homeodomain residues as important to developmental function, as transformations in the larval cuticle are observed when these critical residues are altered. Larval denticle patterning may, however, provide limited criteria for assessing functional specificity. Several gnathal HOM-C proteins (Dfd, Scr, and Lab), when ectopically expressed, are sufficient to transform the second and third thoracic denticle belts to a first thoracic identity (Kuziora and McGinnis, 1988b; Zeng *et al.*, 1993; T.C. Kaufman, personal communication), though in normal development, only Scr patterns the first thoracic segment. Thus, the larval denticle pattern, alone, may prove insufficient to evidence altered HOM-C functional specificities. Further, the biological specificity of isoforms of the same HOM-C protein (i.e., Ubx) may not result from differences in target DNA sequence recognition, as the homeodomains are unchanged between isoforms. This seeming incongruence between the specificity of HOM-C protein function and the apparent lack of specificity in DNA target recognition is frequently referred to as the “HOX paradox.” It seems likely then, that functional specificity results from spatial and/or temporal differences in expression and differential protein interactions. The N-terminal region of the homeodomain and the highly variable regions outside of the conserved portion of the homeodomain may guide cofactor interactions to direct the developmental specificity brought about by the homeotic selector genes.

1.5. Cooperative Interactions between HOM-C Proteins and Other Factors

1.5.1. A Case for Cofactors

The results from DNA binding studies in *Drosophila*, investigating the specificity of HOM-C protein target recognition, do not appear to provide the degree of specificity required for developmentally distinct cell fates. As described above (see Section 1.4.3), Antp and Ubx recognize identical preferential binding sequences and have overlapping expression

domains, yet have very different biological functions (e.g., *Antp* patterns the wing, where *Ubx* patterns the haltere), implying the need for additional mechanisms or factors to direct their target recognition and resulting biological specificity.

Virtually all Q50 homeodomains bind, with varying affinity, to a consensus core sequence of 5'-TAAT-3' (Gehring *et al.*, 1994), and this motif would be expected to occur, on average, once in approximately every 256 bp of DNA in the *Drosophila* genome. Given the estimates of protein concentrations within nuclei, the homeodomain containing proteins Eve and Ftz (Q50 homeodomains) are estimated to bind to high and medium affinity sites at a density between one monomer per 4 kb of DNA (for weakly bound genes) to five monomers per 1 kb of DNA (for strongly bound genes) (Biggin and McGinnis, 1997). One would expect only a portion of these potential binding sites to be required for spatial and temporal regulation of specific target genes. Thus, the simple presence of a HOM-C protein, bound to DNA, is unlikely to bestow target status to a gene. This is nicely illustrated by studies utilizing the HOM-C protein Dfd and a known Dfd autoregulatory sequence.

Multiples of a small Dfd binding fragment, derived from a Dfd autoactivation enhancer, is sufficient to drive β -galactosidase reporter expression in the maxillary segment of *Drosophila* embryos. However, the loss of an imperfect inverted repeat sequence removes the maxillary reporter expression (Zeng *et al.*, 1994), suggesting that other proteins must also bind to this fragment to allow activation of transcription. Further, the Dfd protein binds strongly, *in vitro*, to a tandem repeat of its consensus recognition site, but can only significantly activate transcription of a reporter construct containing this site, when it is accompanied by the constitutive VP16 activation domain (Li *et al.*, 1999a). Thus, in spite of strong binding, Dfd alone cannot activate the reporter without the addition of an activation sequence, further indicating a requirement for additional factors. Additionally, the Dfd-VP16 fusion activates ectopic expression of *Scr* *in vivo*, whereas the wild-type Dfd protein does not regulate *Scr*. This demonstrates that the HOM-C proteins likely bind *in vivo* to sites that do not represent their normal target gene promoters or enhancers.

The presence of cooperatively binding proteins, or cofactors, provides a resolution to the conflict between the lack of binding specificity and developmental precision. Biggin and McGinnis (1997) described two potential models of HOM-C/Co-factor interaction, the co-selective binding model and the

widespread binding model. The co-selective model proposes that a cofactor is required to direct HOM-C proteins to the appropriate target gene regulatory sites, while the widespread binding model predicts that HOM-C proteins alone are able to bind to many recognition sites throughout the genome, but are only able to activate transcription in conjunction with appropriate, available cofactors. Cooperative transcriptional control mechanisms, such as those the HOM-C proteins appear to require, are not novel and have been described in other organisms. The yeast homeodomain transcription factors MAT α 1 and MAT α 2 form cooperative heterodimers, which bind DNA to repress the transcription of haploid-specific mating type genes (Goutte and Johnson, 1993; Mak and Johnson, 1993). The mammalian octamer transcription factors (OCT) also contain a POU class homeodomain, which recognizes an octamer sequence motif. Activation of RNA polymerase II mediated transcription at the octamer binding site requires the heterodimerization of OCT factors (Bürglin, 1994). Yeast also provides an example of a homeodomain/zinc finger interaction between the PHO2 and SWI5 proteins during regulation of the HO endonuclease gene (Brazas and Stillman, 1993).

HOM-C gene cofactors would be expected to have properties fulfilling several genetic and biochemical criteria:

- absence of the cofactors would duplicate full or partial HOM-C loss-of-function phenotypes;
- a cofactor would act in parallel to the HOM-C protein, neither functioning as an upstream regulator, nor as a downstream target (although some regulatory “cross-talk” between the two may be expected);
- HOM-C target gene expression would be reduced or absent in the absence of the cofactor; and
- the cofactor may increase the DNA target specificity and/or the DNA binding affinity of its HOM-C partner.

Several *Drosophila* gene products have been described, which meet many, or all, of these criteria. The homeodomain encoding gene *extradenticle* (*exd*), initially described by Peifer and Weischaus (1990), is the best-characterized HOM-C cofactor. Other genes proposed to encode HOM-C cofactors include *teashirt*, (*tsh*) *disconnected* (*disco*), and the partially redundant *disco-related* (*disco-r*), whose proteins include a zinc finger DNA binding motif; *cap'n'collar* (*cnc*), a member of the bZip family; and two genes encoding putative novel transcription regulators, *lines* (*lin*) and *apontic* (*apt*).

1.5.2. Extradenticle is a HOM-C Protein Cofactor

The *extradenticle* gene maps cytogenetically to position 14A5 on the X-chromosome and encodes a 378 amino acid protein required for proper embryonic and adult patterning. The *exd* gene is homologous to the *pbx* group of vertebrate genes (Rauskolb *et al.*, 1993) and its protein product is a member of the Pbx class of homeodomain proteins (Bürglin, 1994), which share 95% similarity within their homeodomains and 71% similarity across their entire sequences. The Exd homeodomain diverges from those of the *HOM-C* genes, having three extra residues between the first and second α -helix. Atypical homeodomains of this type are referred to as three amino acid loop extension (TALE) homeodomains. A second conserved domain is the PBC-A domain, through which Exd interacts with a second homeodomain-containing protein, Homothorax (Hth) (Ryoo *et al.*, 1999). This interaction is discussed in detail below.

Exd is expressed and packaged into the egg during oogenesis (Rauskolb *et al.*, 1993). Loss of maternal and zygotic *exd* expression disrupts embryonic development, even more so than does the loss of zygotic *exd* alone. Maternal Exd protein is uniformly distributed and cytoplasmically localized in early embryogenesis and persists until germ band extension (Aspland and White, 1997). Zygotic transcription initiates thereafter, with transcripts initially found uniformly throughout the embryo, and then being modulated thereafter (Rauskolb *et al.*, 1995). The initiation of zygotic *exd* expression is unaltered in *HOM-C* mutants, and conversely, the loss of *exd* expression does not affect the expression of the *HOM-C* genes, indicating that these genes act in parallel developmental pathways (Peifer and Wieschaus, 1990; Rauskolb *et al.*, 1995). By germ band contraction, expression of *exd* is lost in abdominal segments 7–10, the remaining Exd protein becomes localized to the nucleus, and this nuclear localization is spatially regulated (Aspland and White, 1997). Expression is also evident in the somatic and visceral mesoderm, the CNS and brain, and the larval imaginal discs (Rauskolb *et al.*, 1993; Aspland and White, 1997).

Embryos lacking zygotic Exd function die during late embryogenesis and have defects in overall pattern formation (Peifer and Wieschaus, 1990). Development of some head structures is disrupted, and the thoracic segments are altered, with the second thoracic segment adopting a first thoracic-like denticle pattern, and the third thoracic segment showing characteristics of both the first thoracic and second abdominal segments. The identities of

the abdominal segments are transformed to those two to three segments more posterior. For example, the first abdominal segment resembles the third or fourth. No effect is detected in the most posterior segments. Weaker alleles are postembryonic lethals, often forming pharate adults with cuticular defects, and clonal analysis demonstrates that lack of Exd also causes transformation of adult structures (González-Crespo and Morata, 1995; Rauskolb *et al.*, 1995). Null *exd* clones result in homeotic transformation of adult head structures, legs, and abdominal segments, resembling the phenotypes observed in some *HOM-C* mutants. Antenna and arista are transformed to leg, while the head capsule is altered to resemble the dorsal thorax or notum. Abdominal clones in the first through fourth segments transform towards the fifth or sixth abdominal segments. Ubiquitous overexpression of *exd* has no effect on segment identities (Rauskolb *et al.*, 1995). Because *exd* mutants appeared to have alterations in *HOM-C* target specificity, Peifer and Wieschaus proposed that Exd acts as a *HOM-C* cofactor.

Exd homologs have been identified in the cricket *Gryllus bimaculatus*, the cricket *A. domesticus*, the pine sawfly *Diprion similis*, and the grasshopper *S. americana* (Abzhanov and Kaufman, 2000; Jockusch *et al.*, 2000; Suzuki and Palopoli, 2001; Inoue *et al.*, 2002). These studies examined Exd expression as it is involved in leg development, and found that like *Drosophila*, nuclear Exd is present in the proximal developing leg. There are currently no published data assessing the function of Exd with the *HOM-C* proteins of other insects. However, the highly conserved nature of the interacting regions of these proteins across several animal taxa (Rauskolb *et al.*, 1993; Pöpperl *et al.*, 1995; Liu and Fire, 2000) leads us to expect that the protein serves a similarly conserved role across insect taxa. This, however, remains to be demonstrated.

To explore the interaction and contribution of the *HOM-C* proteins and Exd to development, embryos mutant for *exd* were compared to embryos singly mutant for various *HOM-C* genes and doubly mutant for both a *HOM-C* gene and *exd*. Double mutants differed from either single mutant, indicating that the *HOM-C* genes continued to be active within their normal domains in the absence of *exd*, although they were unable to specify segmental identity properly (Peifer and Wieschaus, 1990). For example, single *exd* mutants exhibit a transformation of the first abdominal segment to the third, the second abdominal segment to the fourth, and the third abdominal segment to the fifth. An *abd-A*

mutant exhibits a transformation of these same segments to an A1 identity. The double *exd;abd-A* mutant transforms all of these segments to an A3 identity. These results further indicate that *exd* functions in a parallel pathway to the HOM-C genes and is required for the normal specification of segment identity by the HOM-C genes examined.

Consistent with the expectation of some cross-regulation between the HOM-C proteins and their cofactors, late stage *exd* expression is modulated by HOM-C proteins (Rauskolb *et al.*, 1993). Embryos deficient for the BX-C genes have increased posterior expression of *exd* late in embryogenesis, indicating the BX-C proteins downregulate *exd* expression in the posterior abdomen. Further, Ubx, Abd-A, and Abd-B modulate the subcellular localization of Exd protein, albeit to different extents (Azpiazu and Morata, 1998).

Genetic and biochemical studies support a cooperative transcriptional activation by a HOM-C/Exd heterodimer. The HOM-C genes *Ubx*, *abd-A*, and *Antp* are required for normal midgut development and directly modulate the expression of genes within this developing tissue, including *wingless* (*wg*), *teashirt* (*tsh*), and *decapentaplegic* (*dpp*) (Immerglück *et al.*, 1990; Reuter *et al.*, 1990). The regulation of *wg* expression within the visceral mesoderm of parasegment 8 is controlled by Abd-A, and Antp positively regulates *tsh* within the visceral mesoderm of parasegments 5 and 6. Expression of *wg* and *tsh* is lost in these regions when embryos lack Exd, although there is no alteration of the expression of either Abd-A or Antp (Rauskolb and Wieschaus, 1994). The requirement of Exd for proper HOM-C protein function is illustrated again with Ubx. The *dpp* gene, a member of the transforming growth factor (TGF)- β -encoding gene super family, is a direct target of Ubx *in vivo* and is also required for midgut morphogenesis (Capovilla *et al.*, 1994; Chan *et al.*, 1994). Ubx directly activates the transcription of a *dpp* minimal enhancer construct in parasegment 7 within the visceral mesoderm, and this region-specific activation requires Exd (Rauskolb and Wieschaus, 1994; Sun *et al.*, 1995). Even the ectopic expression of Ubx in embryos lacking Exd fails to activate the *dpp* enhancer element.

Exd is also required for epidermal function of the HOM-C gene products. Removing both maternal and zygotic Exd appears to abolish Dfd function, as the mandibular and maxillary derived larval structures are eliminated (Peifer and Wieschaus, 1990). Further, some Dfd response elements require Exd to emulate normal expression *in vivo* (Pinsonneault *et al.*, 1997). Unlike Antp, Abd-A,

and Ubx, however, Dfd expression is severely reduced when both maternal and zygotic Exd are removed. Although initially normal, Dfd protein accumulation dramatically decreases during the germ band stage, the time at which Dfd begins to maintain its own transcription through autoactivation. Therefore, it appears that Exd is necessary for Dfd autoactivation. Similarly, Lab requires Exd for maintenance of its own expression *via* an autoactivation circuit (Chan *et al.*, 1996), and Ubx requires Exd for transcription maintenance in the haltere imaginal discs (Azpiazu and Morata, 1998). It is suggested that Exd may not only cooperatively activate transcription by the HOM-C proteins, but may also alleviate the repressive action of some bound HOM-C factors (Pinsonneault *et al.*, 1997; Li *et al.*, 1999b) or even promote the repressive properties of HOM-C proteins (Ryoo and Mann, 1999; Merabet *et al.*, 2003).

The evidence supporting Exd/HOM-C interactions is not only based on results from genetic studies. *In vitro* binding studies demonstrate that the Exd and HOM-C proteins interact directly and can bind DNA cooperatively with greater affinity than the HOM-C proteins alone (van Dijk and Murre, 1994; Chan and Mann, 1996; Pinsonneault *et al.*, 1997; Ryoo and Mann, 1999). Exd can also selectively enhance the specific affinity of one HOM-C protein relative to others for binding to a particular target region. Scr and Exd bind a response element derived from the Scr target gene *fork head* (*fkf*) cooperatively and specifically (Ryoo and Mann, 1999). The Exd/Scr heterodimer binds effectively to this element *in vitro*, and both proteins are required for the activation of a reporter construct carrying this element *in vivo*. Other Exd/HOM-C heterodimers fail to activate this element *in vivo* and fail to bind to it with high affinity *in vitro*, while Exd is unable to bind the element alone. Two half-sites are required for the Exd/HOM-C heterodimer to bind DNA, one recognized by the HOM-C protein homeodomain, and one recognized by the Exd homeodomain (van Dijk and Murre, 1994). Although these sites vary slightly for different heterodimers, a consensus sequence of 5'-ATNNATCA-3' has been described (Mann and Chan, 1996).

The ability of the Exd mammalian homologue Pbx to bind cooperatively with mammalian HOX proteins and some of their *Drosophila* homologs *in vitro* and *in vivo*, illustrates the level of conservation of the Exd/PXB family (Chan *et al.*, 1994; van Dijk and Murre, 1994; Chang *et al.*, 1995; Phelan *et al.*, 1995; Pöpperl *et al.*, 1995; Rauskolb and Wieschaus, 1994; Sun *et al.*, 1995; Chang *et al.*,

1997; Azpiazu and Morata, 1998). This strong conservation across phyla, combined with the similar interactions between the yeast factors MATA1 and MAT α 2, suggests that the Exd/HOM-C(HOX) interaction could be quite ancient, dating back to the earliest origins of multicellularity.

1.5.3. The DNA Bound Exd/Ubx Crystal Structure

Chan and Mann (1996) used a series of point mutations to characterize the Exd/Lab/DNA interaction, and proposed a structural model for HOM-C/Exd interactions, much of which was later confirmed by structural resolution. X-ray crystallography was used to resolve the crystal structure of a DNA-bound Ubx/Exd heterodimer (Passner *et al.*, 1999).

The Ubx and Exd homeodomains bind opposite faces of the DNA molecule, aligned in a head to tail orientation in close proximity (Figure 11a). As indicated by *in vitro* and *in vivo* data, the HOM-C hexapeptide or YPWM motif and the Exd homeodomain are the sites of protein–protein interaction. Able to form a reverse turn, the YPWM motif

extends, via a flexible linker, to contact Exd within a hydrophobic pocket (Figure 11b). This pocket is formed, in part, by the additional three residues between alpha helices 1 and 2 of the Exd homeodomain. The tryptophan (W) residue is significant to Exd/Ubx binding, mediating hydrophobic contacts within the Exd hydrophobic pocket. The contacts made by the tyrosine (Y), proline (P), and methionine (M) residues are less extensive and serve to stabilize the binding of the tryptophan (W) residue. The proximity of the two proteins results in highly overlapping binding sites, with more than half of the Exd contacts to DNA occurring within the Ubx recognition site. The binding of the Exd/Ubx dimer to DNA induces very little conformational change in the proteins, however the minor groove of the bound DNA region broadens significantly, when compared to the crystal structure of Antp bound to DNA complex (Fraenkel and Pabo, 1998). This broadening may represent a “preconfiguring” of the DNA by Exd to allow Ubx recruitment to its target site. A similar mechanism of cooperative binding has been suggested for Oct1 (Clemm and Pabo, 1996).

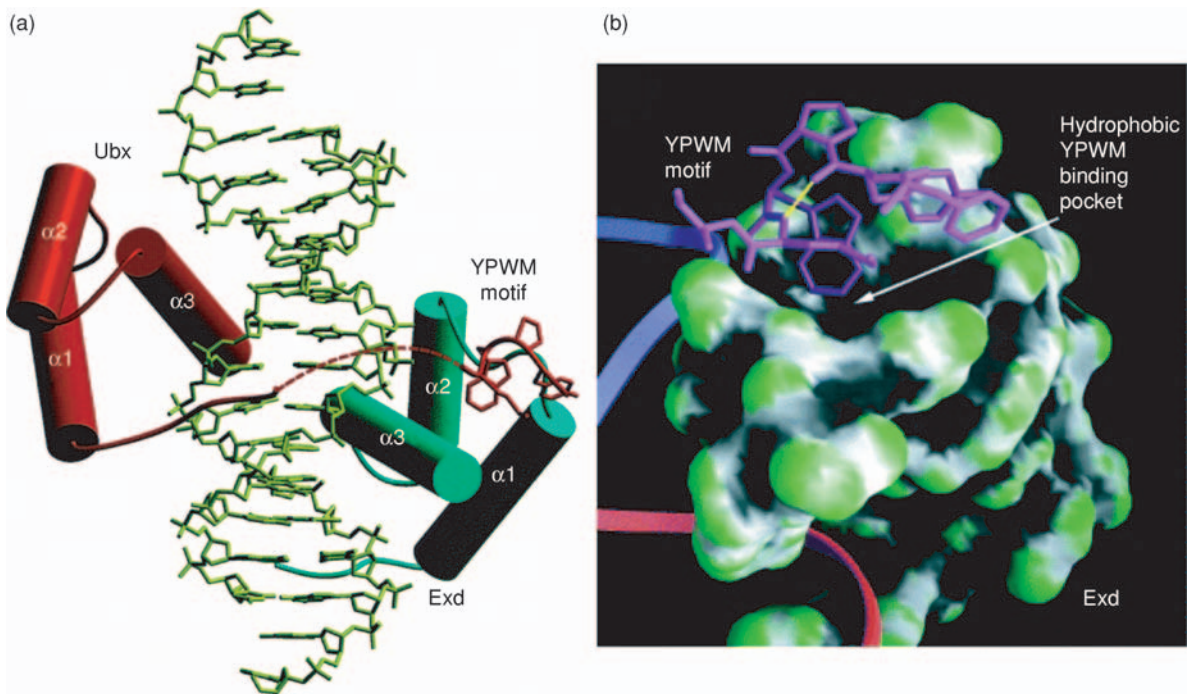


Figure 11 The Ubx/Exd/DNA ternary complex. (a) The Ubx/Exd complex binding DNA and (b) Ubx/Exd protein–protein interaction. (a) The diagram shows the Ubx (red) and Exd (green) homeodomains binding cooperatively on opposite faces of the DNA (green). The flexible linker between the Ubx homeodomain and its YPWM motif is represented by a dashed red line. (b) The YPWM motif (magenta) of Ubx forms a reverse curve to reach into a hydrophobic pocket on the Exd homeodomain. The yellow line indicates a hydrogen bond between the first and fourth residues of YPWM. The colored surface of Exd illustrates its curvature: convex patches are shaded green and concave patches are shaded gray. The DNA backbone (purple and red) is shown as a reference. (Adapted with permission from Nature Publishing Group from Passner, J.M., Ryoo, H.D., Shen, L., Mann, R.S., Aggarwal, A. 1999. Structure of a DNA-bound Ultrabithorax–Extradenticle homeodomain complex. *Nature* 397, 714–719; <http://www.nature.com/nature/>)

1.5.4. The YPWM (Hexapeptide) Motif and Exd/HOM-C Heterodimer Formation

Variouly called the hexapeptide motif, the pentapeptide motif, the tetrapeptide motif, and the YPWM motif, this short, highly conserved region N-terminal to the HOM-C homeodomain is integral to the Exd/HOM-C interaction (Chan and Mann, 1996) (Figure 9). Yeast two-hybrid, mutational, and structural analyses reveal that the region of interaction between Pbx/Exd and its HOX/HOM-C partners is the conserved YPWM motif (Chang *et al.*, 1995; Johnson *et al.*, 1995; Phelan *et al.*, 1995). NMR studies involving the highly conserved vertebrate Pbx homeodomain, describe a “lock and key” interaction, with the YPWM motif of the HOX protein (key) inserting into the hydrophobic pocket (lock) of the Pbx homeodomain (Sprules *et al.*, 2003).

Despite the apparent genetic and structural importance of the YPWM motif for Exd/HOM-C interaction and DNA binding, however, there are also examples of its dispensability. Abd-B, which lacks this conserved motif, fails to cooperatively interact *in vitro* with Exd (van Dijk and Murre, 1994), although a murine Abd-B homolog does appear to be able to interact with Pbx, despite the lack of the YPWM sequence (Izpisua-Belmonte *et al.*, 1991). Recent work with Abd-A demonstrates that the YPWM motif is neither required for Exd recruitment, nor for DNA binding and target selection (Merabet *et al.*, 2003). Elimination of the YPWM motif, or a second, short conserved linker-region sequence, does not affect Abd-A’s epidermal patterning functions, its binding site selection on a *dpp* response element, or its ability to interact with Exd and Hth *in vitro*. The sequence was, however, proven to be required for correct regulation of the Abd-A target genes *wg* and *dpp* within the visceral mesoderm, where the YPWM motif confers both target activating and target repressing properties to Abd-A, in a gene-specific manner. The *wg* and *dpp* genes are differentially affected by the loss of the YPWM motif, demonstrating the importance of this motif in the control of Abd-A *trans*-regulatory functions.

1.5.5. Homothorax Forms a Trimer with Exd and HOM-C Proteins

In order for Exd to function as a coregulator of the HOM-C proteins, it must move from the cytoplasm into the nucleus. Interestingly, Exd has no nuclear localization signal (NLS), but relies on yet another homeodomain-containing protein, Homothorax (Hth), to translocate to the nucleus (Rieckhof

et al., 1997; Ryoo *et al.*, 1999). This relationship is also conserved among the vertebrate Pbx proteins, which require interaction with Hth homologs, the MEIS (named after the founding member, myeloid ecotropic viral integration site 1 or MEIS1; Moscow *et al.*, 1995) proteins, for nuclear localization (Knoepfler *et al.*, 1997). The absence of *hth* in embryos results in posterior-directed transformations, just as observed with embryos lacking *exd*. Hth has a conserved N-terminal domain, the HM (*hth/meis*) motif, which binds Exd at the PBC-A domain, allowing the formation of a trimeric Hth/Exd/HOM-C (Ryoo *et al.*, 1999). This binding structure leaves the Hth homeodomain available to interact with DNA, perhaps further increasing the specificity of the Exd/HOM-C interaction. A trimeric Hth/Exd/Lab complex was shown to bind DNA at an endodermal *lab* autoregulatory enhancer (*lab48/95*). Hth is required for the *in vivo* function of a *lac-Z* reporter construct containing this enhancer, while ectopic expression of a form of Hth lacking the homeodomain acts in a dominant negative fashion, nearly abolishing reporter activity (Ryoo *et al.*, 1999). DNA footprinting analysis of an Hth/Exd/Lab/DNA complex indicates that Hth binds approximately 12 bases away from the overlapping Exd/Lab binding site, and that high-affinity Hth binding requires the Exd/Lab binding as well. Lab and Hth do not bind DNA cooperatively in the absence of Exd.

1.5.6. Extradenticle Is Insufficient to Fully Explain HOM-C Function

An interesting series of studies illustrates the difficulties in interpreting and correlating *in vitro* binding results with *in vivo* function, particularly when examining minimal response elements. The *HOXB-1* gene is a vertebrate homolog of the *Drosophila lab* gene. A well-characterized 20 bp *HOXB-1* element (derived from the 5' *HOXB-1* promoter) is bound cooperatively and specifically by Exd and Lab and is able to recapitulate endogenous *lab* expression patterns in *Drosophila* embryos (Pöpperl *et al.*, 1995; Chan *et al.*, 1996). The *in vivo* function of this reporter requires both Exd and Lab. The consensus Exd/HOM-C recognition site has been described as 5'-ATNNATCA-3' (Mann and Chan, 1996). A change in the two central base pairs (the NN “specificity residues”) of the consensus Exd/HOM-C binding site, from GG to TA, alters the *in vitro* specificity from Exd/Lab, to Exd/Dfd and results in strong *in vivo* reporter expression in a Dfd-like pattern (Chan *et al.*, 1997). At first glance, it appears that a simple change in a few base pairs is sufficient to direct *in vivo* HOM-C specificity, but

further studies have shown, that, although Exd/Ubx also binds the altered 20 bp *HOXB-1* response element *in vitro*, it is unable to activate this element *in vivo* (Chan and Mann, 1996; Chan *et al.*, 1997). Additional, difficult to interpret results involve a 26 bp element from the regulatory region of the *Drosophila* gene *Distal-less* (*Dll*), an element negatively regulated by Ubx and Abd-A, and bound by both HOM-C proteins at the same site (White *et al.*, 2000). This element was altered to resemble the known Exd/Dfd consensus sequence, and placed into a reporter construct. Interestingly, while the altered *Dll* element continued to be negatively regulated by Ubx and Abd-A, it was strongly activated by Scr and only weakly activated by Dfd. Further, Li *et al.* (1999a) demonstrated that an Exd/Lab responsive element could be altered to a Dfd responsive element by the addition of a naturally derived 21 bp region that included neither an Exd/HOM-C binding site nor a Dfd binding site. Results such as these serve to reinforce the complexity of the Exd/HOM-C protein mechanism of target recognition. Obviously, a simple consensus sequence with two variable nucleotides cannot fully explain the complex action of these genes.

Although Exd certainly functions as a coregulator with HOM-C proteins, it is clear that Exd alone does not define HOM-C target specificity. Not all Dfd response elements require Exd for *in vivo* function, suggesting there are multiple regulatory mechanisms at work for a single HOM-C protein (Pederson *et al.*, 2000). *In vitro* binding studies demonstrate the ability for multiple Exd/HOM-C heterodimers to bind similar sequences (i.e., with Ubx and Abd-A), suggesting that Exd alone cannot explain the functional specificity of the HOM-C proteins (van Dijk and Murre, 1994). Abd-B appears necessary to repress *exd* activation, rather than utilize it as a cofactor, and Pb represses *exd* expression during the development of the adult proboscis (Abzhanov *et al.*, 2001). Further, in the case of Scr, which is expressed in both the head (labial segment) and the trunk (first thoracic segment), coregulation with Exd is insufficient to explain this HOM-C protein's ability to differentially pattern segments from two different tagmata, suggesting that other cooperative factors must be at work. In fact, cooperative interactions between HOM-C proteins and a cofactor may not be limited to Exd. Several other DNA binding proteins appear to coregulate HOM-C function, but in a more region-specific manner than the coregulation observed with Exd. Direct interaction between these factors and a HOM-C protein, however, has yet to be demonstrated.

1.5.7. Zinc Finger HOM-C Protein Cofactors

Several other *Drosophila* proteins are proposed to function as HOM-C cofactors, including the C2H2 zinc finger proteins encoded by *tsh*, *disco*, and the partially redundant *disco-r* genes, and perhaps the *buttonhead* (*btd*) gene. Of these, *tsh*, located on the left arm of chromosome 2, is well characterized in *Drosophila*, while some information is also available from other insects. The *tsh* gene was initially identified in an enhancer trap screen for embryonic segmentation genes (Fasano *et al.*, 1991). Embryos homozygous for null alleles of *tsh* die before hatching, and have a homeotic alteration of the ventral trunk segments to a gnathal- or head-like structure. The denticle belts are reduced, laterally narrowed, disorganized, and lack segment-specific characteristics. Small patches of sclerotized material, reminiscent of the cephalopharyngeal skeleton, are present in the ventral cuticle, and the anal opening is also sclerotized. The expression of *tsh* overlaps expression of *Scr*, *Antp*, *Ubx*, *abd-A*, and *Abd-B*, but is not required for transcriptional activation of these genes (Röder *et al.*, 1992). Conversely, initiation and early regulation of *tsh* is independent of the HOM-C proteins, although *Antp*, *Ubx*, and *Abd-A* modulate *tsh* expression later, following germ band extension (Mathies *et al.*, 1994; McCormick *et al.*, 1995). The expression of *Scr* is somewhat affected by the loss of *tsh*, exhibiting ectopic activation in the ventral prothoracic segment (Fasano *et al.*, 1991). Röder *et al.* (1992) concluded that *tsh* acts on the same hierarchical level as the HOM-C genes, with the two functioning together to promote proper trunk development while suppressing head identity. The *tsh* mutant phenotype and its expression in all of the trunk segments except for the most posterior abdominal, has led to its characterization as a trunk tagma specifier.

Further work by de Zulueta *et al.* (1994), supports a cooperative mechanism between *tsh* and the HOM-C genes of the trunk. The role of Tsh during development is highlighted by its connection with the HOM-C gene *Scr*. As discussed previously (see Section 1.2.2.4), *Scr* controls identity in the labial gnathal segment and in the adjoining first thoracic trunk segment. Part of this dual role is specified by *tsh*. *Scr* is unable to produce a first thoracic identity in the absence of *tsh*, as evidenced by the failure of *tsh* null embryos to differentiate normal first thoracic denticle belts. Ectopic activation of *tsh* in the labial segment transforms that segment toward a prothoracic identity with first thoracic-type denticles. Tsh is also required for repression of salivary gland formation in the trunk segments in embryos ectopically

expressing *Scr*, again demonstrating that *Tsh* helps to distinguish the gnathal versus the trunk roles of *Scr* (Andrew *et al.*, 1994).

Embryos null for the partially redundant genes *disco* and *disco-r* exhibit gnathal patterning defects quite similar to those observed in embryos lacking *Dfd* and *Scr* (Mahaffey *et al.*, 2001). These genes map to the 14B1 and 14A9 regions, respectively, of the X chromosome and encode paired zinc finger-containing proteins. Although *disco* had been previously characterized based upon neural and circadian rhythm phenotypes (Steller *et al.*, 1987; Dushay *et al.*, 1989; Helig *et al.*, 1991), its embryonic patterning role remained unrecognized until the creation of a deficiency removing both *disco* and *disco-r*; either gene alone is sufficient for normal embryonic gnathal patterning (Mahaffey *et al.*, 2001). The expression of *disco* and *disco-r* overlaps that of *Dfd* and *Scr* in the mandibular, maxillary, and labial lobes, and the former are also expressed in the larval limb primordia of the first through third thoracic segments and the clypeolabrum. In addition, *disco* is expressed in the optic lobe, where it is required for normal migration of Bolwig's nerve, and in the proctodeum, where it overlaps with *Abd-B* expression. Loss of *disco* and *disco-r* has no effect on the expression of *Dfd* or *Scr*, and the converse loss of *Dfd* and *Scr* has no significant effect on the expression of the *disco* or *disco-r* genes (Mahaffey *et al.*, 2001), further demonstrating that *disco/disco-r* null embryos exhibit reduced expression of three known *Dfd* target genes—*1.28*, *Serrate* (*Ser*), and *Dll*—and supporting the role of these two proteins as HOM-C cofactors.

The *btd* gene also encodes a zinc finger transcription factor required for embryonic patterning (Wimmer *et al.*, 1993). Mutations in *btd* disrupt development of the anterior head segments, including the antennal, intercalary and mandibular segments. The Btd protein interacts with and functions as a cofactor with the HOM-C-like protein, Empty Spiracles (EMS) (Dalton *et al.*, 1989), and overlaps the expression of *Lab* and perhaps *Dfd*. *ems* is a HOM-C like gene required for patterning the *Drosophila* larval head and brain, and for the development of the filzkörper material in the posterior spiracles (Dalton *et al.*, 1989; Jones and McGinnis, 1993). Although commonly referred to as a head gap gene (Cohen and Jürgens, 1990; Walldorf and Gehring, 1992), *ems* encodes a HOM-C type homeodomain and a somewhat diverged upstream YPWM motif (Figure 9). It has been suggested that *ems* may represent a “lost” HOM-C gene, separated from the HOM-C cluster, but retaining HOM-C like patterning functions (Macías and Morata, 1996).

Interestingly, ectopic *ems* expression causes little disruption to development, only transforming the mandibular segment toward an intercalary identity. Ectopic *btd* expression, on the other hand, is more detrimental, causing segmentation defects. However, ectopic expression of *ems* with *btd* appears to transform the trunk segments toward an intercalary identity, demonstrating that the region of Btd accumulation defines where EMS can function, much as would be expected for a cofactor (Schöck *et al.*, 2000). This property is similar to the observations of HOM-C proteins with *Tsh* and *Disco*.

There are few studies with homologs of these zinc finger cofactors in other insects, yet this could be a very interesting and informative venture. Unlike the HOM-C genes, there appear to be differences in these putative zinc finger cofactors between *Drosophila* and other insects. Somewhat surprisingly, all of the zinc finger genes mentioned above have been duplicated in the *Drosophila* genome (the *Drosophila* homolog of mammalian SP1 (Kadonaga *et al.*, 1987); *tsh* is related to *tiptop*, and *btd* is related to *D-Sp1*), and *disco*'s duplicate, *disco-r* has already been mentioned above. Only *disco* and *disco-r* have been shown to be functionally redundant for their role with HOM-C proteins. The *D-Sp1* gene can substitute for *btd* in the mandibular segment (Schöck *et al.*, 2000), but little is known about the role of *tiptop*.

With several insect genome projects well under way, it is clear now that these duplications are not found in all insects. In the genomes of *A. gambiae* and *A. mellifera* only homologs of *disco-r* and *tiptop* are found. Further, a search of Genbank reveals several homologs of *tsh* cloned from other insects, and although many of these are only partial sequences, they appear to be more closely related to *tiptop* than to *teashirt*. This generates several questions. Since only *disco* and *disco-r* appear to be redundant, what are the roles of the paralogous genes in *Drosophila*, and further, what roles will be found for the genes in other insects? For example, will *tiptop* from other insects retain the roles of *tsh* and *tiptop* in *Drosophila*? Additionally, it will be important to determine which roles are ancestral and which are derived, whether or not the cofactor role for these genes is particular to *Drosophila* or whether it extends to other insects as well.

Recently, Robertson *et al.* (2004) proposed a model in which the C2H2 zinc finger transcription factors described above play a larger role during *Drosophila* development and HOM-C function. In this model, these transcription factors contribute to the regionalization of the embryo: *Disco* establishing the gnathal region and *Tsh* the trunk (Figure 12). *Btd* may have a similar role in the anterior head, as we

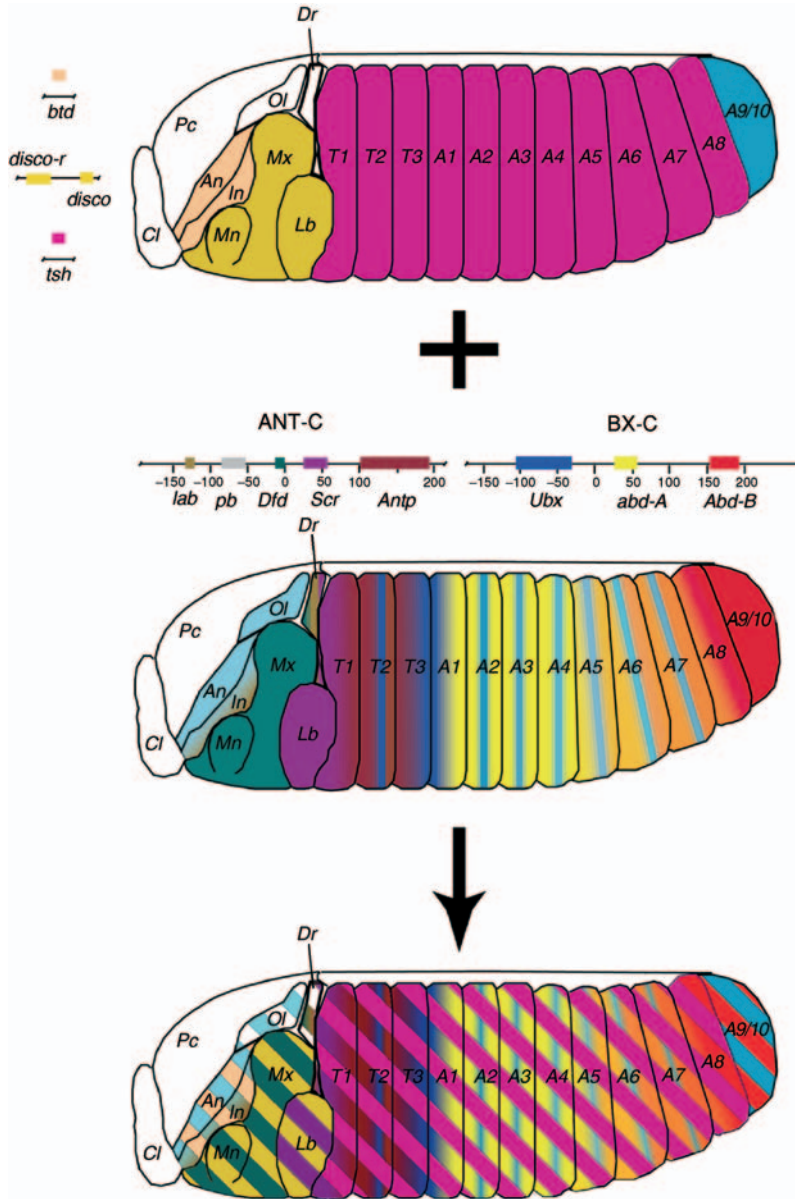


Figure 12 A model for regional specification and establishment of HOM-C protein function. The regionally expressed zinc finger transcription factors (upper image) participate in the regionalization of the *Drosophila* embryo. Some details of the expression patterns and function were intentionally left out for clarity. The anterior head region is postulated to be controlled by *bitd*, the gnathal region by *disco* and *disco-r*, and the trunk region by *tsh*. At present, the identity of a similar posterior abdominal factor is not known, although there are several candidates. A gene map and diagram representing the domains of HOM-C gene expression and control are shown in the middle. These expression patterns are described in the text. Finally, in the lower panel, the two gene expression patterns are merged and the zinc finger expression patterns are represented by the angular views through the HOM-C expression pattern. Unique combinations of C2H2 zinc finger transcription factors and HOM-C proteins in each segment specify individual segment identities.

have suggested in Figure 12. These authors have shown that this is an interactive network, where the Tsh protein can repress expression of the *disco* genes, restricting *disco* and *disco-r* expression to the gnathal region. In the absence of Tsh, *disco* and *disco-r* are ectopically expressed in the trunk

segments, which appears to be responsible for the trunk to gnathal transformation seen in homozygous *tsh* mutant embryos. In addition to this regionalization role, these zinc finger proteins participate with the HOM-C proteins during the establishment of the anterior–posterior axial pattern of

the *Drosophila* embryo. Robertson *et al.* (2004) show that *disco* expression establishes the regions where cells can respond to Dfd and Scr proteins. When embryos lack *disco* and *disco-r*, they do not respond to ectopic activation of these HOM-C genes, neither by activating gnathal HOM-C target genes nor by producing ectopic mouthpart structures, as do embryos expressing Disco (either endogenously or ectopically).

The picture now emerging is one in which the C2H2 zinc finger proteins help regionalize the embryo and define domains, in which specific HOM-C proteins can function. This does leave several important questions that need to be resolved. For example, are these zinc finger proteins biochemical cofactors for the HOM-C proteins? In addition, it is not known if the zinc finger proteins have the same HOM-C partners during adult development as they do during embryogenesis, and further, it is not clear whether or not this mechanism is functioning in other insects (see below). Indeed, this may be a crucial question, since there are indications that the relative expression of these zinc finger genes may be conserved in vertebrates, suggesting that function may be conserved as well. In either case, this opens a new avenue of study for the function of insect HOM-C proteins.

Although little is currently known regarding the role of related zinc finger proteins in other insects, expression of a homolog of *tsh/tektop* (*Tdtsb*) has been examined in *Thermobia*, and some information may be inferred regarding *Tribolium*. Expression analysis for *Tdtsb* reveals a pattern altered from that observed in the fly, with *Tdtsb* being expressed primarily in the thoracic and ventral gnathal segments, with only weak expression visible in the abdominal segments (Peterson *et al.*, 1999). Peterson *et al.* (1999) suggest that *Tdtsb* may specify the thoracic tagmata in *Thermobia*, rather than the larger trunk region as proposed for the *Drosophila* larva. The gnathal expression in *Thermobia* embryos may reflect *tsh*'s role in adult *Drosophila* head patterning, where it is required to suppress the activity of *Antp* (Bhojwani *et al.*, 1997). *Tribolium* embryos lacking the HOM-C genes differentiate antennae in each thoracic and abdominal segment (Peterson *et al.*, 1999, Brown *et al.*, 2002b), indicating that *tsh* may not act to specify the trunk tagmata as in *Drosophila*. It is unclear, however, whether the trunk to head transformation observed in *Tribolium* is limited to the appendages, or applies to an entire segment. Thus, *tsh* may retain a role as a HOM-C cofactor in a more basal insect species, but further work is needed to determine if this is the case.

1.5.8. Other Potential HOM-C Protein Cofactors

The proteins encoded by *cap 'n'collar* (*cnc*), *lines* (*lin*), and *apontic* (*apt*) have also been suggested as putative HOM-C protein cofactors. The *cnc* gene, located on the right arm of chromosome 3, encodes a member of the bZip protein family and produces three protein isoforms (A, B, and C) ranging in size from 533 to 1296 amino acids (McGinnis *et al.*, 1998). Its activity is required for normal patterning of the embryonic mandibular and labral segments. Loss of *cnc* function causes a transformation of the mandibular lobe towards a maxillary identity, demonstrated by the formation of ectopic mandibular mouth hooks and cirri (Mohler *et al.*, 1995). The CNC isoform B (CNC-B) localizes to the mandibular and labral segments, overlapping with Dfd expression in the mandibular segment. In contrast to the role of *disco* and *disco-r*, which appear necessary for Dfd activation of target genes, normal CNC-B function diminishes the activity of Dfd response elements by antagonizing the Dfd autoregulatory pathway. Mutants of *cnc* have persistent Dfd accumulation in the anterior mandibular lobe (where Dfd protein levels decrease in wild-type embryos during germ band contraction), and the overexpression of *cnc* during embryonic development produces a phenotype very similar to that of null Dfd embryos (McGinnis *et al.*, 1998). Taken together, these results support the cospecification of mandibular identity by CNC and Dfd. The gnathal pattern of *cnc* expression is very similar in *Thermobia* and *Oncopeltus*, thus the *cnc* homologs in these insects may be functioning in a similar way to *Drosophila* (Rogers *et al.*, 2002).

The putative HOM-C cofactor encoded by *apontic* (*apt*) was identified in a second chromosome screen for modifiers of Dfd function (Gellon *et al.*, 1997). Embryos mutant for *apt* share some phenotypic characteristics with both Dfd and Scr mutant embryos, including the extreme reduction of the dorsal pouch and the loss of the dorsal bridge. Other cephalopharyngeal structures lost in Dfd and Scr mutant embryos are, however, retained in *apt* mutants. Zygotic *apt* transcripts localize to the dorsal acron, clypeo-labrum, and ventral and anteriolateral gnathal lobes. A segmentally repeated pattern is observed in the trunk epidermis and CNS. Because loss of *apt* has no apparent effect on Dfd and Scr transcription, and these HOM-C genes do not affect the transcription of *apt*, these genes likely function in parallel to pattern portions of the gnathal region. Like *disco* and *disco-r*, *apt* does not

affect the *Dfd* autoregulatory loop, and thus may directly coregulate some *Dfd* target genes.

Another region of embryonic *apt* expression includes the developing dorsal vessel and heart, and *apt* is required for appropriate heart morphogenesis and function (Su *et al.*, 1999). The homeotic proteins Antp, Ubx, and Abd-A determine the anterior–posterior portioning of the dorsal vessel into the aorta and heart, and Abd-B represses cardiogenesis (Lo *et al.*, 2002; Lovato *et al.*, 2002). An interesting question is whether APT might also function in concert with one or more of these HOM-C proteins in the development of the larval circulatory system.

In the eighth abdominal segment, identity is primarily controlled by the HOM-C gene *Abd-B*, and requires the activity of the *Abd-B* target genes, *empty spiracles* (*ems*), *spalt major* (*salm*), and *cut* (*ct*). The segment polarity gene *lines* (*lin*) (Volk *et al.*, 1994; Bokor and DiNardo, 1996) is also required for the proper patterning of the posteriormost abdomen and may function as a cofactor for *Abd-B* in this domain. Expression of *Abd-B* is normal in *lin* mutants (Castelli-Gair, 1998), but the posteriormost segment fails to develop normally and lacks posterior spiracles, mirroring a phenotypic effect of the loss of *Abd-B* function (Sánchez-Herrero *et al.*, 1985). The LIN protein appears to function cooperatively with *Abd-B* in the transcriptional activation of an *ems* reporter construct and both *salm* and *ct*. Another function of *Abd-B* is the repression of the more anterior *BX-C* genes, *Ubx* and *abd-A* (Struhl and White, 1985; Macías *et al.*, 1990). Gene *lin* mutants have no effect upon the expression of these HOM-C genes, indicating *Abd-B* does not require LIN for this repressive function. Current information indicates that LIN only coregulates *Abd-B* targets, as there is no evidence of alteration in *Abd-A*, *Ubx*, or *Dfd* downstream target expression. As *Abd-B* appears not to require *Exd* as a cofactor, *lin* is currently the only gene known to produce a putative *Abd-B* transcriptional coregulator.

1.5.9. HOM-C Protein Function in the Absence of Cofactors: Cooperative Binding

There is much evidence to support that HOM-C proteins function while interacting with cofactors, but is there evidence that target gene regulation can occur without cofactors? Some *Drosophila* promoter and enhancer regions contain clustered recognition sites for a single transcription factor (for examples, see Laughon *et al.*, 1988; Yang *et al.*, 2001; Markstein *et al.*, 2002), and *in vitro* studies demonstrate that homeodomain proteins can bind

cooperatively to clustered sites (Desplan *et al.*, 1988). Such clustering may provide the means for spatially and temporally specific gene activation based on the concentration of a given factor(s). A mechanism such as this may provide some explanation for the biological specificity directed by the HOM-C genes, in spite of their overlapping DNA binding specificities. Indeed, this type of transcriptional regulation was shown to be true for other early-acting patterning genes (reviews: Pankratz and Jaekle, 1990; Small and Levine, 1991; St Johnston and Nüsslein-Volhard, 1991). Numerous studies demonstrate HOM-C protein binding to multiple, clustered recognition sites. Multiple binding sites were required for a measurable response in a yeast two-hybrid screen for transcriptional activation, even when using high-affinity HOM-C protein recognition sites (Ekker *et al.*, 1992). Known *Ubx* target sequences contain multiple 5'-TAAT-3' repeats, and the *Ubx* protein can protect regions of DNA up to 87 kb (Beachy *et al.*, 1988), although the minimum consensus sequence for *Ubx* binding is only 9 bp (Ekker *et al.*, 1992). Further, *Ubx* binding is increasingly stabilized by cooperative interactions resulting from clustered binding (Beachy *et al.*, 1993). Interacting sites are not, however, always closely clustered. They may lie as much as 200 bp apart, with their interaction involving a DNA looping mechanism. An example of cooperative binding, important to development, occurs during haltere/wing development. *Ubx* represses *spalt major* (*salm*), a gene involved in wing patterning, to promote haltere formation, and it does so in the absence of either *Exd* or *Hth* through a number of *Ubx* monomer binding sites (Galant *et al.*, 2002). Taken together, these studies provide evidence for HOM-C target regulation in the absence of known cofactors. Concentration-dependent HOM-C target activation may provide an additional mechanism to guide developmental specificity, whether acting alone or in concert with currently unrecognized factors.

For the HOM-C proteins to direct the specification of segment identities, it is apparent that additional factors or mechanisms are required beyond the simple binding of a HOM-C protein to a recognition site. How might cofactors contribute to target gene selection? We mentioned above two models proposed by Biggin and McGinnis (1997) for the role of cofactors during HOM-C protein target regulation. Although there is evidence for both models, the widespread binding model appears to have the most support. But it should be pointed out that this is not likely to be a simple process involving one, two, or even three factors. More likely, the process of target gene selection involves multiple factors,

with perhaps some, like the gap gene products, being expressed prior to the HOM-C proteins. Furthermore, when thinking of cofactor function, one might expect direct interaction with the HOM-C proteins, but this need not be the case. Some proteins functioning as cofactors may not even be DNA binding proteins, but may serve as “linkers” required to stabilize interactions between the basal transcription machinery and the HOM-C proteins. Clearly, this is an area where much more investigation is needed, and because there are differences between different insect species, perhaps a comparative study can yield some of the sought-after answers.

1.6. Targets of the HOM-C Proteins and Functional Specificity

Adam Wilkins (1986), challenges:

Isn't it a rather large leap from transcription-regulation, the assumed forte of homeo box-containing genes, to explaining the large-scale interaction and movements of cells that comprise morphogenesis and pattern formation? ... the rush to pin so much on the homeo box ... reveals the eagerness, even the desperation of the molecular-developmental biologists to find universal or, at least, general principles of genetic control in development.

The *HOM-C* genes are of great interest because they drive developmental specificity, both throughout an individual organism and across phyla. Those studying animal development and evolution desire to understand the mechanisms allowing this specification to take place. Graba *et al.* (1997) state:

The goal is to identify functions that are required to translate the positional information delivered by HOX proteins into diversified morphogenetic programmes.

Although characterization of HOM-C binding and the interactions of possible cofactors certainly contribute to understanding HOM-C function, the fact that they are so conserved and yet specify pattern in such different animal body plans is perplexing. Deciphering how this is accomplished in divergent phyla is the ultimate task. An understanding of downstream targets and how they are integrated to yield different body plans is crucial to the understanding the plasticity of development.

García-Bellido has described a model by which homeotic genes might function. As “selector” genes, HOM-C products would control developmental pathways by regulating the action of “realizator” genes. The “realizator” gene products would directly define and control “morphogenic cell properties” (such as mitosis, cell recognition, cell adhesion

properties, and cell death), to produce specific cell types and morphologies (García-Bellido, 1977). In such a way, these target, “realizator” genes could allow individual, regionally expressed *HOM-C* genes to direct developmentally specific characteristics through a sequence of steps similar to those of a metabolic pathway.

Despite over 20 years of intensive research into the nature and function of the *HOM-C* genes, identification of target genes has proven illusive. In a 1997 review, Graba *et al.* (1997) identify only 19 known HOM-C protein targets. Since that time, at least 13 additional HOM-C target genes have been identified, and a compilation of known HOM-C target genes appears in Table 2. On the basis of functional characterization, these genes fall into four categories. The first one corresponds to García-Bellido's “realizator” genes, being directly involved in modulating cellular processes. A second category contains genes whose products are involved in signal transduction. There are a few target genes whose functions are unknown, and finally, the majority of known HOM-C target genes are, themselves, transcription factors. Based on the number of developmental processes likely to be modulated by the *HOM-C* genes, and experimental results from molecular studies, Akam (1998) proposes that these “versatile generalists” might direct as many as 1000 downstream targets, each.

1.6.1. The Search for Target Genes

There are characteristics inherent to the *HOM-C* genes that make the identification and characterization of target genes difficult. As discussed previously, the HOM-C proteins have similar *in vitro* binding properties, recognizing a consensus core sequence of 5'-TAAT-3'. This can prove problematic when attempting *in vitro* screens for target promoter sequences, as promiscuous binding to sites lacking *in vivo* significance can occur. Several of the *HOM-C* genes have overlapping expression domains, and target gene regulation may require input from multiple *HOM-C* genes. In such a case, the removal of one HOM-C protein may not significantly effect target gene action. A single target gene also may be regulated in adjacent regions by different HOM-C proteins, as is illustrated by the activation of *tsh* activity in the anterior midgut by Antp and in the central midgut by Ubx and Abd-A (Mathies *et al.*, 1994). Cross-regulation between the *HOM-C* genes also muddies the waters of target gene identification. It can be unclear whether an observed effect results directly from the loss of one HOM-C protein, or indirectly because of the ability of the *HOM-C* genes to cross-regulate one another. The

control regions of potential target genes can be quite large, often up to 100 kb, making systematic evaluation of potential regulatory sites challenging, and also making it difficult to ascertain if an observed HOM-C protein's effect is direct or indirect. HOM-C protein action can be non cell autonomous, acting through signaling cascades and leading to effects outside of the HOM-C protein's expression domain, and in adjacent germ layers. Finally, some *HOM-C* genes have received more attention, and thus may appear more amenable to the search for target genes. Genes under the control of Antp, Ubx and/or Abd-A make up the majority of the characterized target genes, while the more anterior HOM-C proteins (LB, Pb, Dfd, Scr) have far fewer or no characterized targets outside of the *HOM-C* genes themselves.

Many of the identified HOM-C target genes were previously characterized genes whose expression or function gave cause to suspect HOM-C protein control. There have been, however, a number of *in vitro* and *in vivo* screening techniques that have been employed to identify other HOM-C protein targets, with varying degrees of success (for examples, see Gould *et al.*, 1990; Gould and White, 1992; Graba *et al.*, 1992; Mahaffey *et al.*, 1993; Feinstein *et al.*, 1995; Mastick *et al.*, 1995; Merabet *et al.*, 2002; Marchetti *et al.*, 2003). Common *in vitro* screening techniques include protein–DNA interaction screens in yeast and immunoprecipitation of bound DNA fragments with HOM-C protein antibodies. *In vivo* techniques include the use of enhancer trapping and deficiency or mutagenesis screens. Other screening techniques combine elements of both, such as subtractive hybridization and chromatin immunoprecipitation. Each approach has both advantages and drawbacks. *In vivo* studies can be quite time consuming, and sometimes produce target genes that are difficult to characterize further as to their function and the directness of HOM-C protein regulation.

Yeast reporter assays can be useful in screening a large number of genomic fragments quickly and efficiently, but nonphysiological conditions, alterations in chromatin structure, and the lack of other interacting proteins can lead to imprecise results. In one such study, Mastick *et al.* (1995) sought to identify genes directly regulated by the Ubx protein. Fifteen per cent of the genome was searched, and based on fortuitous sequence alone, at least 500 interacting sites were expected. In fact, only 53 genomic fragments were recovered, and of those, only three appeared to be associated with Ubx target regulation.

Chromatin immunoprecipitation, when utilizing DNA–protein complexes cross-linked *in vivo* at

physiological conditions, can be useful in identifying novel targets of direct HOM-C regulation, but requires an antibody against the HOM-C protein of interest, and results can be affected by cloning efficiencies. Also, if true, the widespread binding model would predict the recovery of a large number of DNA sites to which the HOM-C proteins bind, but many will have no endogenous regulatory effect.

Subtractive hybridization allows the sorting of transcripts that differ between normal individuals and individuals with altered HOM-C function, although hybridization conditions and cDNA synthesis can strongly influence results. Further, if target gene activation requires several HOM-C proteins, it may not be identified by such an approach.

Marchetti *et al.* took a cytological approach to identifying targets of the *BX-C* genes through immunostaining of polytene chromosomes taken from larval fat bodies (Marchetti *et al.*, 2003). Although not as endoreplicated as the salivary gland polytene chromosomes, adequate *BX-C* protein binding was detected to allow the mapping of more than 300 binding sites. Each site bound one or more of the *BX-C* proteins, and several of these sites corresponded to known *BX-C* target genes. Differential patterns of *BX-C* binding were observed when polytene chromosomes from the fat bodies and salivary glands were compared, suggesting tissue-specific activity. As the *BX-C* genes are normally expressed in the fat bodies, this approach may be more likely to identify genuine binding targets, when compared to studies utilizing Ubx overexpression in the salivary glands (Botas and Auwers, 1996).

One remaining strategy that has not yet provided additional HOM-C targets is that of whole genome microarray analysis. This technique has been used to identify candidate genes involved in dorsal–ventral patterning, circadian rhythms, and immune response (Irving *et al.*, 2001; McDonald and Rosbash, 2001; Stathopoulos *et al.*, 2001), and may hold the same promise for further HOM-C target discovery.

1.6.2. Possible Modes of HOM-C Target Activation

The HOM-C proteins could regulate, directly, a vast number of individual target genes in combinations required for regional specification. Alternately, the HOM-C proteins could function primarily through the regulation of a few downstream transcription factors to activate regional, developmentally specific genetic cascades. In most cases, the line of control between the *HOM-C* genes and downstream targets is unclear. Of the target genes summarized in Table 2, only eight have been demonstrated to be directly regulated by a HOM-C protein. Of those

Table 2 *Drosophila* HOM-C target genes

Target gene	HOM-C regulator	Molecular type ^b	Biological function ^b	Identification method ^c	References
<i>1.28 (1.28)^a</i>	Dfd	Unknown	Unknown	ET	Mahaffey <i>et al.</i> (1993)
<i>apterous(ap)^a</i>	Antp	Lim-homeodomain transcription factor	Wing patterning, neuronal fasciculation, myogenesis	PC	Capovilla <i>et al.</i> (2001)
<i>belt (belt)</i>	Antp, Ubx	Unknown	Unknown function	SH	Feinstein <i>et al.</i> (1995)
β 3-tubulin (<i>βtub60D^a</i>)	Ubx	Cytoskeletal protein	Visceral mesoderm differentiation	PC	Hinz <i>et al.</i> (1992)
<i>blistered (bs)</i>	Ubx	MADS-box transcription factor	Wing vein patterning, tracheal patterning	PC	Weatherbee <i>et al.</i> (1998)
<i>canoe (cno)</i>	Ubx, Abd-A	GLGF/DHR motif	Actin binding protein dorsal closure	SH	Feinstein <i>et al.</i> (1995)
<i>centrosomin (cnn)</i>	Antp, Ubx	Microtubule binding, myosin ATPase	Midgut morphogenesis, CNS development	CIP	Megraw <i>et al.</i> (1999)
<i>Connectin (Con)^a</i>	Ubx	Cell adhesion	Neuronal fasciculation, synaptic target attraction	CIP	Gould and White (1992)
<i>dachshund (dac)</i>	Pb, Abd-B	Novel transcription factor	Eye disc development	PC	Estrada and Sánchez-Herrero (2001), Abzhanov <i>et al.</i> (2001)
<i>dlarp</i>	Scr, Ubx	Novel protein of unknown function	Unknown function	CIP	Chauvet <i>et al.</i> (2000)
<i>decapentaplegic (dpp)^a</i>	Antp, Ubx	TGF- β receptor ligand	Embryonic, imaginal axial patterning, midgut morphogenesis	PC	Capovilla <i>et al.</i> (1994), Sun <i>et al.</i> (1995)
<i>Distal-less (Dll)^a</i>	Pb, Dfd, Scr, Ubx, Abd-A, Abd-B	Homeodomain transcription factor	Appendage development, sensory structures	PC	Estrada and Sánchez-Herrero (2001), Abzhanov <i>et al.</i> (2001), Gebelein <i>et al.</i> (2002), O'Hara <i>et al.</i> (1993), Castelligair and Akam (1995), Vachon <i>et al.</i> (1992)
<i>Dwnt-4 (Wnt4)^a</i>	Antp, Ubx, Abd-A	Signaling molecule	Epidermal, visceral mesoderm development	CIP	Graba <i>et al.</i> (1995)
<i>empty spiracles (ems)</i>	Abd-B	Homeodomain transcription factor	Head, brain, and tracheal development	PC	Jones and McGinnis (1993)
<i>extradenticle (exd)</i>	Pb, Ubx, Abd-A, Abd-B	Homeodomain transcription factor	Embryonic and imaginal patterning	PC	Abzhanov <i>et al.</i> (2001), Azpiazu and Morata (1998)

<i>fork head (fkh)</i>	Scr	“Forkhead” domain transcription factor	Salivary gland development	PC	Ryoo and Mann (1999), Panzer <i>et al.</i> (1992)
<i>homothorax (hth)</i>	Scr, Antp, Abd-A, Abd-B	Homeodomain-HM transcription factor	Embryonic and imaginal patterning	PC	Estrada and Sánchez-Herrero (2001), Yao <i>et al.</i> (1999)
<i>modulo (mod)</i>	Scr, Ubx	DNA/RNA binding	Protein position effect variegation	PC	Graba <i>et al.</i> (1994), Alexandre <i>et al.</i> (1996)
<i>nervy (nvy)</i>	Antp, Ubx, Abd-A	Zinc finger transcription factor	Unknown function	SH	Feinstein <i>et al.</i> (1995)
<i>nessy (nes)</i>	Antp, Ubx, Abd-A	Transmembrane protein	Unknown function	CIP	Maurel-Zaffran <i>et al.</i> (1999)
<i>odd-paired (odd)</i>	Antp, Ubx, Abd-A	C2H2 zinc finger transcription factor	Midgut morphogenesis	PC	Cimbora and Sakonju (1995)
<i>paired (prd)</i>	Dfd	Homeodomain transcription factor	Sense organ development, segmentation	PC	Li <i>et al.</i> (1999a)
<i>pointed (pnt)</i>	Abd-A	Type II TGF- β receptor protein	Epidermal, CNS, and tracheal patterning	ET	Pribyl <i>et al.</i> (1988)
<i>reaper (rpr)^a</i>	Dfd, Abd-B	Apoptosis activating factor	Programmed cell death	PC	Lohmann <i>et al.</i> (2002)
<i>retrotransposon 412</i>	Abd-A	Transposable element	Gonadal mesoderm	CIP	Brookman <i>et al.</i> (1992)
<i>scabrous (sca)^a</i>	Ubx, Abd-A, Abd-B	Fibrinogen family signaling protein	Eye morphogenesis, neurogenesis	CIP	Graba <i>et al.</i> (1992)
<i>Serrate (Ser)</i>	Dfd, Ubx, Abd-A	Signaling protein	Embryonic patterning	PC	Wiellette and McGinnis (1999)
<i>spalt (salm)</i>	Pb, Scr, Antp	C2H2 zinc finger transcription factor	Trachea, appendage, oenocyte patterning	ET	Wagner-Bernholz <i>et al.</i> (1991), Galant <i>et al.</i> (2002), Abzhanov <i>et al.</i> (2001)
<i>spalt related (salr)</i>	Ubx	C2H2 zinc finger transcription factor	Haltere patterning	PC	Weatherbee <i>et al.</i> (1998)
<i>teashirt (tsh)</i>	Antp, Ubx, Abd-A	C2H2 zinc finger transcription factor	Midgut morphogenesis	ET	McCormick <i>et al.</i> (1995), Mathies <i>et al.</i> (1994)
<i>T48</i>	Ubx, Abd-A	Transmembrane protein	Unknown	CIP	Strutt and White (1994)
<i>unplugged (unpg)</i>	Ubx, Abd-A	Homeodomain transcription factor	CNS and tracheal development	ET	Chiang <i>et al.</i> (1995)
<i>wingless (wg)</i>	Ubx, Abd-A	Signaling molecule	Embryonic and imaginal patterning	PC	Weatherbee <i>et al.</i> (1998)
<i>vestigial (vg)</i>	Ubx	Novel transcription factor	Wing/haltere patterning	PC	Weatherbee <i>et al.</i> (1998)

^aEvidence supports a direct regulation by the HOM-C protein(s).

^bFunctions as indicated in the reference and/or FlyBase (FlyBase Consortium (2003)).

^cET, enhancer trap; SH subtractive hybridization (Hedrick *et al.*, 1984; Davis, 1986); CIP, chromatin immunoprecipitation (Gould *et al.*, 1990; Graba *et al.*, 1992); YRA, yeast reporter assay (Mastick *et al.*, 1995); PC, previously characterized gene.

with characterized direct interactions, most are either transcription factors or signaling molecules, and not the “realizator” genes envisioned by García-Bellido (1977). Based on the known functions of target genes currently characterized, it appears that some combination of these two modes of action is likely at work.

1.6.3. The Concentration of a HOM-C Protein Can Direct Target Activation

The concentration of a HOM-C protein can influence its downstream effects, as illustrated by phenotypic differences between null and hypomorphic alleles. For example, null *pb* mutants have a strong transformation of mouthparts to leg, while a hypomorphic *pb* allele results in the transformation of mouthparts to antenna or a combination of leg and antenna (Kaufman, 1978; Pultz *et al.*, 1988). Using a transgenic heat shock-inducible *pb* construct, Cribbs *et al.* (1995) further demonstrated that a defined threshold concentration of Pb is required to overcome the dominant antennae to leg phenotype of an *Antp* mutant.

Ubx provides a second example of concentration dependent action. Ubx is expressed, endogenously, at different levels in parasegments 5 and 6, with the higher concentration in parasegment 6. Increased expression of Ubx in parasegment 5 can alter the identity of that region towards the identity of parasegment 6, as evidenced in the larval denticle belts and abdominal imaginal histoblast cells (Smolik-Utlaut, 1990; Frayne and Sato, 1991).

Because variation in HOM-C protein concentration can alter the morphological outcome, it is likely that activation of some specific target genes is concentration dependent, possibly acting via cooperative binding at multiple, clustered recognition sites (see Section 1.5.9). Varying the number of HOM-C monomer binding sites in target promoters and/or enhancers could define, in part, a concentration dependency for gene regulation. What is apparent is that the simple presence or absence of a HOM-C protein, at least in some cases, is insufficient to control differing morphological specificity.

1.6.4. Beyond Targets: HOM-C Protein Regulation of Developmental Processes

To understand the mechanisms by which the HOM-C proteins control body patterning, it is essential to look beyond single target genes, and attempt to discern their function in the context of a larger developmental process. Insect appendage development provides such an opportunity (see Chapter 2), as the adult insect has appendages on different segments, arising from both dorsal and

ventral body regions, and specified by different HOM-C genes. The ability of a HOM-C gene product to specify the patterning of entire appendages is evidenced by the famous homeotic phenotypes that led to their discovery. Morata (2001) presents a model for appendage specification in which the regional expression of HOM-C proteins provides segment or regional specific identity (i.e., antennae vs. leg), while the signaling molecules provide the dorsal/ventral pattern cues (i.e., wing/haltere vs. leg). In Morata’s model, *pb* acts to specify the proboscis, *Scr* to specify the first thoracic leg, *Antp* to specify the second thoracic leg, and *Ubx* to specify the third thoracic leg, while also repressing wing formation in favor of haltere development.

The HOM-C proteins can also act regionally, across segments, in appendage specification. For example, *Antp* is required to repress antennal development in all leg discs, and does so by repressing the expression of *hth*. This repression is required for the appropriate patterning of the distal and medial leg regions, as one *hth* function is that of an antennal selector gene (Casares and Mann, 2001). Ubx blocks the entire appendage development program in the anterior *Drosophila* abdomen via the direct repression of the leg selector gene *Dll* (Vachon *et al.*, 1991). This requirement is demonstrated by the appearance of abdominal legs in some Ubx mutants (Lewis, 1978).

Ubx also has multiple levels of hierarchical control in specifying the third thoracic haltere. Weatherbee *et al.* (1998) investigated the control exerted by *Ubx* activity in the haltere over known wing patterning genes, while attempting to better define the developmental processes differentiating the haltere and the wing. Not surprisingly, they found that Ubx represses wing-specific genes; however, control beyond that initial level of repression was also observed. Ectopic expression of *vestigial* (*vg*) (required for wing formation and repressed by Ubx in the haltere) does not create ectopic wing tissue in the haltere, suggesting that Ubx is continuing to repress genes downstream of *vg*. Furthermore, normal haltere patterning requires Ubx function throughout larval development, indicating that Ubx does not simply function as an upstream initiator of the haltere developmental cascade.

We have discussed the probability of a large number of target genes for each HOM-C protein, and given examples in which a number of target genes are known to affect a single developmental process. Studies on the *Drosophila* larval oenocyte provide a well-characterized example of how a single HOM-C protein can direct the fate of a single cell. The larval oenocyte is a secretory cell derived from the dorsal ectoderm, and is found in clusters of

about six cells in the first through seventh abdominal segments. Oenocyte precursor cells delaminate from the dorsolateral ectoderm at the extended germ band stage, and differentiate in response to induction by C1 precursor cells, which also give rise to chordotonal sensory organs. A simplified diagram of this pathway is depicted in Figure 13. Although the C1 precursor cells and the ectodermal oenocyte precursor cells, prepatterned by Spalt major (*Salm*) are present in both the thoracic and abdominal segments, epidermal growth factor receptor (EGFR) activity is present only around the abdominal C1 cells, limiting oenocyte formation to these segments. The *spitz* (*spi*) gene product, encoding an EGFR ligand, is secreted by the abdominal C1 cells, and received by the oenocyte precursor cells, leading to the oenocyte specific gate (Elstob *et al.*, 2001; Rusten *et al.*, 2001). The *rhomboid* (*rho*) gene encodes a processing factor for the Spi ligand, modifying a membrane bound form of Spi (mSpi) to a secreted form (sSpi). Although *rho* is initially activated by *atonal* (*ato*) in the C1 cells, a burst of *abd-A* and *exd* expression in the abdominal segments is required to maintain *rho* transcription and allow the processing of membrane bound Spi in the Golgi apparatus (by Rho) to the secreted form (Brodu *et al.*,

2002). The processed Spi is then secreted, can activate EGFR and induce the *salm*-prepatterned ectodermal cells to form oenocytes. As oenocytes are found in a wide variety of both long and short germ insects, this system may provide a nice model for comparative insect studies of HOM-C target regulation.

Comparative studies of developmental processes in other insects are critical to the further understanding *HOM-C* gene function. The ability of the *HOM-C* genes to direct developmental processes is well documented in other insects (for examples, see Beeman *et al.*, 1989, 1993; Nagata *et al.*, 1996). Identifying the *Drosophila* *HOM-C* gene homologs, and their downstream targets directing these developmental processes, is beginning to provide interesting data regarding homeotic gene control of development. The direction of gnathal appendage development by the *HOM-C* genes provides an example.

In *Drosophila*, the *HOM-C* genes *Scr* and *pb* are required for appropriate patterning of the proboscis, and this specification requires suppression or down-regulation of antennal and leg-specific genes in the labial disc, such as *exd*, *dachshund* (*dac*), *salm*, and *Dll*. For example, *pb* limits the expression of *Dll* to a compact crescent-shaped domain within the developing labial appendage (Abzhanov *et al.*, 2001). The loss of *pb* causes an increase and an expansion of *Dll* expression, leading to the leg or partial antennal transformations observed in *pb* mutants. The loss of both *pb* and *Scr* causes a transformation of the proboscis to the “ground state” of antenna. Studies undertaken in *Tribolium* indicate that these genes function similarly to direct proboscis development as well. The loss of *mxp* (*pb* homolog) in the labial appendage bud causes a transformation of the labial palp to leg, and double mutants for *Cx* (*Scr* homolog) and *mxp*, develop antenna in place of the palps, similar to the fly. Unlike *Drosophila*, however, *Dll* expression in the *Tribolium* labial appendage is not significantly altered in *mxp* mutants (DeCamillis *et al.*, 2001). It will be interesting to see the effects of *mxp* and *Cx* loss on other leg/antennal patterning genes.

Appropriate *Dll* expression is required for the distal development of all *Drosophila* appendages, and *Dll* is also expressed in the developing appendages of other insects. Its pattern of expression is well conserved, as observed in *Tribolium*, *Thermobia*, *Acheta*, *Oncopeltus*, *Precis*, and *Neodiprion*, but there do appear to be some differences in both expression and regulation (Weatherbee *et al.*, 1999; Beermann *et al.*, 2001; Suzuki and Palopoli, 2001; Rogers *et al.*, 2002). Like in *Drosophila*, *Tribolium* requires the repression of *Dll* to suppress the

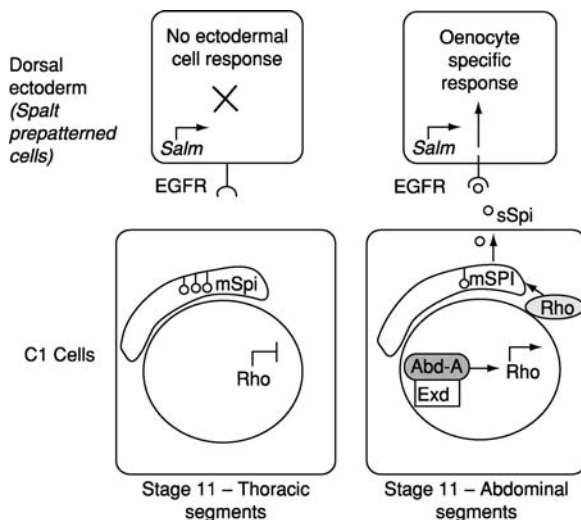


Figure 13 *Drosophila* Abd-A regulates a single principal target, *rho*, to direct larval oenocyte differentiation. The *spalt major* gene (*salm*) is transcribed in the dorsal embryonic ectoderm throughout the thoracic and abdominal segments. Rho is a processing factor, which acts in the Golgi apparatus to modify the membrane-bound form of the Spitz ligand (mSpi) to the secreted form (sSpi). In the abdominal segments at the germ band extended stage (Stage 11), a burst of *abd-A* and *exd* expression is required for *rho* transcription, allowing the production of sSpi and resulting in oenocyte-specific epidermal growth factor receptor (EGFR) output. Because Abd-A is not expressed in the thoracic cells and *rho* activity is absent, sSpi is not present to activate the oenocyte differentiation pathway.

appendage development pathway, but rather than utilizing Utx (Ubx homolog) and A (Abd-A homolog) for this function, A alone prevents *Dll* expression in the *Tribolium* larval abdomen (Lewis *et al.*, 2000). Utx, instead, appears to modulate patterning of a first abdominal appendage, the pleuropodia. Lepidopteran larvae, which have abdominal prolegs not found in *Drosophila* or *Tribolium* larvae, express *Dll* in the abdominal regions, in which these legs will form. Correspondingly, there are “holes” in the BX-C gene expression domains in the abdomen, allowing *Dll* expression and indicating that the lepidopteran BX-C genes may have a limb-repressing role. Studies in *Neodiprion* larvae reveal the conservation of BX-C control of *Dll* expression, even though the developmental process is altered (Suzuki and Palopoli, 2001). In contrast to lepidopteran larvae, *Neodiprion* larvae lack abdominal *Dll* expression even though these larvae develop abdominal prolegs. Concomitantly, the expression of the BX-C genes is found throughout the abdomen, including the developing prolegs. This reflects a conservation of the BX-C/*Dll* regulatory relationship, but a possibly convergent *Neodiprion* developmental pathway for abdominal prolegs, when compared to Lepidoptera. *Dll* is also a target of Ubx in the lepidopteran hindwing, which derives from the third thoracic segment. In contrast to the highly modified haltere of *Drosophila*, butterflies have well developed hindwings. As might be expected, several of the wing-specific genes that are repressed by Ubx in the *Drosophila* haltere remain under Ubx control in the butterfly, but are differentially regulated to allow hindwing development and patterning (Weatherbee *et al.*, 1999). These examples illustrate how differences in HOM-C gene target regulation may correlate to different developmental outcomes between insect species.

The HOM-C proteins can, in some developmental processes, directly regulate a large number of target genes to dictate regional specification. In other processes, however, far fewer targets may be required, with the primary targets being other transcription factors or signaling molecules. Specification of body regions, such as the wing or midgut, appears to be complex and involve a large number of downstream factors. In contrast, specifying the identity of only a few cells, as illustrated by the oenocyte case, can require only a single HOM-C responding gene.

1.7. Looking Forward

Our further understanding of how HOM-C gene function directs axial patterning requires a thorough

understanding of the biological processes guided by the HOM-C genes, the target genes responding to HOM-C protein regulation, and comparative studies involving nondrosophilid insects. A three-step approach begins with the identification of additional HOM-C guided developmental processes. These may be as large and complex as neural development (see Chapter 3), or may be as specific as the patterning of a single sensory cell, or even the regulation of a single gene. For example, recent studies into the process of larval salivary gland development, are providing insights into how Scr function establishes cell fate and tissue morphology (Abrams *et al.*, 2003; Haberman *et al.*, 2003). Next, characterizing the manner in which the HOM-C proteins then direct specific processes through downstream targets may shed light on the various mechanisms by which they govern pattern formation. Dfd, for example, was recently shown to directly regulate expression of the proapoptotic gene, *reaper*, in sculpting the *Drosophila* embryonic gnathal lobes through the initiation of programmed cell death (Lohmann *et al.*, 2002; see also Chapter 5).

Finally, comparisons of developmental processes and genetic cascades between insect species and noninsect arthropods are critical for addressing long-standing questions regarding the role of HOM-C/HOX genes in both development and evolution. The success of RNAi is now allowing the characterization of gene function in a growing number of nonmodel insects (Brown *et al.*, 1999; Denell and Shippy, 2001) such as *Thermobia* and *Onco-peltus*, without the generation of mutant strains. Recent studies by Galant and Carroll (2002) and Ronshaugen *et al.* (2002) correlate functional changes in Ubx, between insect and other arthropods, to the transition, in arthropods, from legs on every segment to legs limited to only the thoracic segments. Studies such as these will certainly continue and grow to include a wide variety of insects and other arthropods. When expanded to include downstream target genes, this work should provide valuable insights correlating changes in HOM-C directed genetic cascades to differing morphologies or developmental outcomes.

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References

- Abbott, M.K., Kaufman, R.C., 1986. The relationship between the functional complexity and the molecular organization of the *Antennapedia* locus of *Drosophila melanogaster*. *Genetics* 114, 919–942.
- Abrams, E.W., Vining, M.S., Andrew, D.J., 2003. Constructing an organ: the *Drosophila* salivary gland as a model for tube formation. *Trends Cell Biol.* 13, 247–254.
- Abzhanov, A., Holtzman, S., Kaufman, T.C., 2001. The *Drosophila* proboscis is specified by two Hox genes, *proboscipedia* and *Sex combs reduced*, via repression of leg and antennal appendage genes. *Development* 128, 2803–2814.
- Abzhanov, A., Kaufman, T.C., 2000. Homologs of *Drosophila* appendage genes in the patterning of arthropod limbs. *Devel. Biol.* 227, 673–689.
- Ades, S.E., Sauer, R.T., 1994. Differential DNA-binding specificity of the engrailed homeodomain: the role of residue 50. *Biochemistry* 33, 9187–9194.
- Affolter, M., Percival-Smith, A., Müller, M., Leupin, W., Gehring, W.J., 1990. DNA binding properties of the purified *Antennapedia* homeodomain. *Proc. Natl Acad. Sci. USA* 87, 4093–4097.
- Akam, M., 1998. Hox genes: from master genes to micro-managers. *Curr. Biol.* 8, R676–R678.
- Akam, M., Dawes, R., 1992. More than one way to slice an egg. *Curr. Biol.* 2, 395–398.
- Akam, M., Dawson, I., Tear, G., 1988. Homeotic genes and the control of segment diversity. *Development* 104, 123–134.
- Akam, M.E., Martinez-Arias, A., 1985. The distribution of *Ultrabithorax* transcripts on *Drosophila* embryos. *EMBO J.* 4, 1689–1700.
- Alexandre, E., Graba, Y., Fasano, L., Gallet, A., Perrin, L., et al., 1996. The *Drosophila* Teashirt Homeotic protein is a DNA-binding protein and *modulo*, a HOM-C regulated modifier of variegation, is a likely candidate for being a direct target gene. *Mech. Devel.* 59, 191–204.
- Andrew, D.J., 1995. The *Sex combs reduced* gene of *Drosophila melanogaster* has multiple transcripts. *Gene* 152, 149–155.
- Andrew, D.J., Horner, M.A., Petitt, M.G., Smolik, S.M., Scott, M.P., 1994. Setting limits on homeotic gene function: restraint of *Sex combs reduced* activity by *teashirt* and other homeotic genes. *EMBO J.* 13, 1132–1144.
- Appel, B., Sakonju, S., 1993. Cell-type-specific mechanisms of transcriptional repression by the homeotic gene products UBX and ABD-A in *Drosophila* embryos. *EMBO J.* 12, 1099–1109.
- Aspland, S.E., White, R.A.H., 1997. Nucleocytoplasmic localization of *extradenticle* protein is spatially regulated throughout development in *Drosophila*. *Development* 124, 741–747.
- Azpiazu, N., Morata, G., 1998. Functional and regulatory interactions between Hox and *extradenticle* genes. *Genes Devel.* 12, 261–273.
- Barges, S., Mihaly, J., Galloni, M., Hagstrom, K., Muller, M., et al., 2000. The *Fab-8* boundary defines the distal limit of the bithorax complex *iab-7* domain and insulates *iab-7* from initiation elements and a PRE in the adjacent *iab-8* domain. *Development* 127, 779–790.
- Bateson, W., 1894. Materials for the Study of Variation. Macmillan, New York.
- Beachy, P.A., Helfand, S.L., Hogness, D.S., 1985. Segmental distribution of bithorax complex proteins during *Drosophila* development. *Nature* 313, 545–551.
- Beachy, P.A., Krasnow, M.A., Gavis, E.R., Hogness, D.S., 1988. An *Ultrabithorax* protein binds sequences near its own and the *Antennapedia* P1 promoters. *Cell* 55, 1069–1081.
- Beachy, P.A., Varkey, J., Young, K.E., von Kessler, D.P., Sun, B.I., et al., 1993. Cooperative binding of an *Ultrabithorax* homeodomain protein to nearby and distant DNA sites. *Mol. Cell. Biol.* 13, 6941–6956.
- Beeman, R.W., 1987. A homeotic gene cluster in the red flour beetle. *Nature* 327, 247–249.
- Beeman, R.W., Stuart, J.J., Brown, S.J., Denell, R.E., 1993. Structure and function of the homeotic gene complex (*HOM-C*) in the beetle, *Tribolium castaneum*. *BioEssays* 15, 439–444.
- Beeman, R.W., Stuart, J.J., Hass, M.S., Denell, R.E., 1989. Genetic analysis of the homeotic gene complex (*HOM-C*) in the beetle *Tribolium castaneum*. *Devel. Biol.* 133, 196–209.
- Beermann, A., Jay, D., Beeman, R., Hulskamp, M., Tautz, D., et al., 2001. The *Short antennae* gene of *Tribolium* is required for limb development and encodes the orthologue of the *Drosophila* Distal-less protein. *Development* 128, 287–297.
- Bender, W., Akam, M., Karch, F., Beachy, P.A., Peifer, M., et al., 1983. Molecular genetics of the *Bithorax Complex* in *Drosophila melanogaster*. *Science* 221, 23–29.
- Bennett, R.L., Brown, S.J., Denell, R.E., 1999. Molecular and genetic analysis of the *Tribolium Ultrabithorax* ortholog, *Ultrathorax*. *Devel. Genes Evol.* 209, 608–619.
- Bergson, C., McGinnis, W., 1990. An autoregulatory enhancer element of the *Drosophila* homeotic gene *Deformed*. *EMBO J.* 9, 4287–4297.
- Birmingham, J.R., Jr., Martinez-Arias, A., Petitt, M.G., Scott, M.P., 1990. Different patterns of transcription from the two *Antennapedia* promoters during *Drosophila* embryogenesis. *Development* 109, 553–566.
- Birmingham, J.R., Jr., Scott, M.P., 1988. Developmentally regulated alternative splicing of transcripts from the *Drosophila* homeotic gene *Antennapedia* can produce four different proteins. *EMBO J.* 7, 3211–3222.
- Berry, M., Gehring, W., 2000. Phosphorylation status of the Scr homeodomain determines its functional activity: essential role for protein phosphatase 2A,B'. *EMBO J.* 19, 2946–2957.
- Bhojwani, J., Shashidhara, L.S., Sinha, P., 1997. Requirement of *teashirt* (*tsb*) function during cell fate specification in developing head structure in *Drosophila*. *Devel. Genes Evol.* 207, 137–146.

- Bienz, M., 1997. Endoderm induction in *Drosophila*: the nuclear targets of the inducing signals. *Curr. Opin. Genet. Devel.* 7, 683–688.
- Bienz, M., Müller, J., 1995. Transcriptional silencing of homeotic genes in *Drosophila*. *BioEssays* 17, 775–784.
- Bienz, M., Tremml, G., 1988. Domain of *Ultrabithorax* expression in *Drosophila* visceral mesoderm from autoregulation and exclusion. *Nature* 333, 576–578.
- Biggin, M.D., McGinnis, W., 1997. Regulation of segmentation and segmental identity by *Drosophila* homeoproteins: the role of DNA binding in functional activity and specificity. *Development* 124, 4425–4433.
- Billeter, M., Qian, Y., Otting, G., Muller, M., Gehring, W.J., et al., 1990. Determination of the three-dimensional structure of the *Antennapedia* homeodomain from *Drosophila* in solution by 1H nuclear magnetic resonance spectroscopy. *J. Mol. Biol.* 214, 183–197.
- Bokor, P., DiNardo, S., 1996. The roles of *hedgehog*, *wingless* and *lines* in patterning the dorsal epidermis in *Drosophila*. *Development* 122, 1083–1092.
- Botas, J., Auwers, L., 1996. Chromosomal binding sites of Ultrabithorax homeotic proteins. *Mech. Devel.* 56, 129–138.
- Boulet, A.M., Lloyd, A., et al., 1991. Molecular definition of the morphogenetic and regulatory functions and the *cis*-regulatory elements of the *Drosophila* Abd-B homeotic gene. *Development* 111, 393–405.
- Bourbon, H.M., Martin-Blanco, E., Rosen, D., Kornberg, T.B., 1995. Phosphorylation of the *Drosophila* engrailed protein at a site outside its homeodomain enhances DNA binding. *J. Biol. Chem.* 270, 11130–11139.
- Brand, A.H., Perrimon, N., 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118, 401–415.
- Brazas, R.M., Stillman, D.J., 1993. Identification and purification of a protein that binds DNA cooperatively with the yeast SWI5 protein. *Mol. Cell. Biol.* 13, 5524–5537.
- Breen, T.R., Harte, P.J., 1993. *trithorax* regulates multiple homeotic genes in the *Bithorax* and *Antennapedia* complexes and exerts different tissue-specific, parasegment-specific and promoter specific effects on each. *Development* 117, 119–134.
- Bridges, C., Dobzhansky, T., 1933. The mutant “*proboscipedia*” in *Drosophila melanogaster*: a case of hereditary homöosis. *Roux’s Arch. Devel. Biol.* 127, 575–590.
- Brodu, V., Elstob, P.R., Gould, A.P., 2002. *abdominal A* specifies one cell type in *Drosophila* by regulating one principal target gene. *Development* 129, 2957–2963.
- Brookman, J.J., Toosy, A.T., Shashidhara, L.S., White, R.A., 1992. The 412 retrotransposon and the development of gonadal mesoderm in *Drosophila*. *Development* 116, 1185–1192.
- Brown, S.J., DeCamillis, M., Gonzalez-Charneco, K., Denell, M., Beeman, R., et al., 2000. Implications of the *Tribolium Deformed* mutant phenotype for the evolution of Hox gene function. *Proc. Natl Acad. Sci. USA* 97, 4510–4514.
- Brown, S.J., Fellers, J.P., Shippy, T.D., Richardson, E.A., Maxwell, M., et al., 2002a. Sequence of the *Tribolium castaneum* Homeotic Complex: the region corresponding to the *Drosophila melanogaster* Antennapedia Complex. *Genetics* 160, 1067–1074.
- Brown, S.J., Hilgenfeld, R.B., Denell, R.E., 1994. The beetle *Tribolium castaneum* has a *fushi tarazu* homolog expressed in stripes during segmentation. *Proc. Natl Acad. Sci. USA* 91, 12922–12926.
- Brown, S.J., Holtzman, S., Kaufman, T., Denell, R.E., 1999. Characterization of the *Tribolium Deformed* ortholog and its ability to directly regulate *Deformed* target genes in the rescue of a *Drosophila Deformed* null mutant. *Devel. Genes Evol.* 209, 389–398.
- Brown, S.J., Parrish, J.K., Beeman, R.W., Denell, R.E., 1997. Molecular characterization and embryonic expression of the *even-skipped* ortholog of *Tribolium castaneum*. *Mech. Devel.* 61, 165–173.
- Brown, S.J., Shippy, T.D., Beeman, R.W., Denell, R.E., 2002b. *Tribolium* Hox genes repress antennal development in the gnathos and trunk. *Mol. Phylogenet. Evol.* 24, 384–387.
- Bürglin, T.R., 1994. A comprehensive classification of homeobox genes. In: Duboule, D. (Ed.), *Guidebook to Homeobox Genes*. Oxford University Press, New York, pp. 27–71.
- Busturia, A., Casonova, J., Sánchez-Herrero, E., González, R., Morata, G., 1989. Genetic structure of the *abd-A* gene of *Drosophila*. *Development* 107, 575–583.
- Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., et al., 2002. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science* 298, 1039–1043.
- Capovilla, M., Brandt, M., Botas, J., 1994. Direct regulation of *decapentaplegic* by *Ultrabithorax* and its role in *Drosophila* midgut morphogenesis. *Cell* 76, 461–475.
- Capovilla, M., Kambris, Z., Botas, J., 2001. Direct regulation of the muscle-identity gene *apterous* by a Hox protein in the somatic mesoderm. *Development* 128, 1221–1230.
- Carrasco, A.E., McGinnis, W., Gehring, W.J., Robertis, E.M., 1984. Cloning of an *X. laevis* gene expressed during early embryogenesis that codes for a protein domain homologous to *Drosophila* homeotic genes. *Cell* 37, 409–414.
- Carroll, S.B., Laymon, R.A., McCutcheon, M.A., Riley, P.D., Scott, M.P., 1986. The localization and regulation of Antennapedia protein expression in *Drosophila* embryos. *Cell* 47, 113–122.
- Casonova, J., Sánchez-Herro, E., Morata, G., 1986. Identification and characterization of a parasegment specific regulatory element of the *Abdominal-B* gene of *Drosophila*. *Cell* 47, 627–636.
- Casares, F., Mann, R.S., 2001. The ground state of the ventral appendage in *Drosophila*. *Science* 293, 1477–1480.
- Casares, F., Sánchez-Herrero, E., 1995. Regulation of the *infraabdominal* regions of the bithorax complex

- of *Drosophila* by gap genes. *Development* 121, 1855–1866.
- Castelli-Gair, J., 1998. The *lines* gene of *Drosophila* is required for specific functions of the Abdominal-B Hox protein. *Development* 125, 1269–1274.
- Castelli-Gair, J., Akam, M., 1995. How the Hox gene *Ultrabithorax* specifies two different segments: the significance of spatial and temporal regulation within metameres. *Development* 121, 2973–2982.
- Castelli-Gair, J.E., Garcia-Bellido, A., 1990. Interactions of *Polycomb* and *trithorax* with *cis* regulatory regions of *Ultrabithorax* during the development of *Drosophila melanogaster*. *EMBO J.* 9, 4267–4275.
- Celniker, S.E., Keelan, D.J., Lewis, E.B., 1989. The molecular genetics of the bithorax complex of *Drosophila*: characterization of the products of the *Abdominal-B* domain. *Genes Devel.* 3, 1424–1436.
- Chan, S., Jaffe, L., Capovilla, M., Botas, J., Amann, R.S., 1994. The DNA binding specificity of *Ultrabithorax* is modulated by cooperative interactions with *Extradenticle*, another homeoprotein. *Cell* 78, 603–615.
- Chan, S., Mann, R.S., 1993. The segment identity functions of *Ultrabithorax* are contained within its homeo domain and carboxy-terminal sequences. *Genes Devel.* 7, 796–811.
- Chan, S., Mann, R.S., 1996. A structural model for a homeotic protein–*Extradenticle*–DNA complex accounts for the choice of HOX protein in the heterodimer. *Proc. Natl Acad. Sci. USA* 93, 5223–5228.
- Chan, S., Pöpper, H., Krumlauf, R., Mann, R.S., 1996. An *Extradenticle* induced conformational change in a HOX protein overcomes an inhibitory function of the conserved hexapeptide motif. *EMBO J.* 15, 2476–2487.
- Chan, S., Ryoo, H.D., Gould, A., Krumlauf, R., Mann, R.S., 1997. Switching the *in vivo* specificity of a minimal Hox-responsive element. *Development* 124, 2007–2014.
- Chang, C., Shen, W., Rozenfeld, S., Lawrence, J.H., Largman, C., et al., 1995. Pbx proteins display hexapeptide-dependent cooperative DNA binding with a subset of Hox proteins. *Genes Devel.* 9, 663–674.
- Chang, C.P., Brocchieri, L., Shen, W.F., Largman, C., Cleary, M.L., et al., 1997. Pbx modulation of Hox homeodomain amino-terminal arms establishes different DNA-binding specificities across the Hox locus. *Mol. Cell. Biol.* 16, 1734–1745.
- Chauvet, S., Maurel-Zaffran, C., Miassod, R., Jullien, N., Pradel, J., et al., 2000. *dlarp*, a new candidate Hox target in *Drosophila* whose orthologue in mouse is expressed at sites of epithelium/mesenchymal interactions. *Devel. Dyn.* 218, 401–413.
- Chiang, C., Young, K.E., Beachy, P.A., 1995. Control of *Drosophila* tracheal branching by the novel homeodomain gene *unplugged*, a regulatory target for genes of the bithorax complex. *Development* 121, 3901–3912.
- Chinwalla, V., Jane, W., Harte, P., 1995. The *Drosophila* *trithorax* protein binds to specific chromosomal sites and is co-localized with *polycomb* at many sites. *EMBO J.* 14, 2056–2065.
- Choi, S.H., Oh, C.T., Kim, S.H., Kim, Y.T., Jeon, S.H., 2000. Effects of *Polycomb* group mutations on the expression of *Ultrabithorax* in the *Drosophila* visceral mesoderm. *Mol. Cells.* 10, 156–161.
- Chouinard, S., Kaufman, T.C., 1991. Control of expression of the homeotic *labial* (*lab*) locus of *Drosophila melanogaster*: evidence for both positive and negative autogenous regulation. *Development* 113, 1267–1280.
- Cimbora, D.M., Sakonju, S., 1995. *Drosophila* midgut morphogenesis requires the function of the segmentation gene *odd-paired*. *Devel. Biol.* 169, 580–595.
- Clarke, N.D., Kissinger, C.R., Desjarlais, J., Gilliland, G.L., Pabo, C.O., 1994. Structural studies of the engrailed homeodomain. *Protein Sci.* 3, 1779–1787.
- Clemm, J.D., Pabo, C.O., 1996. Oct1 POU domain interactions: cooperative binding of isolated subdomains and effects of covalent linkage. *Genes Devel.* 10, 27–36.
- Cohen, S.M., Jürgens, G., 1990. Mediation of *Drosophila* head development by gap-like segmentation genes. *Nature* 346, 482–485.
- Cook, C.E., Smith, M.L., Telford, M.J., Bastianello, A., Akam, M., 2001. Hox genes and the phylogeny of the arthropods. *Curr. Biol.* 11, 759–763.
- Cribbs, D.L., Benassayag, C., Randazzo, F.M., Kaufman, T.C., 1995. Levels of homeotic protein function can determine developmental identity: evidence from low-level expression of the *Drosophila* homeotic gene *proboscipedia* under Hsp70 control. *EMBO J.* 14, 767–778.
- Cribbs, D.L., Pultz, M.A., Johnson, D., Mazzulla, M., Kaufman, T.C., 1992. Structural complexity and evolutionary conservation of the *Drosophila* homeotic gene *proboscipedia*. *EMBO J.* 11, 1437–1449.
- Cumberledge, S., Zaratian, A., Sakonju, S., 1990. Characterization of two RNAs transcribed from the *cis*-regulatory region of the *abd-A* domain within the *Drosophila* bithorax complex. *Proc. Natl Acad. Sci. USA* 87, 3259–3263.
- Curtis, C.D., Brisson, J.A., DeCamillis, M., Shippy, T.D., Brown, S.J., et al., 2001. Molecular characterization of *Cephalothorax*, the *Tribolium* ortholog of *Sex combs reduced*. *Genesis* 30, 12–20.
- Dalton, D., Chadwick, R., McGinnis, W., 1989. Expression and embryonic function of *empty spiracles*: a *Drosophila* homeobox gene with two patterning functions on the anterior–posterior axis of the embryo. *Genes Devel.* 3, 1940–1956.
- Damen, W.G., Tautz, D., 1999. Abdominal-B expression in a spider suggests a general role for Abdominal-B in specifying the genital structure. *J. Exp. Zool.* 285, 85–91.
- Davis, M.M., 1986. Subtractive cDNA hybridization and the T-cell receptor genes. In: Weir, D.M. (Ed.), *Handbook of Experimental Immunology*, vol. 2, Cellular Immunology. Blackwell Scientific Publications, Palo Alto, CA, pp. 76.1–76.13.

- Davenport, M.P., Blass, C., Eggleston, P., 2000. Characterization of the Hox gene cluster in the malaria vector mosquito, *Anopheles gambiae*. *Evol. Devel.* 2, 326–339.
- de Zulueta, P., Alexandre, E., Jacq, B., Kerridge, S., 1994. Homeotic complex and *teashirt* genes co-operate to establish trunk segmental identities in *Drosophila*. *Development* 120, 2278–2296.
- DeCamillis, M.A., French-Constant, R., 2003. *Proboscipedia* represses distal signaling in the embryonic gnathal limb fields of *Tribolium castaneum*. *Devel. Genes Evol.* 213, 55–64.
- DeCamillis, M.A., Lewis, D.L., Brown, S.J., Beeman, R.W., Denell, R.E., 2001. Interactions of the *Tribolium* *Sex combs reduced* and *proboscipedia* orthologs in embryonic labial development. *Genetics* 159, 1643–1648.
- Delorenzi, M., Bienz, M., 1990. Expression of *Abdominal-B* homeoproteins in *Drosophila* embryos. *Development* 108, 323–329.
- Denell, R.E., Shippy, T., 2001. Comparative insect developmental genetics: phenotypes without mutants. *BioEssays* 23, 379–382.
- Desplan, C., Theis, J., O'Farrell, P.H., 1985. The *Drosophila* developmental gene, *engrailed*, encodes a sequence-specific DNA binding activity. *Nature* 318, 630–635.
- Desplan, C., Theis, J., O'Farrell, P.H., 1988. The sequence specificity of homeodomain–DNA interaction. *Cell* 54, 1081–1090.
- Dessain, S., Gross, C.T., Kuziora, M.A., McGinnis, W., 1992. *Antp*-type homeodomains have distinct DNA binding specificities that correlate with their different regulatory functions in embryos. *EMBO J.* 11, 991–1002.
- Diederich, R.J., Merrill, V.K.L., Pultz, M.A., Kaufman, T.C., 1989. Isolation, structure, and expression of *labial*, a homeotic gene of the *Antennapedia* Complex involved in *Drosophila* head development. *Genes Devel.* 3, 399–414.
- Diederich, R.J., Pattatucci, A.M., Kaufman, T.C., 1991. Developmental and evolutionary implications of *labial*, *Deformed* and *engrailed* expression in the *Drosophila* head. *Development* 113, 273–281.
- Drewell, R.A., Bae, E., Burr, J., Lewis, E.B., 2002. Transcription defines the embryonic domains of cis-regulatory activity at the *Drosophila* *Bithorax* Complex. *Proc. Natl Acad. Sci. USA* 99, 16853–16858.
- Duncan, I.M., 1982. *Polycomblike*: a gene that appears to be required for the normal expression of the *Bithorax* and *Antennapedia* gene complexes of *Drosophila melanogaster*. *Genetics* 102, 49–70.
- Dura, J.M., Brock, H.W., Santamaria, P., 1985. *Polyhomeotic*: a gene of *Drosophila melanogaster* required for correct expression of segmental identity. *Mol. Gen. Genet.* 198, 213–220.
- Dushay, M.S., Rosbash, M., Hall, J.C., 1989. The disconnected visual system mutations in *Drosophila melanogaster* drastically disrupt circadian rhythms. *J. Biol. Rhythms* 4, 1–27.
- Ekker, S.C., Jackson, D.G., von Kessler, D.P., Sun, B.I., Young, K.E., et al., 1994. The degree of variation in DNA sequence recognition among four *Drosophila* homeotic proteins. *EMBO J.* 13, 3551–3560.
- Ekker, S.C., von Kessler, D.P., Beachy, P.A., 1992. Differential DNA sequence recognition is a determinant of specificity in homeotic gene action. *EMBO J.* 11, 4059–4072.
- Ekker, S.C., Young, K.E., von Kessler, D.P., Beachy, P.A., 1991. Optimal DNA sequence recognition by the *Ultrabithorax* homeodomain of *Drosophila*. *EMBO J.* 10, 1179–1186.
- Elstob, P.R., Brodu, V., Gould, A.P., 2001. *spalt*-dependent switching between two cell fates that are induced by the *Drosophila* EGF receptor. *Development* 128, 723–732.
- Eresh, S., Riese, J., Jackson, D.B., Bohmann, D., Bienz, M., 1997. A CREB-binding site as a target for *decapentaplegic* signaling during *Drosophila* endoderm development. *EMBO J.* 16, 2014–2022.
- Estrada, B., Sánchez-Herrero, E., 2001. The Hox gene *Abdominal-B* antagonizes appendage development in the genital disc of *Drosophila*. *Development* 128, 331–339.
- Falciani, F., Hausdorf, B., Schroder, R., Akam, M., Tautz, D., et al., 1996. Class 3 Hox genes in insects and the origin of zen. *Proc. Natl Acad. Sci. USA* 93, 8479–8484.
- Fasano, L., Röder, L., Coré, N., Alexandre, E., Vola, C., et al., 1991. The gene *teashirt* is required for the development of *Drosophila* embryonic trunk segments and encodes a protein with widely spaced zinc finger motifs. *Cell* 64, 63–79.
- Feinstein, P.G., Kornfeld, K., Hogness, D., Mann, R.S., 1995. Identification of homeotic target genes in *Drosophila melanogaster* including *nervy*, a proto-oncogene homologue. *Genetics* 140, 573–586.
- Ferrier, D.E., Akam, M., 1996. Organization of the Hox gene cluster in the grasshopper, *Schistocerca gregaria*. *Proc. Natl Acad. Sci. USA* 93, 13024–13029.
- Florence, B., Handrow, R., Laughon, A., 1991. DNA-binding specificity of the fushi tarazu homeodomain. *Mol. Cell. Biol.* 11, 3613–3623.
- FlyBase Consortium, 2003. The FlyBase database of the *Drosophila* genome projects and community literature. *Nucl. Acids Res.* 31, 172–175. <http://flybase.org/>.
- Fraenkel, E., Pabo, C.O., 1998. Comparison of X-ray and NMR structures for the Antennapedia homeodomain–DNA complex. *Nature Struct. Biol.* 5, 692–696.
- Fraenkel, E., Rould, M.A., Chambers, K.A., Pabo, C.O., 1998. *Engrailed* homeodomain–DNA complex at 2.2 Å resolution: a detailed view of the interface and comparison with other engrailed structures. *J. Mol. Biol.* 27, 351–361.
- Franke, A., DeCamillis, M., Zink, D., Cheng, N., Brock, H.W., et al., 1991. *Polycomb* and *polyhomeotic* are constituents of a multimeric protein complex in chromatin of *Drosophila melanogaster*. *EMBO J.* 11, 2941–2950.
- Frayne, E.G., Sato, R., 1991. The *Ultrabithorax* gene of *Drosophila* and the specification of abdominal histoblasts. *Devel. Biol.* 146, 265–277.

- Furukubo-Tokunaga, K., Flister, S., Gehring, W.J., 1993. Functional specificity of the *Antennapedia* homeodomain. *Proc. Natl Acad. Sci. USA* 90, 6360–6364.
- Galant, R., Walsh, C.M., Carroll, S.B., 2002. Hox repression of a target gene: extradenticle-independent, additive action through multiple monomer binding sites. *Development* 129, 3115–3126.
- Galloni, M., Gyurkovics, H., Shedl, P., Karch, F., 1993. The bluetail transposon: evidence for independent cis-regulatory domains and domain boundaries in the bithorax complex. *EMBO J.* 12, 1087–1097.
- Garber, R.I., Kuroiwa, A., Gehring, W.J., 1983. Genomic and cDNA clones of the homeotic locus *Antennapedia* in *Drosophila*. *EMBO J.* 2, 2027–2036.
- García-Bellido, A., 1977. Homoeotic and atavic mutations in insects. *Am. Zool.* 17, 613–629.
- Gebelein, B., Culi, J., Ryoo, H.D., Zhang, W., Mann, R.S., 2002. Specificity of *Distalless* repression and limb primordia development by abdominal Hox proteins. *Devel. Cell* 3, 487–498.
- Gehring, W.J., Affolter, M., Bürglin, T., 1994. Homeodomain proteins. *Annu. Rev. Biochem.* 63, 487–526.
- Gellon, G., Harding, K.W., McGinnis, N., Martin, M.M., McGinnis, W., 1997. A genetic screen for modifiers of *Deformed* homeotic function identifies novel genes required for head development. *Development* 124, 3321–3331.
- Gibson, G., Schier, A., LeMotte, P., Gehring, W.J., 1990. The specificities of *Sex combs reduced* and *Antennapedia* that includes the homeodomain. *Cell* 62, 1087–1103.
- Gindhart, J.G., Kaufman, T.C., 1995. Identification of *Polycomb* and *trithorax* group responsive elements in the regulatory region of the *Drosophila* homeotic gene *Sex combs reduced*. *Genetics* 139, 797–814.
- Glicksman, M.A., Brower, D.L., 1988. Expression of the *Sex combs reduced* protein in *Drosophila* larvae. *Devel. Biol.* 127, 113–118.
- González-Crespo, S., Morata, G., 1995. Control of *Drosophila* adult pattern by *extradenticle*. *Development* 121, 2117–2125.
- González-Reyes, A., Macías, A., Morata, G., 1992. Autocatalysis and phenotypic expression of *Drosophila* homeotic gene *Deformed*: its dependence on polarity and homeotic gene function. *Development* 116, 1059–1068.
- González-Reyes, A., Morata, G., 1990. The developmental effect of overexpressing a UBX product in *Drosophila* embryos is dependent on its interactions with other homeotic products. *Cell* 61, 515–522.
- Gould, A.P., Brookman, J.J., Strutt, D.I., White, R.A.H., 1990. Targets of homeotic gene control in *Drosophila*. *Nature* 348, 308–312.
- Gould, A.P., Morrison, A., Sproat, G., White, R.A., Krumlauf, R., 1997. Positive cross-regulation and enhancer sharing: two mechanisms for specifying overlapping Hox expression patterns. *Genes Devel.* 11, 900–913.
- Gould, A.P., White, R.A.H., 1992. *Connectin*, a target of homeotic gene control in *Drosophila*. *Development* 116, 1163–1174.
- Goutte, C., Johnson, A.D., 1993. Yeast $\alpha 1$ and $\alpha 2$ homeodomain proteins form a DNA-binding activity with properties distinct from those of either protein. *J. Mol. Biol.* 233, 359–371.
- Graba, Y., Aragnol, D., Laurenti, P., Garzino, V., Charnot, D., et al., 1992. Homeotic control in *Drosophila*: the *scabrous* gene is an *in vivo* target of Ultrabithorax proteins. *EMBO J.* 11, 3375–3384.
- Graba, Y., Aragnol, D., Pradel, J., 1997. *Drosophila* Hox complex downstream targets and the function of homeotic genes. *BioEssays* 19, 379–388.
- Graba, Y., Gieseler, K., Aragnol, D., Laurenti, P., Mariol, M.C., et al., 1995. *DWnt-4*, a novel *Drosophila Wnt* gene acts downstream of homeotic complex genes in the visceral mesoderm. *Development* 121, 209–218.
- Graba, Y., Laurenti, P., Perrin, L., Aragnol, D., Pradel, J., 1994. The modifier of variegation *modulo* gene acts downstream of dorsoventral and HOM-C genes and is required for morphogenesis in *Drosophila*. *Devel. Biol.* 166, 704–715.
- Grieder, N.C., Marty, T., Ryoo, H.D., Mann, R.S., Affolter, M., 1997. Synergistic activation of a *Drosophila* enhancer by HOM/EXD and DPP signaling. *EMBO J.* 16, 7402–7410.
- Gyurkovics, H., Gausz, J., Kummer, J., Karch, F., 1990. A new homeotic mutation in the *Drosophila* bithorax complex removes a boundary separating two domains of regulation. *EMBO J.* 9, 2579–2585.
- Haberman, A.S., Isaac, D.D., Andrew, D.J., 2003. Specification of cell fates within the salivary gland primordium. *Devel. Biol.* 258, 443–453.
- Hanes, S.D., Brent, R., 1989. DNA specificity of the bicoid activator protein is determined by homeodomain recognition helix residue 9. *Cell* 57, 1275–1283.
- Hanes, S.D., Brent, R., 1991. A genetic model for interaction of the homeodomain recognition helix with DNA. *Science* 251, 426–430.
- Hanes, S.D., Riddihough, G., Ish-Horowicz, D., Brent, R., 1994. Specific DNA recognition and intersite spacing are critical for action of the bicoid morphogen. *Mol. Cell. Biol.* 14, 3364–3375.
- Harding, K., Levine, M., 1988. Gap genes define the limits of *Antennapedia* and *Bithorax* gene expression during early development in *Drosophila*. *EMBO J.* 7, 205–214.
- Hayward, D.C., Patel, N.H., Rehm, E.J., Goodman, C.S., Ball, E.E., 1995. Sequence and expression of grasshopper *Antennapedia*: comparison to *Drosophila*. *Devel. Biol.* 172, 452–465.
- Hedrick, S.M., Cohen, D.I., Nielsen, E.A., Davis, M.M., 1984. Isolation of cDNA clones encoding T cell-specific membrane associated proteins. *Nature* 308, 149–153.
- Heilig, J.S., Freeman, M., Laverty, T., Lee, K.J., Campos, A.R., et al., 1991. Isolation and characterization of the *disconnected* gene of *Drosophila melanogaster*. *EMBO J.* 10, 809–815.
- Hinz, U., Wolk, A., Renkawitz-Pohl, R., 1992. Ultrabithorax is a regulator of $\beta 3$ tubulin expression in the *Drosophila* visceral mesoderm. *Development* 116, 543–554.

- Hirsch, J.A., Aggarwal, A.K., 1995. Structure of the even-skipped homeodomain complexed to AT-rich DNA: new perspectives on homeodomain specificity. *EMBO J.* 14, 6280–6291.
- Hirth, F., Hartmann, B., Reichert, H., 1998. Homeotic gene action in embryonic brain development of *Drosophila*. *Development* 125, 1579–1589.
- Hirth, F., Loop, T., Egger, B., Miller, D.F.B., Kaufman, T., et al., 2001. Functional equivalence of Hox gene products in the specification of the tritocerebrum during embryonic brain development of *Drosophila*. *Development* 128, 4781–4788.
- Hoey, T., Levine, M., 1988. Divergent homeo box proteins recognize similar DNA sequences in *Drosophila*. *Nature* 332, 858–861.
- Hoppler, S., Bienz, M., 1995. Two different thresholds of wingless signaling with distinct developmental consequences in the *Drosophila* midgut. *EMBO J.* 14, 5016–5026.
- Hovde, S., Abate-Shen, C., Geiger, J.H., 2001. Crystal structure of the Msx-1 homeodomain/DNA complex. *Biochemistry* 40, 12013–12021.
- Hughes, C., Kaufman, T.C., 2000. RNAi analysis of *Deformed*, *proboscipedia* and *Sex combs reduced* in the milkweed bug *Oncopeltus fasciatus*: novel roles for Hox genes in the hemipteran head. *Development* 127, 3683–3694.
- Hughes, C., Kaufman, T.C., 2002. Hox genes and the evolution of the arthropod body plan. *Evol. Devel.* 4, 459–499.
- Immerglück, A.K., Lawrence, P.A., Bienz, M., 1990. Induction across germ layers in *Drosophila* mediated by a genetic cascade. *Cell* 62, 261–268.
- Ingham, P., Whittle, R., 1980. *Trithorax*: a new homeotic mutation of *Drosophila melanogaster* causing transformations of abdominal and thoracic imaginal segments. *Mol. Gen. Genet.* 179, 607–614.
- Ingham, P.W., 1998. *trithorax* and the regulation of homeotic gene expression in *Drosophila*: a historical perspective. *Int. J. Devel. Biol.* 42, 423–429.
- Ingham, P.W., Martinez-Arias, A., 1986. The correct activation of *Antennapedia* and *Bithorax* complex genes requires the *fushi tarazu* gene. *Nature* 324, 592–597.
- Inoue, Y., Mito, T., Miyawaki, K., Matsushima, K., Shinmyo, Y., et al., 2002. Correlation of expression patterns of *homothorax*, *dachshund*, and *Distal-less* with the proximodistal segmentation of the cricket leg bud. *Mech. Devel.* 113, 141–148.
- Irving, P., Troxler, L., Heuer, T.S., Belvin, M., Kopczynski, C., et al., 2001. A genome-wide analysis of immune responses in *Drosophila*. *Proc. Natl. Acad. Sci. USA* 98, 15119–15124.
- Izpisua-Belmonte, J.C., Falkenstein, J.H., Dolle, P., Renucci, A., Duboule, D., 1991. Murine genes related to the *Drosophila Abd-B* homeotic gene are sequentially expressed during development of the posterior part of the body. *EMBO J.* 10, 2279–2289.
- Jack, T., McGinnis, W., 1990. Establishment of the *Deformed* expression stripe requires the combinatorial action of coordinate, gap and pair-rule proteins. *EMBO J.* 9, 1187–1198.
- Jack, T., Regulski, M., McGinnis, W., 1988. Pair-rule segmentation genes regulate the expression of the homeotic selector gene, *Deformed*. *Genes Devel.* 2, 635–651.
- Jacobs, J.J.L., van Lohuizen, M., 1999. Cellular memory of transcriptional states by Polycomb-group proteins. *Cell Devel. Biol.* 10, 227–235.
- Jaffe, L., Ryoo, H.D., Mann, R.S., 1997. A role for phosphorylation by casein kinase II in modulating *Antennapedia* activity in *Drosophila*. *Genes Devel.* 11, 1327–1340.
- Jaynes, J.B., O'Farrell, P.H., 1991. Active repression of transcription by the engrailed homeodomain protein. *EMBO J.* 10, 1427–1433.
- Jockusch, E.L., Nulsen, C., Newfeld, S.J., Nagy, L.M., 2000. Leg development in flies versus grasshoppers: differences in *dpp* expression do not lead to differences in the expression of downstream components of the leg patterning pathway. *Development* 127, 1617–1626.
- Johnson, F.B., Krasnow, M.A., 1990. Stimulation of transcription by an Ultrabithorax protein *in vitro*. *Genes Devel.* 4, 1044–1052.
- Johnson, F.B., Parker, E., Krasnow, M.A., 1995. Extradenticle protein is a selective cofactor for the *Drosophila* homeotics: role of the homeodomain and YPWM amino acid motif in the interaction. *Proc. Natl. Acad. Sci. USA* 92, 739–743.
- Jones, B., McGinnis, W., 1993. The regulation of empty spiracles by Abdominal-B mediates an abdominal segment identity function. *Genes Devel.* 7, 229–240.
- Jorgenson, E.M., Garber, R.L., 1987. Function and misfunction of the two promoters of the *Drosophila Antennapedia* genes. *Genes Devel.* 1, 544–555.
- Jürgens, G., Lehman, R., Scharding, M., Nüsslein-Volhard, C., 1986. Segmental organization of the head in the embryo of *Drosophila melanogaster*. *Roux's Arch. Devel. Biol.* 195, 359–377.
- Kadonaga, J., Carner, K., Masiarz, F., Tjian, R., 1987. Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. *Cell* 51, 1079–1090.
- Karch, F., Bender, W., Weiffenbach, B., 1990. *abd-A* expression in *Drosophila* embryos. *Genes Devel.* 4, 1573–1587.
- Kaufman, T.C., 1978. Cytogenetic analysis of chromosome 3 in *Drosophila melanogaster*: isolation and characterization of four new alleles of the *proboscipedia (pb)* locus. *Genetics* 90, 579–586.
- Kaufman, T.C., Lewis, R., Wakimoto, B., 1980. Cytogenetic analysis of chromosome 3 in *Drosophila melanogaster*: the homeotic gene complex in polytene chromosome interval 84 A–B. *Genetics* 94, 115–133.
- Kaufman, T.C., Seeger, M.A., Olsen, G., 1990. Molecular and genetic organization of the *Antennapedia* gene complex of *Drosophila melanogaster*. *Adv. Genet.* 27, 309–362.
- Kelsh, R., Dawson, I., Akam, M., 1993. An analysis of Abdominal-B expression in the locust *Schistocerca gregaria*. *Development* 117, 293–305.

- Kennison, J.A., 1993. Transcriptional activation of *Drosophila* homeotic genes from distant regulatory elements. *Trends Genet.* 9, 75–79.
- Kissinger, C.R., Liu, B.S., Martin-Blanco, E., Kornberg, T.B., Pabo, C.O., 1990. Crystal structure of an engrailed homeodomain-DNA complex at 2.8 Å resolution: a framework for understanding homeodomain-DNA interactions. *Cell* 63, 579–590.
- Kmita, M., Duboule, D., 2003. Organizing axes in time and space: 25 years of collinear tinkering. *Science* 301, 331–333.
- Knoepfler, P.S., Calvo, K.R., Chen, H., Antonarakis, S.E., Kamps, M.P., 1997. Meis1 and pKnox1 bind DNA cooperatively with Pbx1 utilizing an interaction surface disrupted in oncoprotein E2a-Pbx1. *Proc. Natl Acad. Sci. USA* 94, 14553–14558.
- Kondo, T., Duboule, D., 1999. Breaking collinearity in the mouse HoxD complex. *Cell* 97, 407–417.
- Kopp, A., Duncan, I., 2002. Anteroposterior patterning in adult abdominal segments of *Drosophila*. *Devel. Biol.* 242, 15–30.
- Krasnow, M.A., Saffman, E.E., Kornfeld, K., Hogness, D.S., 1989. Transcriptional activation and repression by *Ultrabithorax* proteins in cultured *Drosophila* cells. *Cell* 57, 1031–1043.
- Kuhn, D.T., Turenchalk, G., Mack, J.A., Packert, G., Kornberg, T.B., 1995. Analysis of the genes involved in organizing the tail segments of the *Drosophila melanogaster* embryo. *Mech. Devel.* 53, 3–13.
- Kuzin, B., Tillib, S., Sedkov, Y., Mizrokhi, L., Mazo, A., 1994. The *Drosophila trithorax* gene encodes a chromosomal protein and directly regulates the region-specific homeotic gene *forkhead*. *Genes Devel.* 8, 2478–2490.
- Kuziora, M.A., McGinnis, W., 1988a. Different transcripts of the *Drosophila Abd-B* gene correlate with distinct genetic sub-functions. *EMBO J.* 7, 3233–3244.
- Kuziora, M.A., McGinnis, W., 1988b. Autoregulation of a *Drosophila* homeotic selector gene. *Cell* 55, 477–485.
- Kuziora, M.A., McGinnis, W., 1989. A homeodomain substitution changes the regulatory specificity of the *Deformed* protein in *Drosophila* embryos. *Cell* 59, 563–571.
- Kuziora, M.A., McGinnis, W., 1991. Altering the regulatory targets of the *Deformed* protein in *Drosophila* embryos by substituting the *Abdominal-B* homeodomain. *Mech. Devel.* 33, 83–94.
- Laughon, A., Scott, M.P., 1984. Sequence of a *Drosophila* segmentation gene: protein structure homology with DNA binding proteins. *Nature* 310, 25–31.
- Laughon, A., Howell, W., Scott, M.P., 1988. The interaction of proteins encoded by *Drosophila* homeotic and segmentation genes with specific DNA sequences. *Development* 104 (Suppl.), 75–83.
- Levine, M., Hafen, E., Garber, R.L., Gehring, W.J., 1983. Spatial distribution of *Antennapedia* transcripts during *Drosophila* development. *EMBO J.* 2, 2037–2046.
- Lewis, D.L., DeCamillis, M., Bennett, R.L., 2000. Distinct roles of the homeotic genes *Ubx* and *abd-A* in beetle embryonic abdominal appendage development. *Proc. Natl Acad. Sci. USA* 97, 4504–4509.
- Lewis, E.B., 1978. A gene complex controlling segmentation in *Drosophila*. *Nature* 276, 565–570.
- Lewis, E.B., 1998. The bithorax complex: the first fifty years. *Int. J. Devel. Biol.* 42, 403–415.
- Lewis, R.A., Kaufman, T.C., Denell, R.E., Tallero, P., 1980a. Genetic analysis of the *Antennapedia* gene complex (*ANT-C*) and adjacent chromosomal regions of *Drosophila melanogaster*. 1. Polytene chromosome segments 84B-D. *Genetics* 95, 367–381.
- Lewis, R.A., Wakimoto, B.T., Denell, R.E., Kaufman, T.C., 1980b. Genetic analysis of the *Antennapedia* gene complex (*ANT-C*) and adjacent chromosomal regions of *Drosophila melanogaster* 2. Polytene chromosome segments 84A-84B1, 2. *Genetics* 95, 383–397.
- Li, X., Murre, C., McGinnis, W., 1999a. Activity regulation of a Hox protein and a role for the homeodomain in inhibiting transcriptional activation. *EMBO J.* 18, 198–211.
- Li, X., Veraksa, A., McGinnis, W., 1999b. A sequence motif distinct from Hox binding sites controls the specificity of a Hox response element. *Development* 126, 5581–5589.
- Lin, L., McGinnis, W., 1992. Mapping functional specificity in the *Dfd* and *Ubx* homeo domains. *Genes Devel.* 6, 1071–1081.
- Liu, J., Fire, A., 2000. Overlapping roles of two Hox genes and the *exd* ortholog *ceb-20* in diversification of the *C. elegans* postembryonic mesoderm. *Development* 127, 5179–5190.
- Lo, P.C.H., Skeath, J.B., Gajewski, K., Schulz, R.A., Frasch, M., 2002. Homeotic genes autonomously specify the anteroposterior subdivision of the *Drosophila* dorsal vessel into aorta and heart. *Devel. Biol.* 251, 307–319.
- Lohmann, I., McGinnis, N., Bodmer, M., McGinnis, W., 2002. The *Drosophila* Hox gene *Deformed* sculpts head morphology via direct regulation of the apoptosis activator *reaper*. *Cell* 110, 457–466.
- Lonie, A., D'Andrea, R., Paro, R., Saint, R., 1994. Molecular characterization of the *Polycomblike* gene of *Drosophila melanogaster*, a *trans*-acting negative regulator of homeotic gene expression. *Development* 120, 2629–2636.
- Lou, L., Bergson, C., McGinnis, W., 1995. *Deformed* expression in the *Drosophila* central nervous system is controlled by an autoactivated intronic enhancer. *Nucl. Acids Res.* 23, 3481–3487.
- Lovato, T.L., Nguyen, T.P., Molina, M.R., Cripps, R.M., 2002. The Hox gene *abdominal-A* specifies heart cell fate in the *Drosophila* dorsal vessel. *Development* 129, 5019–5027.
- Macías, A., Casanova, J., Morata, G., 1990. Expression and regulation of the *abd-A* gene of *Drosophila*. *Development* 110, 1197–1207.
- Macías, A., Morata, G., 1996. Functional hierarchy and phenotypic suppression among *Drosophila* homeotic genes: the *labial* and *empty spiracles* genes. *EMBO J.* 15, 334–343.

- Mahaffey, J.W., Diederich, R.J., Kaufman, T.C., 1989. Novel patterns of homeotic protein accumulation in the head of the *Drosophila* embryo. *Development* 105, 167–174.
- Mahaffey, J.W., Griswold, C.M., Cao, Q., 2001. The *Drosophila* genes *disconnected* and *disco-related* are redundant with respect to larval head development and accumulation of mRNAs from *Deformed* target genes. *Genetics* 157, 225–236.
- Mahaffey, J.W., Jones, D.F., Hickel, J., Griswold, C.M., 1993. Identification and characterization of a gene activated by the *Deformed* homeoprotein. *Development* 118, 203–214.
- Mahaffey, J.W., Kaufman, T.C., 1987. Distribution of the *Sex combs reduced* gene products in *Drosophila melanogaster*. *Genetics* 117, 51–60.
- Mak, A., Johnson, A.D., 1993. The carboxy-terminal tail of the homeo domain protein alpha 2 is required for function with a second homeo domain protein. *Genes Devel.* 7, 1862–1870.
- Mann, R.S., Chan, S., 1996. Extra specificity from extradenticle: the partnership between HOX and PBX;EXD homeodomain proteins. *Trends Genet.* 12, 258–262.
- Marchetti, M., Fanti, L., Berioco, M., Pimpinelli, S., 2003. Differential expression of the *Drosophila BX-C* in polytene chromosomes in cells of the larval fat bodies: a cytological approach to identifying *in vivo* targets of the homeotic Ubx, Abd-A and Abd-B proteins. *Development* 130, 3683–3689.
- Markstein, M., Markstein, P., Markstein, V., Levine, M.S., 2002. Genome-wide analysis of clustered Dorsal binding sites identifies putative target genes in the *Drosophila* embryo. *Proc. Natl Acad. Sci. USA* 99, 763–768.
- Martin, C.H., Mayeda, C.A., Davis, C.A., Ericsson, C.L., Knafels, J.D., *et al.*, 1995. Complete sequence of the bithorax complex of *Drosophila*. *Proc. Natl Acad. Sci. USA* 92, 8398–8402.
- Martin, E.C., Adler, P.N., 1993. The Polycomb group gene Posterior Sex Combs encodes a chromosomal protein. *Development* 117, 641–655.
- Martinez, P., Amemiya, C.T., 2002. Genomics of the HOX gene cluster. *Comp. Biochem. Physiol. B* 133, 571–580.
- Martinez-Arias, A., Ingham, P.W., Scott, M.P., Akam, M.E., 1987. The spatial and temporal deployment of *Dfd* and *Scr* transcripts throughout development of *Drosophila*. *Development* 100, 673–683.
- Martinez-Arias, A., Lawrence, P.A., 1985. Parasegments and compartments in the *Drosophila* embryo. *Nature* 313, 639–642.
- Martinez-Arias, A., White, R.A.H., 1988. *Ultrabithorax* and *engrailed* expression in *Drosophila* embryos mutant for segmentation genes of the pair-rule class. *Development* 102, 325–338.
- Mastick, G.S., McKay, R., Oligino, T., Donovan, K., Lopez, A.J., 1995. Identification of target genes regulated by homeotic proteins in *Drosophila melanogaster* through genetic selection of Ultrabithorax protein binding sites in yeast. *Genetics* 139, 349–363.
- Mathies, L.D., Kerridge, S., Scott, M.P., 1994. Role of the *teashirt* gene in *Drosophila* midgut morphogenesis: secreted proteins mediate the action of homeotic genes. *Development* 120, 2799–2809.
- Mathog, D.R., 1991. Suppression of abdominal legs in *Drosophila melanogaster*. *Roux's Arch Devel. Biol.* 199, 449–457.
- Maurel-Zaffran, C., Chauvet, S., Jullien, N., Miassod, R., Pradel, J., *et al.*, 1999. *nessy*, an evolutionary conserved gene controlled by Hox proteins during *Drosophila* embryogenesis. *Mech. Devel.* 86, 159–163.
- McCall, K., O'Connor, M. B., Bender, W., 1994. Enhancer traps in the *Drosophila* bithorax complex mark parasegmental domains. *Genetics* 138, 389–399.
- McCormick, A., Coré, N., Kerridge, S., Scott, M.P., 1995. Homeotic response elements are tightly linked to tissue-specific elements in a transcriptional enhancer of the *teashirt* gene. *Development* 121, 2799–2812.
- McDonald, M.J., Rosbash, M., 2001. Microarray analysis and organization of circadian gene expression in *Drosophila*. *Cell* 107, 567–578.
- McGinnis, N., Kuziora, M.A., McGinnis, W., 1990. Human *Hox-4.2* and *Drosophila Deformed* encode similar regulatory specificities in *Drosophila* embryos and larvae. *Cell* 63, 969–976.
- McGinnis, W., Levine, M.S., Hafen, E., Kuroiwa, A., Gehring, W.J., 1984. A conserved DNA sequence in homeotic genes of the *Drosophila* Antennapedia and bithorax complexes. *Nature* 308, 428–433.
- McGinnis, N., Ragnhildstveit, E., Veraksa, A., McGinnis, W., 1998. A cap'n'collar protein isoform contains a selective Hox repressor function. *Development* 125, 4553–4564.
- McGinnis, W., Garber, R.L., Wirz, J., Kuroiwa, A., Gehring, W.J., 1984b. A homologous protein-coding sequence in *Drosophila* homeotic genes and its conservation in other metazoans. *Cell* 37, 403–408.
- McGinnis, W., Hart, C.P., Gehring, W.J., Ruddle, F.H., 1984a. Molecular cloning and chromosome mapping of a mouse DNA sequence homologous to homeotic genes of *Drosophila*. *Cell* 38, 675–680.
- McGinnis, W., Levine, M.S., Hafen, E., Kuroiwa, A., Gehring, W.J., 1984c. A conserved DNA sequence in homeotic genes of the *Drosophila* Antennapedia and bithorax complexes. *Nature* 308, 428–433.
- Megraw, T.L., Li, K., Kao, L.R., Kaufman, T.C., 1999. The centrosomin protein is required for centrosome assembly and function during cleavage in *Drosophila*. *Development* 126, 2829–2839.
- Merabet, S., Catala, F., Pradel, J., Graba, Y., 2002. A green fluorescent protein reporter genetic screen that identifies modifiers of Hox gene function in the *Drosophila* embryo. *Genetics* 162, 189–202.
- Merabet, S., Kambris, Z., Capovilla, M., Bérenger, H., Pradel, J., *et al.*, 2003. The hexapeptide and linker regions of the AbdA Hox protein regulate its activation and repressive functions. *Devel. Cell* 4, 761–768.
- Merrill, V.K.L., Diederich, R.J., Turner, F.R., Kaufman, T.C., 1989. A genetic and developmental analysis of

- mutations in *labial*, a gene necessary for proper head formation in *Drosophila melanogaster*. *Devel. Biol.* 135, 376–391.
- Merrill, V.K.L., Turner, F.R., Kaufman, T.C., 1987. A genetic and developmental analysis of mutations in the *Deformed* locus in *Drosophila melanogaster*. *Devel. Biol.* 122, 379–395.
- Mihaly, J., Hogga, I., Barges, S., Galloni, M., Mishra, R.K., *et al.*, 1998. Chromatin domain boundaries in the Bithorax complex. *Cell. Mol. Life Sci.* 54, 60–70.
- Mihaly, J., Hogga, I., Gausz, J., Gyurkovics, H., Karch, F., 1997. *In situ* detection of the *Fab-7* region of the bithorax complex into a chromatin domain boundary and a *Polycomb*-response element. *Development* 124, 1809–1820.
- Miller, D.F.B., Holtzman, S.L., Kalkbrenner, A.L., Kaufman, T., 2001a. Homeotic complex (Hox) gene regulation and homeosis in the mesoderm of the *Drosophila melanogaster* embryo: the roles of signal transduction and cell autonomous regulation. *Mech. Devel.* 102, 17–32.
- Miller, D.F.B., Rogers, B.T., Kalkbrenner, A.L., Hamilton, B., Holtzman, S.L., *et al.*, 2001b. Cross-regulation of Hox genes in the *Drosophila melanogaster* embryo. *Mech. Devel.* 102, 3–16.
- Mlodzik, M., Fjose, A., Gehring, W.J., 1988. Molecular structure and spatial expression of a homeobox gene from the labial region of the Antennapedia-complex. *EMBO J.* 7, 2569–2578.
- Mohler, J., Mahaffey, J.W., Deutsch, E., Vani, K., 1995. Control of *Drosophila* head segment identity by the bZip homeotic gene *cnc*. *Development* 121, 237–247.
- Morata, G., 2001. How *Drosophila* appendages develop. *Nature Rev. Mol. Cell. Biol.* 2, 89–97.
- Morata, G., Kerridge, S., 1981. Sequential functions of the bithorax complex of *Drosophila*. *Nature* 290, 778–781.
- Moskow, J., Bullrich, F., Huebner, K., Daar, I., Buchberg, A., 1995. Meis1, a PBX1-related homeobox gene involved in myeloid leukemia in BXH-2 mice. *Mol. Cell. Biol.* 15, 5434–5443.
- Müller, J., 2000. Transcriptional control: the benefits of selective insulation. *Curr. Biol.* 10, R241–R244.
- Müller, J., Gaunt, S., Lawrence, P., 1995. Function of the Polycomb protein is conserved in mice and flies. *Development* 121, 2847–2852.
- Müller, M., Affolter, M., Leupin, W., Otting, G., Wüthrich, K., *et al.*, 1988. Isolation and sequence-specific DNA binding of the Antennapedia homeodomain. *EMBO J.* 7, 4299–4304.
- Nagata, R., Suzuki, Y., Ueno, K., Kokubo, H., Xu, X., *et al.*, 1996. Developmental expression of the *Bombyx* Antennapedia homologue and homeotic changes in the Nc mutant. *Genes Cells* 1, 555–568.
- Nagy, L.M., Carroll, S., 1994. Conservation of *wingless* patterning functions in the short germ embryos of *Tribolium castaneum*. *Nature* 367, 460–463.
- Negre, B., Ranz, J.M., Casals, F., Caceres, M., Ruiz, A., 2003. A new split of the *Hox* gene complex in *Drosophila*: relocation and evolution of the gene *labial*. *Mol. Biol. Evol.* 20, 2042–2054.
- Nelson, H.B., Laughon, A., 1990. The DNA binding specificity of the *Drosophila* fushi tarazu protein: a possible role for DNA bending in homeodomain recognition. *New Biol.* 2, 171–178.
- Nie, W., Stronach, B., Panganiban, G., Shippy, T., Brown, S., *et al.*, 2001. Molecular characterization of *Tclabial* and the 3' end of the *Tribolium* homeotic complex. *Devel. Genes Evol.* 211, 244–251.
- O'Conner, M.B., Binary, R., Perkins, L.A., Bender, W., 1988. Alternative RNA products from the *Ultra-bithorax* domain of the bithorax complex. *EMBO J.* 7, 435–445.
- O'Hara, E., Cohen, B., Cohen, S.M., McGinnis, W., 1993. *Distal-less* is a downstream gene of *Deformed* required for ventral maxillary identity. *Development* 117, 847–856.
- Ohkuma, Y., Horikoshi, M., Roeder, R.G., Desplan, C., 1990. Binding site-dependent direct activation and repression of *in vitro* transcription by *Drosophila* homeodomain proteins. *Cell* 61, 475–484.
- Otting, G., Qian, Y.Q., Billeter, M., Müller, M., Affolter, M., *et al.*, 1990. Protein–DNA contacts in the structure of a homeodomain–DNA complex determined by nuclear magnetic resonance spectroscopy in solution. *EMBO J.* 9, 3085–3092.
- Pankratz, M.J., Jaeckle, H., 1990. Making stripes in the *Drosophila* embryo. *Trends Genet.* 6, 287–292.
- Panzer, S., Weigel, D., Beckendorf, S.K., 1992. Organogenesis in *Drosophila melanogaster*: embryonic salivary gland determination is controlled by homeotic and dorsoventral patterning genes. *Development* 114, 49–57.
- Paro, R., Zink, B., 1992. The *Polycomb* gene is differentially regulated during oogenesis and embryogenesis of *Drosophila melanogaster*. *Mech. Devel.* 40, 37–46.
- Passner, J.M., Ryoo, H.D., Shen, L., Mann, R.S., Aggarwal, A., 1999. Structure of a DNA-bound Ultrabithorax–Extradenticle homeodomain complex. *Nature* 397, 714–719.
- Patel, N.H., 1994. Developmental evolution: insights from studies of insect segmentation. *Science* 266, 581–590.
- Patel, N.H., Ball, E.E., Goodman, C.S., 1992. Changing role of *even-skipped* during the evolution of insect pattern formation. *Nature* 357, 339–342.
- Patel, N.H., Condrón, B.G., Zinn, K., 1994. Pair-rule expression patterns of *even-skipped* are found in both short and long-germ beetles. *Nature* 367, 429–434.
- Pattatucci, A.M., Kaufman, T.C., 1991. The homeotic gene *Sex combs reduced* of *Drosophila melanogaster* is differentially regulated in the embryonic and imaginal stages of development. *Genetics* 129, 443–461.
- Pattatucci, A.M., Otterson, D.C., Kaufman, T.C., 1991. A functional and structural analysis of the *Sex combs reduced* locus of *Drosophila melanogaster*. *Genetics* 129, 423–441.

- Pederson, J.A., LaFollette, J.W., Gross, V. A., McGinnis, W., Mahaffey, J.W., 2000. Regulation by homeoproteins: a comparison of deformed-responsive elements. *Genetics* 156, 677–686.
- Pederson, J.D., Kiehart, D.P., Mahaffey, J.W., 1996. The role of HOM-C genes in segmental transformations: reexamination of the *Drosophila Sex combs reduced* embryonic phenotype. *Devel. Biol.* 180, 131–142.
- Peifer, M., Wieschaus, E., 1990. Mutations in the *Drosophila* gene *extradenticle* affect the way specific homeo domain proteins regulate segmental identity. *Genes Devel.* 4, 1209–1223.
- Pelaz, S., Urquía, N., Morata, G., 1993. Normal and ectopic domains of the homeotic gene *Sex combs reduced* of *Drosophila*. *Development* 117, 917–923.
- Percival-Smith, A., Müller, M., Affolter, M., Gehring, W.J., 1990. The interaction with DNA of wild-type and mutant *fushi tarazu* homeodomains. *EMBO J.* 9, 3967–3974.
- Percival-Smith, A., Weber, J., Gilfoyle, E., Wilson, P., 1997. Genetic characterization of the role of the two HOX proteins, Proboscipedia and Sex Combs Reduced, in determination of adult antennal tarsal, maxillary palp, and proboscis identities in *Drosophila melanogaster*. *Development* 124, 5049–5062.
- Peterson, M.D., Rogers, B.T., Popadić, A., Kaufman, T.C., 1999. The embryonic expression pattern of *labial*, posterior homeotic complex genes and the *teashirt* homologue in an apterygote insect. *Devel. Genes Evol.* 209, 77–90.
- Phelan, M.L., Rambaldi, I., Featherstone, M.S., 1995. Cooperative interactions between Hox and PBX proteins mediated by a conserved peptide motif. *Mol. Cell. Biol.* 15, 3989–3997.
- Pinsonneault, J., Florence, B., Vaessin, H., McGinnis, W., 1997. A model for extradenticle function as a switch that changes HOX proteins from repressors to activators. *EMBO J.* 16, 2032–2042.
- Pöpperl, H., Bienz, M., Studer, M., Chan, S., Aparicio, S., et al., 1995. Segmental expression of *Hoxb-1* is controlled by a highly conserved autoregulatory loop dependent upon *exd/pbx*. *Cell* 81, 1031–1042.
- Powers, T.P., Hogan, J., Ke, Z., Dymbrowski, K., Wang, X., et al., 2000. Characterization of the Hox cluster from the mosquito *Anopheles gambiae* (Diptera: Culicidae). *Evol. Devel.* 2, 311–325.
- Pribyl, L.G., Watson, D.K., McWilliams, M.J., Ascione, R., Papas, T.S., 1988. The *Drosophila ets-2* gene: molecular structure, chromosomal location, and developmental expression. *Devel. Biol.* 127, 45–53.
- Pultz, M.A., Diederich, R.J., Cribbs, D.L., Kaufman, T.C., 1988. The *proboscipedia* locus of the Antennapedia Complex: a molecular and genetic analysis. *Genes Devel.* 2, 901–920.
- Qian, S., Capovilla, M., Pirrotta, V., 1993. Molecular mechanisms of pattern formation by the BRE enhancer of the *Ubx* gene. *EMBO J.* 12, 3865–3877.
- Qian, Y.Q., Billeter, M., Otting, G., Müller, M., Gehring, W.J., et al., 1989. The structure of the Antennapedia homeodomain determined by NMR spectroscopy in solution: comparison with prokaryotic repressors. *Cell* 59, 573–580.
- Qian, Y.Q., Otting, G., Furukubo-Tokunaga, K., Affolter, M., Gehring, W.J., et al., 1992. NMR structure determination reveals that the homeodomain is connected through a flexible linker to the main body in the *Drosophila* Antennapedia protein. *Proc. Natl Acad. Sci. USA* 89, 10738–10742.
- Qian, Y.Q., Resendez-Perez, D., Gehring, W.H., Wüthrich, K., 1994. The des(1–6)Antennapedia homeodomain: comparison of the NMR solution structure and the DNA-binding affinity with the intact Antennapedia homeodomain. *Proc. Natl Acad. Sci. USA* 91, 4091–4095.
- Quinn, T.C., Craig, G.B., Jr, 1971. Phenogenetics of the homeotic mutant *proboscipedia* in *Aedes albopictus*. *J. Hered.* 62, 3–12.
- Rauskolb, C., Peifer, M., Wieschaus, E., 1993. *extradenticle*, a regulator of homeotic gene activity, is a homologue of the homeobox-containing human proto-oncogene *pbx1*. *Cell* 74, 1101–1112.
- Rauskolb, C., Smith, K.M., Peifer, M., Wieschaus, E., 1995. *extradenticle* determines segmental identities throughout *Drosophila* development. *Development* 121, 2117–2125.
- Rauskolb, C., Wieschaus, E., 1994. Coordinate regulation of downstream genes by extradenticle and the homeotic selector proteins. *EMBO J.* 13, 3561–3569.
- Regulski, M., Dessain, S., McGinnis, N., McGinnis, W., 1991. High-affinity binding sites for the *Deformed* protein are required for the function of an autoregulatory enhancer of the *Deformed* gene. *Genes Devel.* 5, 278–286.
- Regulski, M., Harding, K., Kostriken, R., Karch, F., Levine, M., et al., 1985. Homeo box genes of the Antennapedia and Bithorax complexes of *Drosophila*. *Cell* 43, 71–80.
- Regulski, M., McGinnis, N., Chadwick, R., McGinnis, W., 1987. Developmental and molecular analysis of *Deformed*, a homeotic gene controlling *Drosophila* head development. *EMBO J.* 6, 767–777.
- Reintz, J., Levine, M., 1990. Control of the initiation of homeotic gene expression by the gap genes giant and tailless in *Drosophila*. *Devel. Biol.* 140, 57–72.
- Reuter, R., Panganiban, G.E.F., Hoffman, F.M., Scott, M.P., 1990. Homeotic genes regulate the spatial expression of putative growth factors in the visceral mesoderm of *Drosophila* embryos. *Development* 110, 1031–1040.
- Reuter, R., Scott, M.P., 1990. Expression and function of the homeotic genes *Antennapedia* and *Sex combs reduced* in the embryonic midgut of *Drosophila*. *Development* 109, 289–303.
- Rieckhof, G.E., Casares, F., Ryoo, H.D., Abu-Shaar, M., Mann, R.S., 1997. Nuclear translocation of *extradenticle* requires *homothorax*, which encodes an *extradenticle*-related homeodomain protein. *Cell* 91, 171–183.
- Riley, P.D., Carroll, S.B., Scott, M.P., 1987. The expression and regulation of Sex combs reduced protein in *Drosophila* embryos. *Genes Devel.* 1, 716–730.

- Robertson, L.K., Bowling, D.B., Mahaffey, J.P., Imiolczyk, B., Mahaffey, J.W., 2004. An interactive network of zinc-finger proteins contributes to regionalization of the *Drosophila* embryo and establishes the domains of HOM-C protein function. *Development* 131, 2781–2789.
- Röder, L., Vola, C., Kerridge, S., 1992. The role of the *teashirt* gene in trunk segmental identity in *Drosophila*. *Development* 115, 1017–1033.
- Rogers, B.T., Kaufman, T.C., 1997. Structure of the insect head in ontogeny and phylogeny: a view from *Drosophila*. *Int. Rev. Cytol.* 74, 1–84.
- Rogers, B.T., Peterson, M.D., Kaufman, T.C., 1997. Evolution of the insect body plan as revealed by the *Sex combs reduced* expression pattern. *Development* 124, 149–157.
- Rogers, B.T., Peterson, M.D., Kaufman, T.C., 2002. The development and evolution of insect mouthparts as revealed by the expression patterns of gnathocephalic genes. *Evol. Devel.* 4, 96–110.
- Ronshaugen, M., McGinnis, N., McGinnis, W., 2002. Nature. Hox protein mutation and macroevolution of the insect body plan. *Nature* 415, 914–917.
- Rusch, D.B., Kaufman, T.C., 2000. Regulation of *proboscipedia* in *Drosophila* by homeotic selector genes. *Genetics* 156, 183–194.
- Rusten, T. E., Cantera, R., Urban, J., Technau, G., Kafatos, F. C., et al., 2001. *Spalt* restricts EGFR mediated induction of chordotonal precursors in the embryonic PNS of *Drosophila*. *Development* 128, 711–722.
- Ruvkun, G., Hobert, O., 1998. The taxonomy of developmental control in *Caenorhabditis elegans*. *Science* 282, 2033–2041.
- Ryoo, H.D., Mann, R.S., 1999. The control of trunk Hox specificity and activity by Extradenticle. *Genes Devel.* 13, 1704–1716.
- Ryoo, H.D., Marty, T., Casares, F., Affolter, M., Mann, R.S., 1999. Regulation of Hox target genes by a DNA bound Homothorax/Hox/Extradenticle complex. *Development* 126, 5137–5148.
- Samson, M., Jackson-Grusby, L., Brent, R., 1989. Gene activation and DNA binding by *Drosophila* Ubx and Abd-A proteins. *Cell* 57, 1045–1052.
- Sánchez-Herrero, E., Akam, M., 1989. Spatially ordered transcription of regulatory DNA in the bithorax complex of *Drosophila*. *Development* 107, 321–329.
- Sánchez-Herrero, E., Vernós, R.M., Morata, G., 1985. Genetic organization of *Drosophila* bithorax complex. *Nature* 313, 108–113.
- Sato, R., Hayes, P.H., Denell, R.E., 1985. Homeosis in *Drosophila*: roles and spatial patterns of expression of the *Antennapedia* and *Sex combs reduced* loci in embryogenesis. *Devel. Biol.* 111, 171–192.
- Schneuwly, S., Kuroiwa, A., Baumgartner, P., Gehring, W.J., 1986. Structural organization and sequence of the homeotic gene *Antennapedia* of *Drosophila melanogaster*. *EMBO J.* 5, 733–739.
- Schöck, F., Reischl, J., Wimmer, E., Taubert, H., Purnell, B.A., et al., 2000. Phenotypic suppression of *empty spiracles* is prevented by *buttonhead*. *Nature* 405, 351–354.
- Scott, M.P., Weiner, A.J., 1985. Structural relationships among genes that control development: sequence homology between the *Antennapedia*, *Ultrabithorax* and *fushi tarazu* loci of *Drosophila*. *Proc. Natl Acad. Sci. USA* 81, 4115–4119.
- Scott, M.P., Weiner, A.J., Hazelrigg, T.I., Polisky, B.A., Pirrotta, V., et al., 1983. The molecular organization of the *Antennapedia* locus of *Drosophila*. *Cell* 35, 763–776.
- Sedkov, Y., Tillib, S., Mizrokhi, L., Mazo, A., 1994. The bithorax complex is regulated by *trithorax* earlier during *Drosophila* embryogenesis than is the Antennapedia complex, correlating with a bithorax like expression pattern of distinct early *trithorax* transcripts. *Development* 120, 1907–1917.
- Sharpe, J., Nonchev, S., Gould, A., Whiting, J., Krumlauf, R., 1998. Selectivity, sharing and competitive interactions in the regulation of *Hoxb* genes. *EMBO J.* 17, 1788–1798.
- Shen, W.F., Rozenfeld, S., Lawrence, H.J., Largman, C., 1997. The Abd-B-like Hox homeodomain proteins can be subdivided by the ability to form complexes with Pbx1a on a novel DNA target. *J. Biol. Chem.* 272, 8198–8206.
- Shimell, M.J., Peterson, A.J., Burr, J., Simon, J.A., O'Connor, M.B., 2000. Functional analysis of repressor binding sites in the *iab-2* regulatory region of the *abdominal-A* homeotic gene. *Devel. Biol.* 218, 38–52.
- Shimell, M.J., Simon, J., Bender, W., O'Connor, M.B., 1994. Enhancer point mutation results in a homeotic transformation in *Drosophila*. *Science* 264, 968–971.
- Shippy, T.D., Brown, S.J., Denell, R.E., 1998. Molecular characterization of the *Tribolium abdominal-A* ortholog and implications for the products of the *Drosophila* gene. *Devel. Genes Evol.* 207, 446–452.
- Shippy, T.D., Guo, J., Brown, S.J., Beeman, R.W., Denell, R.E., 2000. Analysis of *maxillopedia* expression pattern and larval cuticular phenotype in wild-type and mutant *Tribolium*. *Genetics* 155, 721–731.
- Simon, J., 1995. Locking in stable states of gene expression: transcriptional control during *Drosophila* development. *Curr. Opin. Cell. Biol.* 7, 376–385.
- Simon, J., Chiang, A., Bender, W., 1992. Ten different *Polycomb* group genes are required for spatial control of the *abdA* and *AbdB* homeotic products. *Development* 114, 493–505.
- Simon, J., Chiang A., Bender W., Shimell M.J., O'Connor M., 1995. Elements of the *Drosophila* Bithorax complex that mediate repression by *Polycomb* group products. *Devel. Biol.* 158, 131–144.
- Simon, J., Peifer, M., Bender, W., O'Connor, M., 1990. Regulatory elements of the Bithorax complex that control expression along the anterior–posterior axis. *EMBO J.* 9, 3945–3956.

- Simon, J. A., Tamkun, J. W., 2002. Programming off and on states in chromatin: mechanisms of Polycomb and trithorax group complexes. *Curr. Opin. Genet. Devel.* 12, 210–218.
- Slack, J., 1984. A Rosetta stone for pattern formation in animals? *Nature* 310, 364–365.
- Small, S., Levine, M., 1991. The initiation of pair-rule stripes in the *Drosophila* blastoderm. *Curr. Opin. Genet. Devel.* 1, 255–260.
- Smolik-Utlaut, S.M., 1990. Dosage requirements of *Ultrabithorax* and *bithoraxoid* in the determination of segment identity in *Drosophila melanogaster*. *Genetics* 124, 357–366.
- Sommer, R.J., Tautz, D., 1993. Involvement of an orthologue of the *Drosophila* pair-rule gene *hairy* in segment formation of the short germ-band embryo of *Tribolium* (Coleoptera). *Nature* 361, 448–450.
- Sprules, T., Green, N., Featherstone, M., Gehring, K., 2003. Lock and key binding of the HOX YPWM peptide to the PBX Homeodomain. *J. Biol. Chem.* 278, 1053–1058.
- St. Johnston, D., Nüsslein-Volhard, C., 1991. The origin of pattern and polarity in the *Drosophila* embryo. *Cell* 68, 201–219.
- Staehling-Hampton, K., Hoffmann, F.M., Baylies, M.K., Rushton, E., Bate, M., 1994. *dpp* induces mesodermal gene expression in *Drosophila*. *Nature* 372, 783–786.
- Stathopoulos, A., Van Drenth, M., Erives, A., Markstein, M., Levine, M., 2001. Whole-genome analysis of dorsal–ventral patterning in the *Drosophila* embryo. *Cell* 111, 687–701.
- Stauber, M., Jackle, H., Schmidt-Ott, U., 1999. The anterior determinant bicoid of *Drosophila* is a derived *Hox* class 3 gene. *Proc. Natl Acad. Sci. USA* 96, 3786–3789.
- Steller, H., Fischbach, K.F., Rubin, G.M., 1987. *Disconnected*: a locus required for neuronal pathway formation in the visual system of *Drosophila*. *Cell* 50, 1139–1153.
- Struhl, G., 1981. A homeotic mutation transforming leg to antenna in *Drosophila*. *Nature* 13, 635–638.
- Struhl, G., 1982. Genes controlling segmental specification in the *Drosophila* thorax. *Proc. Natl Acad. Sci. USA* 79, 7380–7384.
- Struhl, G., White, R.A.H., 1985. Regulation of the *Ultrabithorax* gene of *Drosophila* by the other bithorax complex genes. *Cell* 43, 507–519.
- Strutt, D.I., White, R.A.H., 1994. Characterisation of T48, a target of homeotic gene regulation in *Drosophila* embryogenesis. *Mech. Devel.* 46, 27–39.
- Stuart, J.J., Brown, S.J., Beeman, R.W., Denell, R.E., 1991. A deficiency of the homeotic complex of the beetle *Tribolium*. *Nature* 350, 72–74.
- Stuart, J.J., Brown, S.J., Beeman, R.W., Denell, R.E., 1993. The *Tribolium* homeotic gene *Abdominal* is homologous to *abdominal-A* of the *Drosophila* bithorax complex. *Development* 117, 233–243.
- Su, M., Venkatesh, T.V., Wu, X., Golden, K., Bodmer, R., 1999. The pioneer gene, *apontic*, is required for morphogenesis and function of the *Drosophila* heart. *Mech. Devel.* 80, 125–132.
- Sulston, I.A., Anderson, K.V., 1996. Embryonic patterning mutants in *Tribolium castaneum*. *Development* 122, 805–814.
- Sulston, I.A., Anderson, K.V., 1998. Altered patterns of gene expression in *Tribolium* segmentation mutants. *Devel. Genet.* 23, 56–64.
- Sun, B., Hursh, D.A., Jackson, D., Beachy, P.A., 1995. Ultrabithorax protein is necessary but not sufficient for full activation of *decapentaplegic* expression in the visceral mesoderm. *EMBO J.* 14, 520–535.
- Suzuki, Y., Palopoli, M.F., 2001. Evolution of insect abdominal appendages: are prolegs homologous or convergent traits? *Devel. Genes Evol.* 211, 486–492.
- Treisman, J., Gönczy, P., Vashishtha, M., Harris, E., Desplan, C., 1989. A single amino acid can determine the DNA binding specificity of homeodomain proteins. *Cell* 59, 553–562.
- Tremml, G., Bienz, M., 1989. Homeotic gene expression in the visceral mesoderm of *Drosophila* embryos. *EMBO J.* 8, 2677–2685.
- Tucker-Kellogg, L., Rould, M.A., Chambers, K.A., Ades, S.E., Sauer, R.T., et al., 1997. *Engrailed* (Gln50 → Lys) homeodomain-DNA complex at 1.9 Å resolution: structural basis for enhanced affinity and altered specificity. *Structure* 5, 1047–1054.
- Tupler, R., Perini, G., Green, M.R., 2001. Expressing the human genome. *Nature* 409, 832–833.
- Ueno, K., Hui, C.C., Fukuta, M., Suzuki, Y., 1992. Molecular analysis of the deletion mutants in the E homeotic complex of the silkworm *Bombyx mori*. *Development* 114, 555–563.
- Vachon, G., Cohen, B., Preifle, C., McGuffin, M.E., Botas, J., et al., 1992. Homeotic genes of the bithorax complex repress limb development in the abdomen of the *Drosophila* embryo through the target gene *Distal-less*. *Cell* 71, 437–450.
- van der Hoeven, F., Zakany, J., Duboule, D., 1996. Gene transpositions in the *HoxD* complex reveal a hierarchy of regulatory controls. *Cell* 85, 1025–1035.
- van Dijk, M.A., Murre, C., 1994. *extradenticle* raises the DNA binding specificity of homeotic selector gene products. *Cell* 78, 617–624.
- Villee, C., 1944. Phenogenetic studies of the homeotic mutants of *Drosophila melanogaster*. 2. The effects of temperature on the expression of *proboscipedia*. *J. Exp. Zool.* 96, 85–102.
- Volk, T., VijayRaghavan, K., 1994. A central role for epidermal segment border cells in the induction of muscle patterning in the *Drosophila* embryo. *Development* 120, 59–70.
- Von Allmen, G., Hogga, I., Spierer, A., Karch, F., 1996. Splits in fruit fly *Hox* gene complexes. *Nature* 380, 116.
- Wagner-Bernholz, J.T., Wilson, C., Gibson, G., Schuh, R., Gehring, W.J., 1991. Identification of target genes of the homeotic gene *Antennapedia* by enhancer activity. *Genes Devel.* 5, 2467–2480.

- Wakimoto, B., Kaufman, T.C., 1981. Analysis of larval segmentation in lethal genotypes associated with the *Antennapedia* gene complex in *Drosophila melanogaster*. *Devel. Biol.* 81, 51–64.
- Wakimoto, B.T., Turner, F.R., Kaufman, T.C., 1984. Defects in embryogenesis in mutants associated with the *Antennapedia* gene complex of *Drosophila melanogaster*. *Devel. Biol.* 102, 147–172.
- Walldorf, U., Binner, P., Fleig, R., 2000. Hox genes in the honeybee *Apis mellifera*. *Devel. Genes Evol.* 210, 483–492.
- Walldorf, U., Gehring, W.J., 1992. *Empty spiracles*, a gap gene containing a homeobox involved in *Drosophila* head development. *EMBO J.* 11, 2247–2259.
- Walter, J., Biggin, M.D., 1996. DNA binding specificity of two homeodomain proteins *in vitro* and in *Drosophila* embryos. *Proc. Natl Acad. Sci. USA* 93, 2680–2685.
- Walter, J., Dever, C.A., Biggin, M.D., 1994. Two homeo domain proteins bind with similar specificity to a wide range of DNA sites in *Drosophila* embryos. *Genes Devel.* 8, 1678–1692.
- Weatherbee, S.D., Halder, G., Kim, J., Hudson, A., Carroll, S., 1998. Ultrabithorax regulates genes at several levels of the wing-patterning hierarchy to shape the development of the *Drosophila* haltere. *Genes Devel.* 12, 1474–1482.
- Weatherbee, S.D., Nijhout, H.F., Grunert, L.W., Halder, G., Galant, R., *et al.*, 1999. Ultrabithorax function in butterfly wings and the evolution of insect wing patterns. *Curr. Biol.* 9, 109–115.
- Wedeen, C., Harding, K., Levine, M., 1986. Spatial regulation of *Antennapedia* and *bithorax* gene expression by the *Polycomb* locus in *Drosophila*. *Cell* 44, 739–748.
- Wharton, K.A., Yedvobnick, B., Finnerty, V.G., Artavanis-Tsakonas, S., 1985. *opa*: a novel family of transcribed repeats shared by the *Notch* locus and other developmentally regulated loci in *D. melanogaster*. *Cell* 40, 55–62.
- White, R.A., Wilcox, M., 1984. Protein products of the bithorax complex in *Drosophila*. *Cell* 39, 163–171.
- White, R.A.H., Aspland, S.E., Brookman, J.J., Clayton, L., Sproat, G., 2000. The design and analysis of a homeotic response element. *Mech. Devel.* 91, 217–226.
- White, R.A.H., Lehmann, R., 1986. A gap gene, *hunchback*, regulates the spatial expression of *Ultrabithorax*. *Cell* 47, 311–321.
- Wiellette, E.L., McGinnis, W., 1999. *Hox* genes differentially regulate *Serrate* to generate segment-specific structures. *Development* 126, 1985–1995.
- Wilkins, A.S., 1986. Homeo box fever, extrapolation and developmental biology. *BioEssays* 4, 147–148.
- Wilson, D.S., Guenther, B., Desplan, C., Kuriyan, J., 1995. High resolution crystal structure of a paired (Pax) class cooperative homeodomain dimer on DNA. *Cell* 82, 709–719.
- Wilson, D.S., Sheng, G., Jun, S., Desplan, C., 1996. Conservation and diversification in homeodomain-DNA interactions: a comparative genetic analysis. *PNAS* 93, 6886–6891.
- Wimmer, E.A., Jäckle, H., Pfeifle, C., Cohen, S.M., 1993. A *Drosophila* homologue of human Sp1 is a head-specific segmentation gene. *Nature* 366, 690–694.
- Wirz, J., Fessler, L.I., Gehring, W.J., 1986. Localization of the *Antennapedia* protein in *Drosophila* embryos and imaginal discs. *EMBO J.* 5, 3327–3334.
- Wolff, C., Sommer, R., Schroder, R., Glaser, G., Tautz, D., 1995. Conserved and divergent expression aspects of the *Drosophila* segmentation gene *hunchback* in the short germ band embryo of the flour beetle *Tribolium*. *Development* 121, 4227–4236.
- Wu, X., Vasisht, V., Kosman, D., Reinitz, J., Small, S., 2001. Thoracic patterning by the *Drosophila* gap gene *hunchback*. *Devel. Biol.* 237, 79–92.
- Yang, D., Lu, H., Hong, Y., Jinks, T.M., Estes, P.A., *et al.*, 2001. Interpretation of X chromosome dose at *Sex-lethal* requires non-E-box sites for the basic helix-loop-helix proteins SISB and Daughterless. *Mol. Cell Biol.* 21, 1581–1592.
- Yao, L., Liaw, G., Pai, C., Sun, Y., 1999. A common mechanism for antenna-to-leg transformation in *Drosophila*: suppression of *homothorax* transcription by four HOM-C genes. *Devel. Biol.* 211, 268–276.
- Zavortink, M., Sakonju, S., 1989. The morphogenetic and regulatory functions of the *Drosophila Abdominal-B* gene are encoded in overlapping RNAs transcribed from separate promoters. *Genes Devel.* 3, 1969–1981.
- Zeng, C., Pinsonneault, J., Gellon, G., McGinnis, N., McGinnis, W., 1994. Deformed protein binding sites and cofactor binding sites are required for the function of a small segment-specific regulatory element in *Drosophila* embryos. *EMBO J.* 13, 2362–2377.
- Zeng, W., Andrew, D.J., Mathies, L.D., Horner, M.A., Scott, M.P., 1993. Ectopic expression and function of the *Antp* and *Scr* homeotic genes: the N terminus of the homeodomain is critical to functional specificity. *Development* 118, 339–352.
- Zhou, J., Levine, M., 1999. A novel *cis*-regulatory element, the PTS, mediates an anti-insulator activity in the *Drosophila* embryo. *Cell* 99, 567–575.
- Zhu, A., Kuziora, M.A., 1996. Functional domains in the Deformed protein. *Development* 122, 1577–1587.
- Zink, B., Paro, R., 1989. *In vivo* binding pattern of a *trans*-regulator of homeotic genes in *Drosophila melanogaster*. *Nature* 337, 468–471.

Relevant Website

<http://flybase.org> – The FlyBase database of the *Drosophila* genome

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2 *Drosophila* Limb Development

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2.1. *Drosophila* Limb Development as a Model System

A central question in developmental biology is how growth and patterning of tissues are controlled at a molecular and genetic level. *Drosophila melanogaster* has become an important model system to approach this problem because of its suitability for genetic and molecular manipulations and its well-described developmental biology. Systematic genetic screens for loss-of-function mutations (Nüsslein-Volhard and Wieschaus, 1980), gain-of-function phenotypes (Rorth, 1996; Rorth *et al.*, 1998), and the detection of gene expression patterns by enhancer trapping (Bellen *et al.*, 1989; Calleja *et al.*, 1996) have revealed many of the genes involved in developmentally important processes (for a recent review, see Chapter 1). In addition, the completion of the genomic sequencing project for *Drosophila melanogaster* (Adams *et al.*, 2000) and, recently, for *D. pseudoobscura*, makes it possible to employ reverse genetic approaches such as RNA-mediated interference (Kennerdell and Carthew, 1998) and targeted gene disruption (Rong and Golic, 2000; Rong *et al.*, 2002) as well as genome-wide expression analyses (Furlong *et al.*, 2001; Jasper *et al.*, 2001; Arbeitman *et al.*, 2002; Klebes *et al.*, 2002; Butler *et al.*, 2003) to address a wide variety of

questions concerning the developmental biology of the fruitfly and related insects.

The appendages of *Drosophila* have proven to be ideal models for the investigation of limb development. Like the appendages of vertebrates, they develop as outgrowths of the body wall (although these outgrowths are initially topologically inside the fly embryo/larva). Early in embryonic development, imaginal cells (i.e., cells that contribute to the adult animal, the “imago”) segregate from their neighbors by invagination from the ectoderm (Bate and Martinez-Arias, 1991). Later, these clusters of cells separate into ventral and dorsal appendage precursors (Wieschaus and Gehring, 1976; Cohen *et al.*, 1991, 1993; Averof and Cohen, 1997) and form saclike structures called imaginal discs (Figure 1). Although each disc is a continuous epithelial monolayer, opposite sides of the sac acquire different properties. Most structures of the adult limb and of the neighboring body wall are derived from one side of the sac, which forms a highly folded pseudostratified epithelium (review: Cohen, 1993). It is in this epithelium that the major patterning events take place. The other side of the sac forms a squamous epithelium called the peripodial membrane, which has recently been reported to contribute to some aspects of patterning and growth

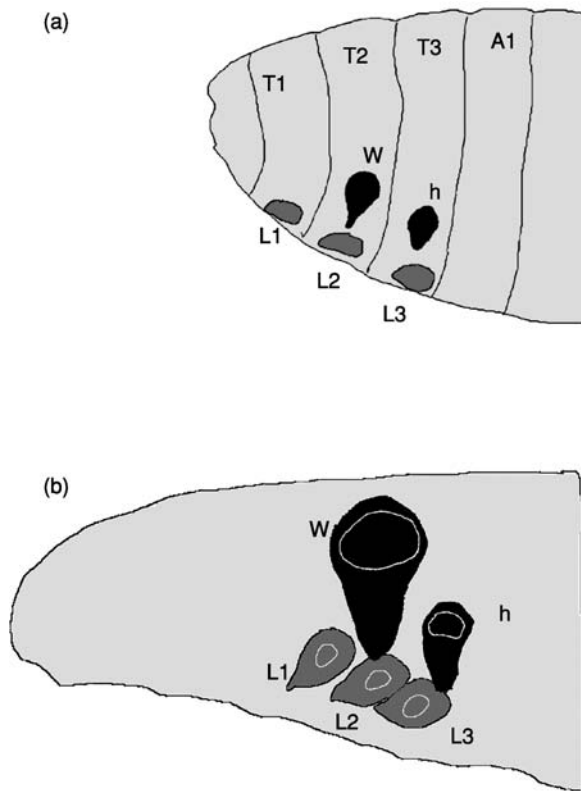


Figure 1 Specification of imaginal discs. (a) Drawing of the anterior half of a stage 17 *Drosophila* embryo (lateral view). Primordia of the imaginal discs are shown in dark gray (ventral appendages) or black (dorsal appendages). T1–3, thoracic segments 1–3; A1, abdominal segment 1; w, wing disc; h, haltere disc; L1–3 – first, second, and third leg discs. (b) Drawing of a third instar larva, showing the different imaginal discs.

control in the pseudostratified epithelial layer (Cho *et al.*, 2000; Gibson and Schubiger, 2000, 2001; Ramirez-Weber and Kornberg, 2000).

Imaginal discs grow considerably during larval stages by exponential cell proliferation (García-Bellido and Merriam, 1971a; Madhavan and Schneiderman, 1977). The wing imaginal disc consists of ~50 cells at the end of embryogenesis and grows to ~50 000 cells at pupariation. Patterning and organ size regulation are, in principle, disc-autonomous processes even though disc growth requires the input of growth factors and hormones from non-imaginal tissues (review: Bryant and Simpson, 1984). *Drosophila* as a higher dipteran insect undergoes complete metamorphosis. During this process the primordium extends along its proximal–distal axis by turning inside out through the lumen (so that the appendages come to lie topologically outside the body wall after disc eversion). Actual differentiation of many adult structures (such as wing margin sensory organs, etc.) does not begin until metamorphosis. However, the structures of the

adult organ reflect patterning and growth processes that occur during development.

2.2. Developmental Subdivisions of the Limb Primordia

The establishment of the three major axes of the limbs takes place during imaginal disc development. The primordia receive the patterning cues needed to generate an anterior–posterior, dorsal–ventral, and proximal–distal axes (Figure 2). Diffusible signals termed morphogens (Turing, 1952) provide cells within the epithelium with positional information (Wolpert, 1969). Axis formation involves a variety of different responses to this positional information. Changes in gene expression control cell identity, with concomitant effects on growth and differentiation.

The establishment of the limb axes depends on subdivision of their primordia into distinct functional units. The first subdivision occurs in embryogenesis in the epithelial cell layer from which the discs derive and so is inherited by the nascent disc primordia (Cohen *et al.*, 1993). Subsequent subdivisions arise *de novo* in the imaginal discs during larval stages. Segregation of these cell populations seems to involve differences in cell affinity (reviews: Dahmann and Basler, 1999; McNeill, 2000; Irvine and Rauskolb, 2001). Two mechanistically distinct modes of subdivision have been described:

1. *Heritable subdivisions.* These subdivisions are based on the establishment of a heritable pattern of transcription factor expression that confers a specific identity on a field of cells. The first such subdivision was detected in the *Drosophila* wing between anterior (A) and posterior (P) “compartments” (García-Bellido *et al.*, 1973, 1976). An analogous AP subdivision exists in the leg imaginal disc (Steiner, 1976). The wing disc is further subdivided into dorsal (D) and ventral (V) compartments (Diaz-Benjumea and Cohen, 1993; Blair *et al.*, 1994; García-Bellido *et al.*, 1976) (Figure 2c and d).
2. *Nonheritable subdivisions.* As an alternative to the stable, heritable mechanism, cell populations can also be subdivided by cell interactions. Continuous input of positional information can instruct cells about their position within a tissue and specify responses that separate cell populations. These cues can be transmitted either through local interactions with neighboring cells or through the reception of long-range morphogen gradient cues (reviews: Strigini and Cohen, 1999; Gurdon and Bourillot, 2001; Freeman and Gurdon, 2002). Cells are able to

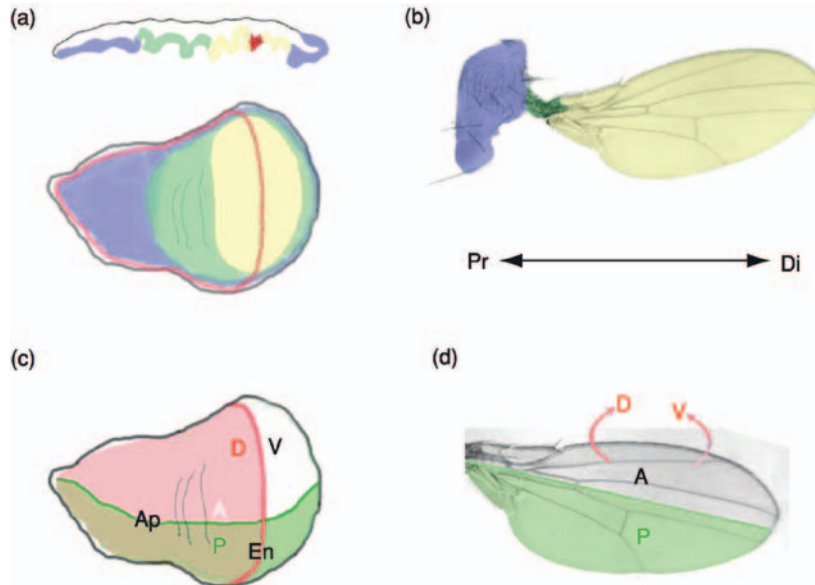


Figure 2 Organization of the axes in the wing imaginal disc. (a) Drawing of a wing imaginal disc, lateral view (upper panel) and apical view (lower panel). In the lateral view the peripodial membrane is depicted as a thin line, over the pseudostratified epithelium, proximal (Pr) structures pointing to the left and distal (Di) ones to the right. The colors represent the different adult structures that develop from the wing imaginal disc. Blue, notum; green, hinge region; yellow, wing pouch; the red line represents the dorsal–ventral boundary. (b) Picture of an adult wing. Color code as in (a). (c) Organization of the anterior–posterior (AP) and dorsal–ventral (DV) boundary within the imaginal disc. The red line represents the DV lineage restriction boundary. Apertous (Ap) is expressed in the D compartment (pink). The green line represents the AP compartment boundary; Engrailed (En) expression in the P compartment is shown in green. (d) Adult wing showing the wing’s AP and DV axes.

move between subdivisions, changing their expression profiles according to the position-dependent cues they receive (Campbell and Tomlinson, 1998; Weigmann and Cohen, 1999; Jungbluth *et al.*, 2001) (Figure 2a and b).

2.2.1. Heritable Subdivisions: Compartments

The first clues to understanding compartmentalization came from using mitotic recombination to genetically mark cells and their descendants in the *Drosophila* wing. Patches of cells homozygous for a recessive, cell-autonomous marker (e.g., cuticle pigmentation, shape of wing hairs) in an otherwise heterozygous background occupy random positions and usually have irregular borders. Clones of cells abutting the border between A and P compartments (slightly anterior to vein four in the adult wing) (Figure 2d) show smooth borders and remain within their compartment of origin, suggesting that cells cannot cross this “compartment boundary,” even if they are given a relative growth advantage (Morata and Ripoll, 1975) (Figure 3). Molecular markers (e.g., green fluorescent protein (GFP) and lacZ) (Xu and Rubin, 1993) have made it possible to investigate in great detail how the compartment boundary is established and maintained (Blair and

Ralston, 1997; Rodriguez and Basler, 1997; reviews: Blair, 1995; Brook *et al.*, 1996).

According to the definition of compartments, these comprise three features:

1. Compartments are subdomains of a tissue that are separated by a boundary of cell lineage restriction.
2. Compartment boundaries show organizing activity that patterns the surrounding tissue.
3. Compartments divide tissues in terms of cell fate/identity.

2.2.1.1. Selector genes The establishment of compartments as units of lineage restriction led to the idea that the activity of certain genes “selects” these units for a specific fate (García-Bellido, 1975). The original definition of a “selector gene” includes the following properties:

1. The functional domain is limited by a lineage restriction boundary (compartment boundary). Selector genes confer cell affinity differences that prevent mixing of cells from different compartments.
2. Selector genes have an instructive developmental role.

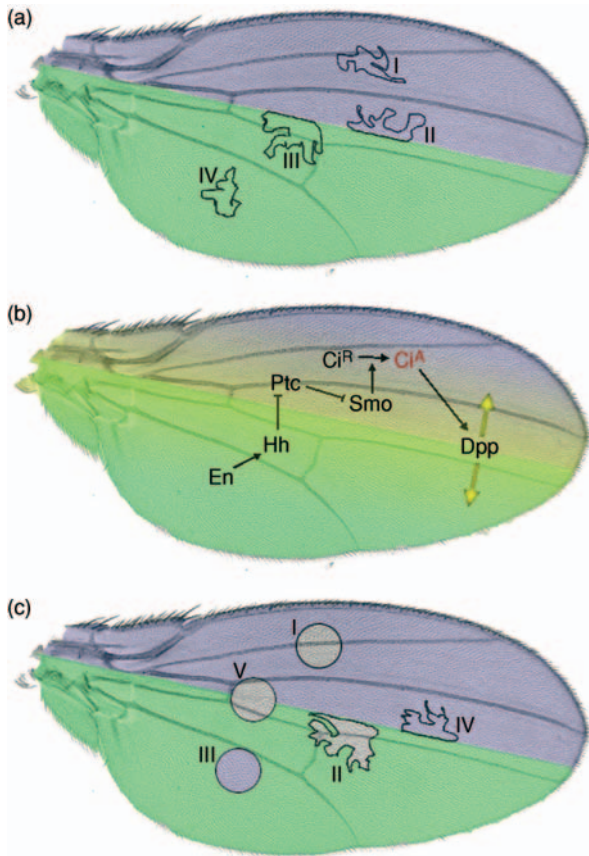


Figure 3 AP compartment formation in the *Drosophila* wing. Anterior cells are shaded blue, posterior cells are shaded green. (a) Clones of wild-type cells have irregular borders and occupy random positions within a compartment (I and IV) but never cross the AP compartment boundary. They show smooth edges when they happen to contact it (II and III). (b) Simplified schematic view of Hedgehog (Hh)-mediated signaling across the AP boundary. Hh signaling ultimately results in conversion of Cubitus interruptus (Ci) from its repressor form (Ci^R) to a transcriptional activator Ci^A. Ci^A activates the long-range morphogen Decapentaplegic (Dpp) (yellow) which spreads from the AP boundary to further pattern the wing. (c) Scheme to show the influence of Engrailed (En) and Hh pathway mutations on clone shape and localization. Posterior clones that lack En (or ectopically express Ci) either sort into the anterior compartment (IV) or round up (III). Anterior clones lacking Smoothed (Smo) (or Ci) form round clones within the anterior compartment (I) or sort into the posterior (II). Clones of cells mutant for both En and Ci have different adhesive properties from both A and P cells and can form round clones at the AP boundary (V). For other protein definitions, see text and appendix. (Modified from Irvine, K.D., Rauskolb, C. 2001. Boundaries in development: formation and function. *Annu. Rev. Cell Devel. Biol.* 17, 189–214.)

- Several selector genes can act in a combinatorial mode.
- Selector genes act strictly autonomously to specify compartment-specific properties (e.g., cell fate).

The term “selector gene” is often misused to describe genes that confer regional identity in a

manner not linked to lineage-restricted compartments (reviews: Mann and Morata, 2000; Mann and Carroll, 2002). In this review, the use of the term selector gene is limited to only those factors that fulfill all the criteria mentioned above. Other genes that specify subdivisions but do not fulfill these criteria will be referred to as “selector-like” or “regional identity” genes. This distinction is considered to be important because of the underlying mechanistic differences between the two modes of regionalization. Compartment boundaries defined by selector genes serve as the sources of organizing signals, which is not usually the case for noncompartmental subdivisions.

2.2.1.1.1. Engrailed/Invected: posterior compartment selector genes During segmentation in the embryo, P cells are programmed to express the homeodomain transcription factors Engrailed (En) and Invected (Inv) (Kornberg *et al.*, 1985) (for a list of all genes and proteins discussed in this chapter, see appendix). Even though the signals that lead to the induction of En and Inv are transient, their expression becomes heritably transmitted and is inherited by the nascent imaginal disc primordia when they form in the embryo (Cohen *et al.*, 1993). This is likely to depend on the epigenetic mechanisms based on chromatin remodeling by Polycomb (Pc) and Trithorax (Trx) group proteins (Ingham, 1981; Busturia and Morata, 1988; see **Chapter 1**). The En/Inv complex controls all aspects of posterior fate in the wing and leg (**Figure 2c** and **d**). Cells mutant for *en* show some anterior character (García-Bellido and Santamaria, 1972; Morata and Lawrence, 1975; Lawrence and Struhl, 1982). However, P cells mutant for both *en/inv* are indistinguishable from A cells (Kornberg, 1981; Tabata *et al.*, 1995; Zecca *et al.*, 1995; Blair and Ralston, 1997; Lawrence *et al.*, 1999a) (**Figure 3**). Inv alone mainly confers P identity in terms of differentiation (Simmonds *et al.*, 1995). En was shown to work as a transcriptional repressor (Jaynes and O’Farrell, 1988, 1991; Han *et al.*, 1989; Ohkuma *et al.*, 1990). The En repressor domain very strongly represses promoters activated by a variety of different activator proteins (Han and Manley, 1993; Smith and Jaynes, 1996). En/Inv are highly conserved and fulfill similar roles in other invertebrate species (Patel *et al.*, 1989).

2.2.1.1.2. Apterous: dorsal compartment selector gene During larval development, the wing imaginal disc is further subdivided by a DV compartment boundary (García-Bellido *et al.*, 1976). The gene responsible for the specification of all aspects of

dorsal cell development is the transcription factor Apterous (Ap) (Blair, 1993; Diaz-Benjumea and Cohen, 1993). The gene *apterous* (*ap*) conforms to the classic definition of a selector gene. Ap activity generates an affinity difference between dorsal and ventral cells and thereby produces the DV lineage boundary. Ap selects between alternative cell fates (D and V) in an autonomous manner and acts in a combinatorial mode with the posterior selector genes *en* and *inve* (Figure 2c and d).

Early in larval development, the wing imaginal disc is patterned by two opposing signaling pathways, the Wingless (Wg) and epidermal growth factor (EGF) pathways. Hedgehog (Hh) induces Wg in the V–A domain, where it is required to specify the primordium of the wing blade (as opposed to the body wall) (Williams *et al.*, 1993; Ng *et al.*, 1996; Klein and Martinez-Arias, 1998) (Figure 4a). The *vein* (*vn*)-dependent epidermal growth factor receptor (EGFR) signaling has a dual role in early wing

disc development (Wang *et al.*, 2000; Zecca and Struhl, 2002a, 2002b). First, it instructs cells to adopt body wall fate by antagonizing wing fate and activating notum-specifying genes. The mutually antagonistic repression of the Wg and EGFR signaling pathways therefore separates the wing from the body wall field. Second, EGRF signaling directs cells to become part of the dorsal compartment by inducing the dorsal selector gene *ap* (Figure 4a). Thus, the antagonism of EGFR and Wg signaling also controls wing blade development through the initiation of DV boundary formation (Figure 4b and c).

2.2.1.1.1. *Specification of dorsal cell fate through Ap* Ap specifies dorsal cell fate. Removal of Ap activity causes complete transformation from D to V identity (Diaz-Benjumea and Cohen, 1993; Blair *et al.*, 1994). D cells differ from V cells in the adult with respect to a set of morphological markers

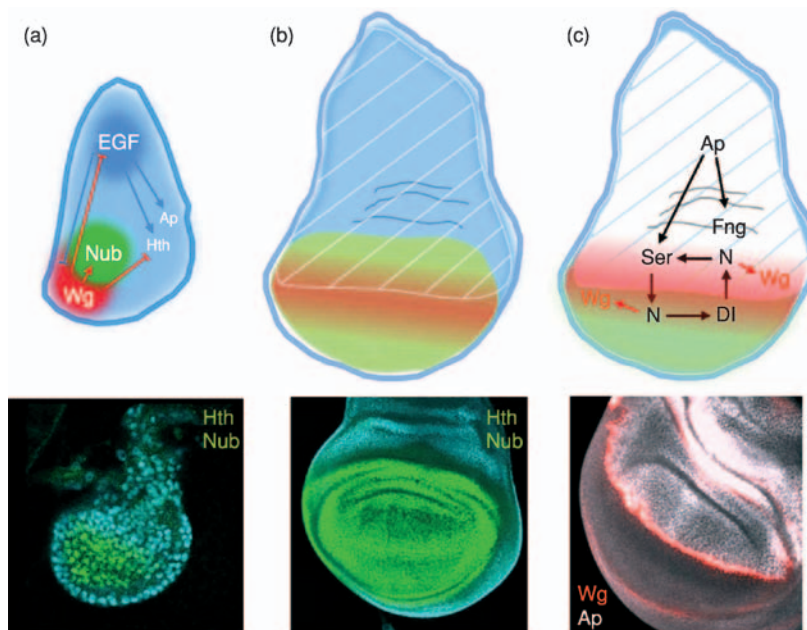


Figure 4 Establishment of the proximal–distal and dorsal–ventral boundaries. (a) (upper panel) The Wingless (Wg) and the epidermal growth factor receptor (EGFR) signaling pathways antagonize each other. Wg specifies the wing pouch through the activation of Nubbin (Nub) and Vestigial (Vg, not shown) and the repression of Vein (Vn, the ligand for the EGFR; not shown), Homothorax (Hth), Apterous (Ap), Teashirt (Tsh, not shown). Vn induces the formation of notum structures and is necessary for the activation of Ap, Hth, and Tsh, and represses the expression of Vg. Both activities together are responsible for establishment of the dorsal–ventral and proximal–distal axes. (lower panel) Single confocal section of a late second instar wing imaginal disc labeled with antibodies against Nub (green), and Hth (cyan). Nub marks the future wing pouch, while Hth marks hinge and body wall tissue. (b) (upper panel) Drawing of late third instar wing disc. The wing pouch is marked in green (Nub), notum tissue is depicted in cyan (Hth). Ap (crosshatched in white) is exclusively expressed in dorsal cells and establishes the dorsal–ventral boundary. Wg (red) is expressed at the dorsal–ventral boundary and spreads along the dorsal–ventral axis into the wing pouch. (lower panel) Late third instar wing disc labeled with anti-Nub (green) and anti-Hth (cyan) antibodies, showing the noncompartmental subdivision in notum and wing tissue. (c) (upper panel) Ap (white) induces the dorsal–ventral compartment boundary through the activation of Fringe (Fng) and Serrate (Ser) in the dorsal compartment and the repression of Delta (DI). This leads to the activation of Notch (N) signaling at the dorsal–ventral boundary and eventually to the expression of Wg (red). (lower panel) Late third instar wing disc labeled with anti-Ap (white) and anti-Wg (red) antibodies, showing dorsal–ventral compartmentalization and dorsal–ventral organizer.

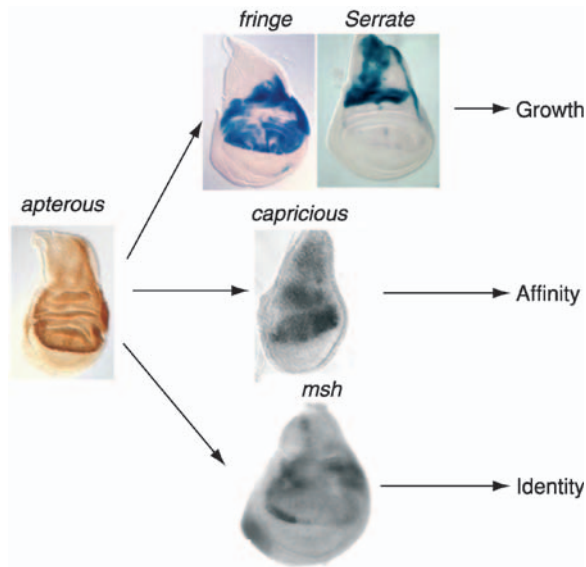


Figure 5 Summary of Apterous functions during wing development. Dorsal–ventral subdivision of the wing imaginal disc depends on the activity of Apterous (Ap) in the dorsal compartment, where it is expressed (*in situ* hybridization with a probe against *apterous*). Ap is responsible for (1) the induction of the dorsal–ventral organizer along the dorsal–ventral compartment boundary through Fringe and Serrate (X-Gal staining of enhancer trap lines inserted at the respective loci); (2) the establishment of a lineage restriction boundary between dorsal and ventral cells through induction of the affinity molecules Capricious (X-Gal staining of an enhancer trap line inserted at the *caps* locus) and Tartan (not shown) in early wing discs; and (3) dorsal specification of dorsal cell fate through Msh (*in situ* hybridization with a probe against *msh*).

such as sensory organs and wing vein structure. It has been claimed that D cell differentiation can take place in the absence of Ap activity (Klein *et al.*, 1998). However, more careful analysis showed that the *ap* mutants used in that study were only partial loss-of-function mutations, and had enough residual Ap activity to confer dorsal cell fate (Milan and Cohen, 1999a, 2003; O’Keefe and Thomas, 2001). The *ap* confers dorsal identity by controlling expression of the homeobox gene *muscle segment homeobox (msh)* (Milan *et al.*, 2001b) (Figure 5). *msh* is expressed in dorsal cells in the embryonic neuroectoderm and muscle precursors (D’Alessio and Frasch, 1996; Isshiki *et al.*, 1997; Lu *et al.*, 2000). In the wing disc, *msh* is expressed in D cells where it is both necessary and sufficient to direct differentiation of dorsal cell characteristics (Milan *et al.*, 2001b). A summary of all known Apterous functions is shown in Figure 5.

2.2.1.1.1.2. Regulation of Ap activity Ap is expressed in the wing and haltere imaginal discs exclusively in D cells (Cohen *et al.*, 1992). Tight

spatial and temporal regulation turned out to be essential for proper Ap activity. Ap belongs to the family of *lin-11*, *islet-1*, *mec-3* (LIM)-homeodomain factors, which shares two tandemly repeated LIM domains and a homeodomain (review: Hobert and Westphal, 2000). LIM-domains are cysteine-rich zinc-finger domains that mediate protein–protein interactions (Jurata *et al.*, 1998). For LIM-homeodomain transcription factors to function, they require a class of proteins known as LIM-domain binding (LDB) or nuclear LIM interactor (NLI) (Agulnick *et al.*, 1996; Jurata *et al.*, 1996; Morcillo *et al.*, 1997; Fernandez-Funez *et al.*, 1998; review: Bach, 2000). In *Drosophila* wing development, Ap activity depends strictly on dLDB/Chip (Fernandez-Funez *et al.*, 1998). A dimer of dLDB/Chip has been shown to bridge two Ap molecules to form a tetrameric complex active *in vivo* (Milan and Cohen, 1999b; van Meyel *et al.*, 1999; Rincon-Limas *et al.*, 2000). The levels of Ap activity are further controlled both spatially and temporally during development (Milan and Cohen, 2000b). Ap regulates its own activity by inducing the expression of its target gene *Beadex/dLMO* (Milan *et al.*, 1998; Shoresch *et al.*, 1998; Zeng *et al.*, 1998). *dLMO* encodes a member of the LIM-domain only (LMO) family and contains two LIM domains highly similar to those of Ap. dLMO is able to downregulate Ap activity by binding to the LIM-interaction domains of dLDB/Chip, competing with Ap itself. Through this mechanism, Ap is inactivated (Milan *et al.*, 1998; Milan and Cohen, 1999b; van Meyel *et al.*, 1999). This limits the time window during which Ap is highly active in the dorsal compartment and during which DV boundary formation can take place. The limitation of Ap activity is essential because some of the genes regulated by Ap are used in a different developmental context later. Late in development, Serrate (Ser) and Delta (Dl) are expressed along the future veins and at both sides of the DV compartment boundary, where they contribute to the establishment of a feedback loop essential for the restriction of Notch (N) activity along veins and the DV boundary (Figure 4c). Continuously, high activity of Ap is not compatible with these later functions (Milan *et al.*, 1998; Milan and Cohen, 2000b; Weihe *et al.*, 2001).

The model that dLMO acts as a competitive inhibitor of Ap suggests that dLMO should displace Ap from Chip. In fact, Ap protein is destabilized in cells expressing dLMO. Interestingly, Ap appears to be destabilized in situations where it is unable to bind to DNA in active tetrameric complexes with Chip (Weihe *et al.*, 2001). dLMO competes effectively with Ap for binding to Chip, whereas

Ap competes poorly with dLMO apparently due to an intrinsic difference in the affinities of the LIM domains of Ap and dLMO. This means that dLMO is a potent inhibitor of Ap activity because it binds Chip more effectively than Ap (Weihe *et al.*, 2001).

dLDB/Chip is also involved in the activation of the *achaete-scute* (*ac-sc*) proneural complex in association with the GATA factor Pannier (Pnr). This process is important for sensory bristle patterning in the thorax. Pnr antagonizes Ap function, presumably by competitively binding to Chip. The correct stoichiometry of these three proteins is crucial for both proneural prepatterning and subdivision of the thorax (Ramain *et al.*, 2000).

2.2.1.2. Mechanisms of boundary formation

2.2.1.2.1. Affinity-based cell separation Because of the signaling interactions that ensue when A and P or D and V cells are confronted (see Section 2.2.1.3.2), a well-defined straight boundary between compartments is essential. Crossing of cells into the other compartments would lead to the establishment of an ectopic organizer within the wing pouch, resulting in severe morphological defects. How are cells from different compartments kept separate? Differences in cell adhesion certainly can contribute to this process. Sorting out of cell populations can be guided by both the amount and types of adhesion proteins that cells express (Steinberg and Takeichi, 1994).

One well-studied example of differential adhesion mediating morphogenesis is the sorting of the oocyte to the posterior of the ovary in *Drosophila*. The oocyte and the posterior follicle cells express higher levels of *Drosophila* EGF-domain containing cadherin (DE-cadherins), which are adhesion molecules, and catenins, which anchor the cadherins to the cytoskeleton and are necessary for strong adhesion. Loss of either cadherins or catenins leads to mislocalization of the oocyte within the ovary (Godt and Tepass, 1998; Gonzalez-Reyes and St. Johnston, 1998). However, cadherins have not been shown to be responsible for boundary formation in the discs. Clones of cells overexpressing cadherins do sort out from surrounding cells in the discs (Dahmann and Basler, 2000), showing that the expression of adhesion molecules can, in principle, separate cell populations in imaginal discs. Affinity molecules involved in cell separation at the AP axis have not been identified yet.

Theoretically, the prospective wing margin cells (zone of nonproliferating cells (ZNCs)) could act as a physical barrier to prevent mixing of D and V compartment cells (O'Brochta and Bryant,

1985). This model predicts that the DV boundary is in fact two boundaries, one on the dorsal and one on the ventral side of the ZNC. It also requires that cells from outside the ZNC are prevented from entering it. Neither requirement was fulfilled however, when clone distribution was analyzed in discs, so the barrier model cannot explain DV boundary formation (Blair, 1993). Thus, Dorsal cells mutant for *ap* fail to respect the DV boundary and tend to sort out into the V compartment (analogous to posterior cells mutant for *en*) (Figure 3c). Therefore, it was hypothesized that Ap could directly regulate the expression of molecules that modify cell adhesion or affinity (Lawrence and Struhl, 1996). Indeed, *ap* controls the expression of two integrin-type adhesion molecules, PS1 and PS2. PS1 expression is activated in the D compartment by Ap activity and PS2 is restricted to V cells by repression through Ap (Blair *et al.*, 1994). However, PS1 and PS2 proved not to be involved in DV compartment formation but rather to regulate the attachment of the D and V wing blades during pupal stages (Brower and Jaffe, 1989; Blair *et al.*, 1994).

Evidence that other Ap-dependent cell interactions contribute to boundary formation has been presented (Milan *et al.*, 2001a). The *capricious* (*caps*) and *tartan* (*trn*) genes are targets of Ap (Figure 5). The genes *caps* and *trn* encode transmembrane proteins with extracellular leucine-rich repeats (LRRs) and are expressed in the D compartment during boundary formation. Caps and Tartan confer affinity for D cells, as assessed by sorting out behavior, and contribute to boundary formation, as assessed by sensitized genetic assays. However, removing both genes does not eliminate the DV boundary, indicating that other *ap* target genes may also confer D cell-affinity properties.

Taken together, the results suggest that *ap* is responsible for the specification of all aspects of D cell development in the *Drosophila* wing and works as a classical selector gene (Figure 5).

2.2.1.2.2. Signaling-based cell separation There are several examples of cell sorting processes that are clearly influenced by cellular signaling, where the mechanism may not be dependent on differences in cellular affinities per se. Rhombomeres in the vertebrate hindbrain are separated by a cell lineage restriction. Ephrin tyrosine kinase receptors and their membrane-bound Ephrin ligands are expressed in complementary rhombomeres. Both receptors and ligand are able to transduce signals. Bidirectional signaling occurs at the interface of their expression domains, causing the restriction of cell intermingling between hindbrain segments.

Unidirectional signaling is not sufficient to establish the cell lineage restriction, indicating that the ligand–receptor interaction is not sufficient to confer a cell–cell affinity difference strong enough to separate cells from different segments (Mellitzer *et al.*, 1999; Xu *et al.*, 1999). Many adhesion proteins form regulated connections with the cytoskeleton and participate in contact-mediated signaling (reviews: Hynes, 1999; Vleminckx and Kemler, 1999). Signaling-based repulsion or deadhesion in this case could promote segregation of cell populations. If signaling promotes reorganization of the cytoskeleton, cell interactions might be destabilized (Brückner and Klein, 1998). Repeated cycles of deadhesion and readhesion could lead to sorting out behavior.

This example is not meant to argue that cell affinity factors do not play a role in these cell sorting processes. However, they do suggest that alternative mechanisms based on signaling are possible. In fact, asymmetric signaling from P to A cells seems to affect AP boundary formation in the *Drosophila* wing. Anterior clones of cells lacking the transmembrane protein Smoothed (Smo), which is essential for *hh* signal transduction, can associate with posterior cells even though they lack En and were of anterior origin (Figure 3c). However, these clones do not mix perfectly with P cells either, suggesting that a contribution of *hh* signaling and En-mediated cell affinity differences, both are crucial for proper sorting of cells into the correct compartment (Blair and Ralston, 1997; Rodriguez and Basler, 1997; Lawrence *et al.*, 1999b; Dahmann and Basler, 2000).

Signaling between dorsal and ventral cells contributes to D–V boundary formation. For example, Fringe-expressing clones (which have “dorsal” Notch signaling properties) are able to cross the boundary from V to D if they happen to contact it (Micchelli and Blair, 1999; Rauskolb and Irvine, 1999). However, it has been shown that Notch activation along the DV boundary in the absence of Ap is insufficient to support DV boundary formation (Milan and Cohen, 1999a). Notch activation at the DV boundary is symmetric (in contrast to the inherently asymmetric signaling from P to A cells) (Figure 4c). It is therefore not obvious how symmetric Notch activation could generate an affinity border. Notch signaling is required, but is not sufficient to generate a boundary without an additional Ap-dependent input. The authors envisage a permissive role for Notch activity in the DV boundary formation, with some other Ap-dependent activity providing the instructive determinant (Milan and Cohen, 2003).

2.2.1.3. Compartment boundaries as organizing centers Cell interactions play a critical role during development. Pioneering experiments in the frog have shown that specific groups of cells can behave as organizing centers that control the developmental fates of nearby cells in a nonautonomous manner (Spemann and Mangold, 1924). Transplantation experiments with dorsal cells from the amphibian blastopore lip showed that these cells were able to induce a complete secondary body axis when located ectopically in ventral tissue. Many developmental organizers have been found since, e.g., the notochord (inducing the floorplate) (Placzek *et al.*, 1990; Yamada *et al.*, 1991), the isthmus organizer between the midbrain and hindbrain (reviews: Joyner *et al.*, 2000; Simeone, 2000) and the AP and DV compartment boundaries in the fly wing and leg (Diaz-Benjumea and Cohen, 1993; Struhl and Basler, 1993; Basler and Struhl, 1994) (Figure 2c).

In the context of compartments, formation of organizers can be separated into three steps: (1) differential cell fate specification in adjacent compartments, (2) leading to asymmetric signaling between the two cell populations, and (3) production of a symmetric organizing signal originating at the compartment boundary (Meinhardt, 1983; Struhl and Basler, 1993; Basler and Struhl, 1994; Diaz-Benjumea *et al.*, 1994; Tabata and Kornberg, 1994; review: Brook *et al.*, 1996).

2.2.1.3.1. The A–P organizer Soon after the discovery that A and P cells are separated by a lineage restriction boundary, the suggestion was made that this boundary might serve as an organizing center in the developing appendages (Crick and Lawrence, 1975). Molecular analyses revealed that specialized cells are established along the AP axis as a consequence of asymmetric signaling by the diffusible protein Hedgehog (Hh) from P to A cells (Basler and Struhl, 1994; Tabata and Kornberg, 1994) (Figure 3b). En generates this asymmetry by inducing expression of Hh in the posterior compartment (Tabata *et al.*, 1992; Zecca *et al.*, 1995) and at the same time repressing the expression of the essential downstream component Cubitus interruptus (Ci) (Eaton and Kornberg, 1990; Schwartz *et al.*, 1995) (Figure 3b). Ci is a transcription factor that is converted to a repressor form in the absence of Hh signaling (Aza-Blanc *et al.*, 1997; Méthot and Basler, 1999). Thus, only A cells that receive the Hh signal across the compartment boundary will respond by stabilization of Ci. Hh signaling leads to the expression of the secreted signaling molecule Decapentaplegic (Dpp) at the AP boundary of wing discs and of Dpp and Wg in leg discs (Struhl and Basler, 1993;

Basler and Struhl, 1994; Diaz-Benjumea *et al.*, 1994). Dpp and Wg act as symmetric long-range morphogens in wing and leg imaginal discs (Brook and Cohen, 1996; Jiang and Struhl, 1996; Lecuit *et al.*, 1996; Nellen *et al.*, 1996; Lecuit and Cohen, 1997; Entchev *et al.*, 2000; Teleman and Cohen, 2000). By this mechanism the originally asymmetric subdivision of the limb primordium eventually leads to the establishment of a symmetric organizing gradient of the morphogen (Figure 3b).

2.2.1.3.2. The DV organizer Ap expression establishes the D–V lineage restriction boundary in the wing disc (Diaz-Benjumea and Cohen, 1993; Blair *et al.*, 1994). Short-range interactions between D and V cells lead to the activation of the Notch signal transduction pathway (Diaz-Benjumea and Cohen, 1995; Kim *et al.*, 1995; Rulifson and Blair, 1995; de Celis *et al.*, 1996b; Doherty *et al.*, 1996). Notch is activated symmetrically on both sides of the DV boundary (Figure 4c). Ap plays an essential role in this process by inducing expression of Serrate and Fringe in dorsal cells (Irvine and Wieschaus, 1994; Speicher *et al.*, 1994; Couso *et al.*, 1995) (Figure 5). Serrate and the ventrally expressed Delta encode transmembrane proteins that act as ligands for the Notch receptor protein (Fleming *et al.*, 1990; Rebay *et al.*, 1991; Doherty *et al.*, 1996). Fringe modulates the sensitivity of Notch to its ligands such that Fringe-expressing cells are sensitive to Delta and refractory to Serrate (Fleming *et al.*, 1997; Johnston *et al.*, 1997; Panin *et al.*, 1997). Fringe acts in the Golgi apparatus as a glycosyltransferase enzyme that modifies the EGF modules of Notch and enhances the ability of Notch to bind to Delta (Brückner *et al.*, 2000; Moloney, 2000; Panin *et al.*, 2002). Consequently, Sersignals to V cells and Delta signals to D cells across the DV boundary. Notch is activated symmetrically and induces Wg and Vestigial (Vg) (through its boundary enhancer) on both sides of the boundary (Figure 4c). Wg spreads along the DV axis of the wing and further organizes growth and patterning of the wing disc (see Section 2.3.3) by inducing and repressing its target genes *vg* (through the quadrant enhancer), *ac-sc*, *Dll*, *fz2*, and *notum* in a concentration-dependent manner (Blair, 1992; Phillips and Whittle, 1993; Couso *et al.*, 1994; Zecca *et al.*, 1996; Neumann and Cohen, 1997; Cadigan *et al.*, 1998; Giraldez *et al.*, 2002) (Figure 7).

2.2.2. Nonheritable Subdivisions of Limb Primordia

Developmental boundaries exist between compartments that separate cell lineages and also between

fields of cells that are subdivided less strictly. The term “territory” has been suggested for subdivisions that are not defined by lineage boundaries but are dynamically maintained by continuous signaling (Theisen *et al.*, 1996). As long as the patterning mechanisms that initiate these subdivisions continue to operate, they can define distinct domains of gene expression.

For example, local cell–cell interactions can stabilize domains of gene expression (e.g., feedback loops with neighboring cells). In these cases, the maintenance of abutting domains of gene expression will be based on position instead of cell lineage. The critical distinction is that cells in the former scenario can move between territories and change their identity accordingly. A number of noncompartmental body subdivisions in *Drosophila* are associated with specific expression of developmental genes (Williams *et al.*, 1993; Calleja *et al.*, 1996). Here the separation of the limb primordia into proximal and distal structures as one example is considered (Figure 2a and b). The wing and – more obviously so – the leg are subdivided along their proximal-distal (PD) axes into segments of morphologically distinct identity. The wing hinge, costa, and wing blade can be considered as separate “segments” (Figure 2b), whereas the leg is subdivided into coxa, trochanter, femur, tibia, and five tarsi (Figure 6c). Several genes are expressed in PD domains of the wing and leg primordia that will give rise to the distinct morphological structures in the adult described above.

2.2.2.1. PD patterning in the *Drosophila* leg The homeodomain proteins Homothorax (Hth) and Extradenticle (Exd) and the Zn-finger protein Teashirt (Tsh) play important roles in defining the proximal territories in legs and wing discs. In the leg, the PD axis is set up under the control of the activities of Dpp and Wg (Diaz-Benjumea *et al.*, 1994; Campbell and Tomlinson, 1995; Lecuit and Cohen, 1997) (Figure 6a). Hh expression in P cells induces Dpp in the dorsal anterior half and Wg in the ventral anterior half of the leg disc (Struhl and Basler, 1993; Diaz-Benjumea *et al.*, 1994). Their combined activities specify a distal region expressing Distal-less (Dll) (Cohen *et al.*, 1989; Cohen, 1990; Diaz-Benjumea *et al.*, 1994) (Figure 6b), in part by repressing Hth and Tsh expressions, which are both required to specify proximal fates (Abu-Shaar and Mann, 1998; Gonzalez-Crespo *et al.*, 1998; Wu and Cohen, 1999). Hth fulfills a dual role in this context: first, it suppresses the action of the Wg and Dpp pathways and, second, it activates the expression of proximal genes such as *tsh* (Abu-Shaar and

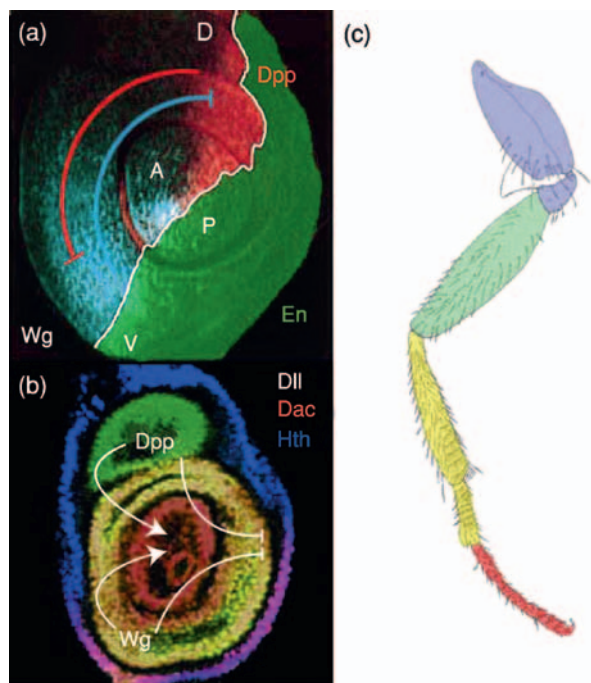


Figure 6 Proximal–distal axis formation during leg development. (a, b) Single confocal images for a third instar leg imaginal disc. (a) Wg protein (cyan) and *dpp-lacZ* (red) are activated in the anterior compartment in the ventral (V) and dorsal (D) regions respectively, by Hh signaling from the posterior compartment (green). The white line denotes the anterior–posterior (AP) boundary. Wg and Dpp repress each other’s expression. (b) Wg and Dpp cooperate to activate the expression of Distal-less (Dll) and Dachshund (Dac) and repress the expression of Hth. (c) Drawing of an adult leg. The colored structures correspond to the different domains of gene expression in (b).

Mann, 1998; Azpiazu and Morata, 2002). Further evidence indicates that Hth but not Tsh is essential for formation of the affinity difference between proximal and distal leg structures (Wu and Cohen, 2000) (Figure 6b).

The expression domain of Hth and Tsh initially forms a sharp border with more distal cells, which express Dll and later Dachshund (Dac) (Lecuit and Cohen, 1997) (Figure 6b). The expression domains appear to be stable but no evidence of a lineage restriction boundary can be found. Indeed, lineage tracing experiments revealed that cells born in the proximal Tsh-expressing region contribute substantially to distal territories in the leg (Weigmann and Cohen, 1999). These cells must have moved from the proximal territory into the distal territory, changing from expression of the proximal genes Tsh and Hth to expression of the distal genes Dac and Dll. These effects can be explained in terms of coupling of growth and patterning of the leg along its PD axis (Wu and Cohen, 1999, 2000).

Dll-expressing cells were also shown to be able to move proximally, provided they are given a relative growth advantage (Weigmann and Cohen, 1999). Interestingly, Dll-expressing cells are able to maintain their distal identity even if Wg and Dpp signaling are inhibited, suggesting that other mechanisms besides the input of these two signals contribute to stabilization of cell fate decisions in the leg (Castelli-Gair and Akam, 1995; Lecuit and Cohen, 1997).

During the third instar, the leg disc is subdivided into presumptive segments. A number of genes expressed in rings in the leg disc prefigure segmentation of the leg (Cohen, 1993). Activation of Notch in cells at the distal end of each forming segment is clearly required for the establishment of segment boundaries, but it remains unclear how the activation of Notch is controlled by the expression of genes along the PD axis (de Celis *et al.*, 1998; Bishop *et al.*, 1999; Rauskolb and Irvine, 1999). Notch activation along the segment boundaries has a nonautonomous effect on proliferation and survival of surrounding cells (de Celis *et al.*, 1998; Kerber *et al.*, 2001). Still later, the presumptive tarsal region of the leg is further subdivided to produce the individual tarsal segments. Recently it has been shown that a gradient of EGFR activity mediated by the distal expression of its ligand Vein (Vn) and the membrane protein Rhomboid (Rho) is necessary for the patterning of these most distal elements (Campbell, 2002; Galindo *et al.*, 2002). At present it is not known how expression of the known Wg and Dpp target genes correlates with the induction of segmentation (Figure 6).

2.2.2.2. PD patterning in the *Drosophila* wing

A similar subdivision between P and D structures takes place in the wing primordium. The notum, wing hinge, costa, and wing blade are easily distinguishable morphological units (Figure 2b). First, the future wing field and the body wall are separated by the mutually exclusive action of Wg and EGFR signaling, respectively (see Section 2.2.1.1.2). Wg signaling activates transcription of the Pit, Oct, Unc (POU)-domain transcription factor *nubbin* (*nub*) and *vestigial* (*vg*) in the wing field and restricts expression of *homothorax* (*hth*), *teashirt* (*tsh*), and *vein* (*vn*) (an EGFR ligand) to the body wall (see Figure 4a). Later, the wing field is further subdivided into domains that will give rise to the wing blade, costa and the hinge articulating the wing with the body wall in the adult animal.

In the wing pouch, Wg and Dpp activate *vg*, which – together with *scalloped* (*sd*) – is required for growth and cell survival of wing blade cells

(Simpson and Schneiderman, 1975; Williams *et al.*, 1994; Kim *et al.*, 1996, 1997b; Halder *et al.*, 1998; Simmonds *et al.*, 1998; Liu *et al.*, 2000). Misexpression and loss-of-function analyses of Vg and Sd revealed that Vg- and Sd-dependent cell affinity differences contribute to the separation of the wing blade from the wing hinge and to a gradient of cell affinities along the PD axis of the wing (Liu *et al.*, 2000). Since Vg expression requires the continuous input of Wg and Dpp signals, the precursor cells of hinge and blade are not strictly separated by cell lineage.

The hinge is a flexible region that is required for wing flapping and for the movement of extension and flexion over the abdomen (Snodgrass, 1935). It originates from rings of tissue surrounding the wing pouch. Wg is required for costa and hinge development (Neumann and Cohen, 1996a). The regulation of Wg expression in the costa and hinge is complex and only partially understood to date. So far only the factors influencing Wg expression in the inner ring (costa) have been analyzed in detail. The *rotund (rn)* gene is a member of the Krüppel family of zinc-finger transcription factors (see **Chapter 1**). Loss-of-function mutations of *rn* lead to a loss of hinge structures among other phenotypes (Del Alamo Rodriguez *et al.*, 2002; St. Pierre *et al.*, 2002). Similarly, the POU-domain transcription factor Nubbin is essential for costa and hinge formation (Ng *et al.*, 1995; Del Alamo Rodriguez *et al.*, 2002). Both *rn* and *nub* are required for Wg expression in the inner ring in a cell-autonomous fashion. Vg expression in the wing pouch is essential for the activation of Wg, Rn, and Nub in the inner ring, indicating that a signal emanating from the Vg-expressing cells in the pouch contributes to patterning in the hinge. The nature of this signal remains to be determined. Interestingly, Wg and Vg coexpression is incompatible with hinge fate (Del Alamo Rodriguez *et al.*, 2002). Maintenance of Wg expression in the hinge is independent of Vg but instead requires Hth. As opposed to the wing blade, Wg activates Hth in the hinge (Azpiazu and Morata, 2000), thereby mediating *wg* autoregulation. In collaboration with Tsh this proximal expression of Hth represses Vg (Casares and Mann, 2000), thereby establishing the new hinge domain.

The notum part of the wing disc will give rise to the thorax (**Figure 2a** and **b**; blue shading). Hth expression is required for notum formation. Vg is required to repress the expression of Hth in distal wing tissue (i.e., the prospective wing blade), thus separating notum and pouch tissues. As in the distal portion of the leg, both Wg and Dpp signaling are responsible for this effect (Azpiazu and Morata, 2000). Hth and Tsh are the only markers of

proximal tissue known to be common in wing and leg primordia. Nub and Dll are required for distal specification in the wing and leg, respectively. Recently, it was found that the *elbow/no ocelli (ell noc)* gene complex is necessary for distal specification in both wings and legs (Weihe *et al.*, 2004), providing evidence for a common molecular basis of wing and leg appendage specification.

2.2.2.3. Formation of vein and intervein regions

Wing veins are the overt morphological readout of the AP patterning system in the adult wing (Sturtevant and Bier, 1995; de Celis, 1998; Bier, 2000; review: Milan and Cohen, 2000a). The establishment of veins and intervein regions depends on both the Hh and Dpp signaling pathways and represents a secondary subdivision of the A and P compartments. The short-range Hh gradient positions vein three (L3) (Mullor *et al.*, 1997; Strigini and Cohen, 1997) and induces expression of the EGFR ligand Vein (Vn), which is in turn required for the establishment of vein four (L4) in nearby P cells (García-Bellido *et al.*, 1994). Hh signaling also controls cell proliferation in this intervein territory (Duman-Scheel *et al.*, 2002).

The veins correspond to four longitudinal stripes of cells, where several genes of the EGFR (*argos*, *veinlet*) and Notch signaling pathways (*Delta*) are expressed. Intervein territories are characterized by the expression of Blistered (Bs), the *Drosophila* homolog of the serum response factor (SRF) transcription factor. Bs activity prevents the formation of vein tissue (Fristrom *et al.*, 1994). The Notch target gene *E(spl)^m* is also expressed in the intervein territories and excluded from the developing veins (review: de Celis, 1998). Dpp signaling along the AP axis leads to the activation of its target genes *spalt-major (salm)* and *spalt-related (slr)* in a broad medial domain of the wing disc (Lecuit *et al.*, 1996; Nellen *et al.*, 1996). The juxtaposition of Spalt-expressing and nonexpressing cells results in the generation of vein L2 and mutations in both genes interfere with formation of vein L5 (de Celis *et al.*, 1996a; Sturtevant *et al.*, 1997).

Differentiation of the veins occurs during the pupal stages through the action of the transcription factor Ventral veinless (Vvl) (de Celis *et al.*, 1995; de Celis and Barrio, 2000). At this stage, it is possible to distinguish a central stripe where EGFR signaling is active, and two adjacent stripes where Notch signaling is active. The cells where EGFR signaling is active will differentiate as veins in the adult wing (Gabay *et al.*, 1997). Activation of Notch in the anterior and posterior lateral stripes limits the width of the vein-forming stripe.

Interestingly, clones of genetically marked cells tend to grow within a particular intervein territory without crossing the presumptive veins (Gonzalez-Gaitan *et al.*, 1994). However, if given a growth advantage over surrounding tissue, clones can cross veins, indicating that veins do not constitute lineage boundaries (Baonza and García-Bellido, 1999). Therefore, intervein regions do not qualify as compartments but are subdivisions separated by differences in gene expression. Differences in cell affinity help to maintain these subdivisions. It has been shown that Hh signaling directly influences cell affinity in the intervein territory between veins 3 and 4 (Blair and Ralston, 1997; Rodriguez and Basler, 1997; Dahmann and Basler, 2000). Activity of Spalt is involved in mediating differential cell affinity between veins 2 and 5 (Milan *et al.*, 2001a).

2.2.2.4. Notum subdivision by Pannier and the Iroquois complex *pannier* and the genes of the *iroquois* complex (*iro-C*) are expressed in a mutually exclusive patterns in the mesothoracic part of the wing imaginal disc. Pannier confers adhesion properties that prevent medial cells from mixing with lateral cells that express Iro-C. This subdivision does not produce a cell lineage boundary (Calleja *et al.*, 1996). The Iro-C complex (consisting of three related homeodomain proteins; Mirror (Mirr), Araucan (Ara), and Caupolican (Caup)) is required to specify lateral thoracic body wall (Gomez-Skarmeta and Modolell, 1996; McNeill *et al.*, 1997; Cavodeassi *et al.*, 2001). Early in development, Iro-C is expressed in the entire thoracic body wall due to the activity of Dpp (Cavodeassi *et al.*, 2002). Cells mutant for *iro-C* transform the lateral body wall into proximal hinge structures (Diez del Corral *et al.*, 1999), suggesting that the genes of the Iro-C complex fulfill selector-like roles for body wall development. Interestingly, the Iro-C complex is also required for DV specification in the developing eye and head (Dominguez and de Celis, 1998; Cavodeassi *et al.*, 2000; Pichaud and Casares, 2000).

2.3. Morphogen Gradients and Pattern Formation

Compartment boundaries are the sources of morphogens. Morphogens are signaling molecules that are produced from a localized source forming long-range concentration gradients that pattern a field of cells (Figure 7a). Cells interpret their position as a function of the amount of signal they receive, thus obtaining “positional information” (Wolpert, 1989, 1996). Signaling molecules have to fulfill two

stringent criteria to qualify as morphogens: (1) their effect must be exerted in a concentration-dependent manner and (2) they must act directly on target cells at a distance from the source (i.e., not through a secondary relay mechanism). When these criteria are met, the local concentration can be interpreted as a measure of distance from the source of the signal (Figure 7a). The three signaling proteins that qualify as morphogens in *Drosophila* wing development are Hedgehog (Hh), Decapentaplegic (Dpp), and Wingless (Wg) (e.g., Wg is shown in Figure 7).

It is generally accepted that cells interpret the gradient by eliciting differential transcriptional responses depending on the concentration of morphogen they are exposed to. This requires cells to make decisions depending on different threshold levels of signaling pathway activity. The concept implies that a single event – namely the production of a secreted molecule at a localized source – can lead to the formation of several different cell types in a correct spatial relationship to each other (Figure 7). This represents a highly efficient way of generating complex patterns in previously uncommitted cells (review: Gurdon and Bourillot, 2001).

Different modes of morphogen movement/transport have been invoked to explain long-range gradient formation: extracellular diffusion of the secreted molecule (van den Heuvel *et al.*, 1989 (discussed in Lander *et al.*, 2002); McDowell *et al.*, 1997; Pfeiffer *et al.*, 2000; Strigini and Cohen, 2000; Teleman and Cohen, 2000; Teleman *et al.*, 2001), cycles of receptor-mediated endocytosis and resecretion (planar transcytosis) (Entchev *et al.*, 2000; Entchev and Gonzalez-Gaitan, 2002), membranous exosomes (argosomes) (Greco *et al.*, 2001), and cytoplasmic extensions (cytonemes) (Ramirez-Weber and Kornberg, 1999). Although there is no compelling evidence to date that mechanisms other than diffusion contribute productively to gradient formation, it is certainly plausible that these mechanisms could do so. Cytonemes and argosomes exist, but have not yet been shown to be required to mediate ligand movement or to transduce signals. Evidence presented in favor of endocytosis and resecretion as a mechanism of ligand transport has been questioned (Lander *et al.*, 2002). Further experimental evidence is needed to support or refute these possibilities.

Several factors have been implicated in shaping morphogen gradients: posttranslational modification of the ligand can influence diffusibility (e.g., acylation of Hh and Wg (Ingham, 2000, 2001)). Regulation of receptor levels can influence ligand

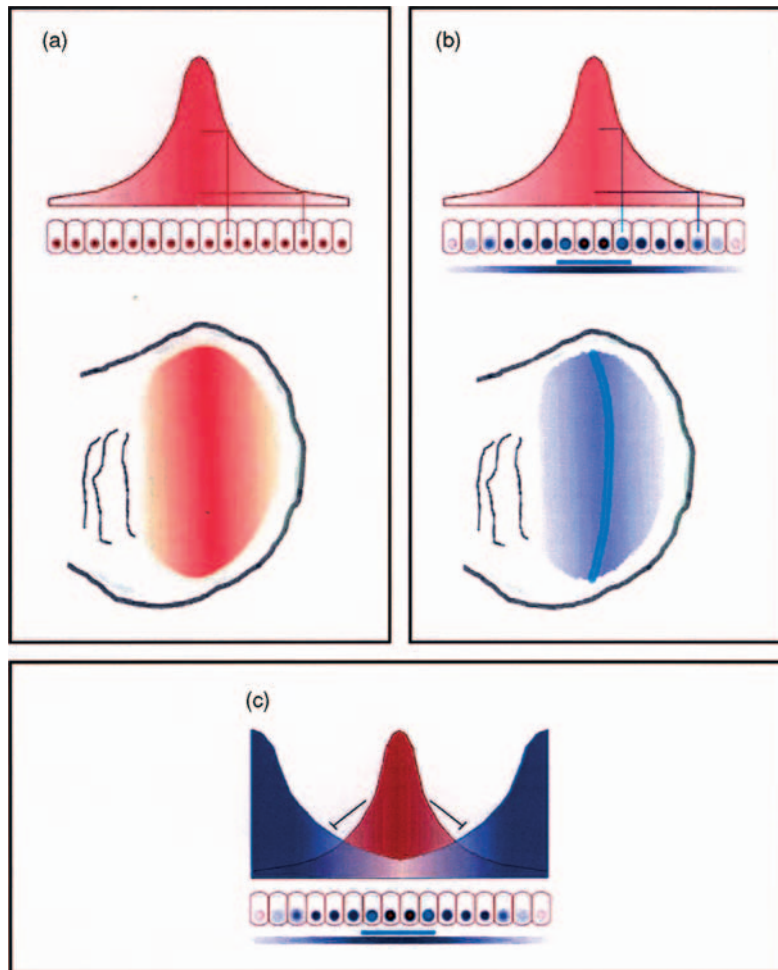


Figure 7 Example of a morphogen gradient: Wingless (Wg). (a) Drawing of a section of the wing pouch epithelium (upper panel) and of the wing pouch in an apical view (lower panel). The cells in the center express Wg which generates a gradient across the adjacent tissue. Cells at different distances receive different levels of Wg. (b) Schematic drawing of the Wg gradient and target gene activation in the epithelium in response to Wg (upper panel). The Wg gradient activates the target genes *hindsight* (cyan) and *distalless* (blue) at different threshold levels. The expression domains of Hindsight and Distalless are depicted in the same colors in the lower panel. (c) Wg downregulates its own receptor Drosophila Frizzled 2 (DFz2; blue), shaping its gradient and rendering the cells at a distance from the source of Wg more sensitive.

movement (Chen and Struhl, 1996; Cadigan *et al.*, 1998; Lecuit and Cohen, 1998) (Figure 7c). Cell surface heparan-sulfate proteoglycans, and secreted enzymes that modify them, can modulate ligand movement (The *et al.*, 1999; Baeg *et al.*, 2001; Giraldez *et al.*, 2002; review: Nybakken and Perrimon, 2002). Secreted ligand binding proteins can influence movement or stability (Bouwmeester *et al.*, 1996; Leyns *et al.*, 1997; Wang *et al.*, 1997; Ashe and Levine, 1999; Ross *et al.*, 2001). In addition, cellular responses to these ligands can also be modulated, contributing to shaping the activity gradients rather than the ligand gradients per se (Tsuneizumi *et al.*, 1997; Teleman and Cohen, 2000; Zeng *et al.*, 2000). These factors will be considered in more detail in the following sections.

2.3.1. Hedgehog

2.3.1.1. Hedgehog function The *hedgehog* (*hh*) mutant and gene were originally characterized in *Drosophila* (Nüsslein-Volhard and Wieschaus, 1980; Lee *et al.*, 1992; Mohler and Vani, 1992; Tabata *et al.*, 1992). Hh proteins are widely conserved within the animal kingdom. Hh signaling is involved in diverse developmental processes, and when misregulated, has been implicated in disease (review: Chuang and Kornberg, 2000) (summarized in Figure 3b). Loss of Hh signaling in wing discs impedes growth and patterning in both A and P compartments and gives rise to severely reduced wings (Basler and Struhl, 1994). Hh acts directly to pattern the central region of the wing (Mullor *et al.*,

1997; Strigini and Cohen, 1997). The long-range effects of reduced Hh are secondary consequences due to loss of Dpp expression along the AP compartment boundary in the wing and loss of Dpp and Wg in the leg. Ectopic expression of Hh in A cells at a distance from the AP boundary or activation of signaling by removing Hh pathway inhibitors such as patched (Ptc), protein kinase A (PKA), Costal-2 (Cos-2), or Slimb (Slmb) causes dramatic reorganization of anterior pattern. Ectopic expression of Hh can lead to complete mirror image duplication of anterior structures. This shows the importance of localized Hh signaling during wing development (Basler and Struhl, 1994; Tabata and Kornberg, 1994; Jiang and Struhl, 1995, 1998; Lepage *et al.*, 1995; Li *et al.*, 1995; Pan and Rubin, 1995; Sisson *et al.*, 1997; Theodosiou *et al.*, 1998).

Asymmetry is critical to the function of the Hh patterning system. P cells, which express Hh, are not capable of transducing the Hh signal. They do not express the transcription factor Ci (due to repression by En). Anterior cells that do not receive Hh, process the full-length activator Ci protein (Ci¹⁵⁵ or Ci^A) into a transcriptional repressor form Ci⁷⁵ or Ci^R (Aza-Blanc *et al.*, 1997). Cells close to the boundary that receive Hh input stabilize Ci¹⁵⁵, leading to the induction of Hh target genes. The distance from the Hh source determines which Hh target genes are activated (Figure 3b). Hh can therefore be defined as a morphogen (Strigini and Cohen, 1997). Hh regulates *dpp*, *wg*, *ptc*, *en*, and *collier* (synonym: *knot*) expression in a concentration-dependent manner (Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Tabata and Kornberg, 1994; Chen and Struhl, 1996; Mullor *et al.*, 1997; Strigini and Cohen, 1997; Vervoort *et al.*, 1999).

2.3.1.2. Hedgehog production Hh protein is produced as a precursor that undergoes autoproteolytic intramolecular cleavage by an intein-like mechanism (Chang *et al.*, 1994; Lee *et al.*, 1994; Porter *et al.*, 1995). This generates a 20 kDa N terminal (Hh-N) and a 25 kDa C terminal fragment (Hh-C). The enzymatic activity required for the cleavage reaction is contained within the C terminal domain (Lee *et al.*, 1994; Porter *et al.*, 1995), while the N terminal domain accounts for all known signaling activity (Porter *et al.*, 1995). During the cleavage reaction, a cholesterol moiety is covalently attached to the C terminal part of Hh-N (Porter *et al.*, 1995). Cholesterol modification may be responsible for the observed tight cell association of Hh-N to Hh producing cells. The range over which Hh-N can move and signal is greatly extended when the cholesterol

modification is prevented (Porter *et al.*, 1996; Burke *et al.*, 1999). In addition, Hh is palmitoylated at its N terminus. This lipid modification is also essential for Hh function. In the absence of the acyltransferase Skinny Hedgehog (Ski), Hh lacks the N terminal palmitate residue and cannot fulfill its embryonic and larval patterning activities (Chamoun *et al.*, 2001; Lee and Treisman, 2001).

2.3.1.3. Hedgehog movement How do these post-translational modifications impact on Hh movement? The cholesterol and palmitate moieties presumably bind Hh to the cell surface and might be expected to preclude diffusion. Yet Hh protein and Hh activity can be detected many cell diameters away from the cells where it is produced (Tabata and Kornberg, 1994; Mullor *et al.*, 1997; Strigini and Cohen, 1997, 1999; Chuang and Kornberg, 2000). The release of cholesterol-modified Hh-N from the producing cells depends on the activity of the protein Dispatched (Disp) in Hh-producing cells (Burke *et al.*, 1999; Caspary *et al.*, 2002; Ma *et al.*, 2002; Pfeiffer *et al.*, 2002). The *disp* mutant cells appear to produce, process, and modify Hh normally but are unable to release Hh-N and instead accumulate the protein to high levels. Intriguingly, *disp* is predicted to encode a sterol-sensing domain (SSD)-containing 12-pass transmembrane protein with sequence homology to the Hh-binding receptor protein Ptc. Ptc has been shown to limit Hh movement, in part, by targeting Hh for internalization and degradation (Chen and Struhl, 1996; Burke *et al.*, 1999; Deneff *et al.*, 2000; Incardona *et al.*, 2000, 2002). Thus, both the sending and the receiving cells require the activity of an SSD-containing protein to regulate the movement of Hh-N. Receiving cells need Ptc to sequester cholesterol modified Hh-N while sending cells need Disp to release it. How exactly Disp works in Hh release remains to be determined. It is also not known how Hh moves across tissues. This may be a case where exovesicle (argosome)-mediated movement may prove to be important.

Once Hh is released, its ability to move is influenced by heparan sulfate proteoglycans (HSPGs) (Bellaiche *et al.*, 1998; Selleck, 2000). The *tout-velu* (*ttv*) gene encodes an enzyme needed for HSPG biosynthesis and its activity is needed to allow movement of cholesterol-modified Hh-N in A cells (The *et al.*, 1999; Toyoda *et al.*, 2000). HSPGs are highly O-glycosylated proteins found abundantly at the cell surface and the extracellular matrix. HSPGs interact with a variety of extracellular proteins such as growth factors, proteases, protease inhibitors, and adhesion molecules. Through

these interactions, they participate in many events during cell adhesion, migration, proliferation, and differentiation. Biochemical analyses have indicated that Hh is a heparin-binding protein (Lee *et al.*, 1994). How exactly Ttv and HSPGs affect the distribution of Hh-N is still unknown. Hh-N may require HSPGs for its stability in the extracellular space. Alternatively, binding of Hh-N to HSPGs could promote its release from producing cells, prevent its reinsertion into the membrane or its sequestration by Ptc. One could speculate that transport of lipid-modified Hh depends on a yet unidentified carrier protein able to mask the lipid moiety. The possibility of a connection between such a protein and the HSPGs remains to be explored.

2.3.1.4. Direct patterning activity of Hedgehog Although the largest morphological changes observed in wings lacking or ectopically activating Hh signaling are caused by changes in the expression of the Hh target gene *Dpp*, Hh also has a more direct effect on patterning. Hh acts directly to pattern the central region of the wing, more specifically the region between the veins 3 and 4 (Mullor *et al.*, 1997; Strigini and Cohen, 1997). This short-range patterning activity of Hh is at least in part mediated through the local induction of En and the transcription factor Collier (Col) (Nestoras *et al.*, 1997; Vervoort *et al.*, 1999). Another important function of Hh, mediated through a Ci-dependent but thus far unidentified target gene, is preventing A and P cells from mixing, thus maintaining the AP compartment boundary (Blair and Ralston, 1997; Rodriguez and Basler, 1997; Dahmann and Basler, 2000) (Figure 3a).

2.3.2. Decapentaplegic

Decapentaplegic (*Dpp*) is a member of the bone morphogenetic protein (BMP) class of transforming growth factor- β (TGF- β) signaling molecules (Hoffmann and Goodman, 1987; Irish and Gelbart, 1987; Padgett *et al.*, 1987; reviews: Padgett *et al.*, 1998; Raftery and Sutherland, 1999; Massague and Chen, 2000). Many different biological processes are mediated by TGF- β ligands. These include the regulation of growth, differentiation, and cell fate determination. Defects in TGF- β signaling components have been implicated in a number of heritable disorders (review: Massague *et al.*, 2000).

2.3.2.1. *Dpp* signaling In *Drosophila*, three BMP-like factors are known: *Dpp*, Glass bottom boat (*Gbb*), and Screw (*Scw*). *Dpp* seems to be the most important factor for limb development. TGF- β factors initiate signaling by assembling receptor

complexes that activate Smad transcription factors. The ligand brings together members from two families of receptor serine/threonine kinases, known as the type I and type II receptors (reviews: Raftery and Sutherland, 1999; Massague and Chen, 2000; Massague *et al.*, 2000). The principal *Dpp* receptors in *Drosophila* are Thickveins (*Tkv*) and Punt (*Pnt*), type I and II, respectively (Brummel *et al.*, 1994; Letsou *et al.*, 1995; Ruberte *et al.*, 1995). Saxophone may also cooperate with *Pnt* to mediate *Dpp* signaling. The only known function of the type II receptor (*Pnt*) is to activate the type I receptor (*Tkv*). *Tkv* propagates the signal by phosphorylating the *Drosophila* Smad, Mad (Mothers against *Dpp*) (Newfeld *et al.*, 1996; Wiersdorff *et al.*, 1996). In addition to Mad, which belongs to the class of receptor-regulated Smads (R-Smads), *Dpp* signaling requires the action of the co-Smad Medea (*Med*) (Das *et al.*, 1998; Hudson *et al.*, 1998; Wisotzkey *et al.*, 1998). Upon phosphorylation of Mad by *Tkv*, it associates with Medea and translocates into the nucleus, where the complex directly binds to DNA and regulates transcription (e.g., Kim *et al.*, 1997a; review: Raftery and Sutherland, 1999). However, activation of the *Dpp* pathway at high levels induces the expression of an inhibitory Smad (*Dad*), which acts to reduce *Dpp* signaling activity in these cells, thereby limiting the maximal extent of pathway activation (Tsuneizumi *et al.*, 1997). The transcription factor Brinker (*Brk*) is another antagonist that modulates cellular responsiveness to *Dpp* signaling. Brinker represses expression of *Dpp* target genes in the wing disc (Campbell and Tomlinson, 1999; Jazwinska *et al.*, 1999; Minami *et al.*, 1999). Interestingly, *brinker* expression is negatively regulated by *Dpp*, providing another level of feedback regulation.

2.3.2.2. Regulation of *Dpp* gradient formation

Dpp is the main organizer of patterning along the AP axis in the wing imaginal disc. *Dpp* is produced by A cells adjacent the AP compartment boundary in the *Drosophila* wing (Figure 3b) and forms a long-range gradient that activates several target genes in a concentration-dependent manner (Lecuit *et al.*, 1996; Nellen *et al.*, 1996; Lecuit and Cohen, 1998; Entchev *et al.*, 2000; Teلمان and Cohen, 2000; Entchev and Gonzalez-Gaitan, 2002). Whether free diffusion alone can explain *Dpp* gradient formation remains unclear. Receptor-mediated endocytosis contributes to shaping the *Dpp* gradient by targeting some *Dpp* for degradation (Entchev *et al.*, 2000; Teلمان and Cohen, 2000). Downregulation of the receptor protein *Tkv* in response to *Dpp* signaling is needed to

allow long-range movement of the ligand (Lecuit and Cohen, 1998; Tanimoto *et al.*, 2000). Tkv downregulation is mediated by *mtv*, itself a target gene of En and Hh (Funakoshi *et al.*, 2001). Receptor-mediated endocytosis has also been proposed to be required for Dpp transport (Entchev *et al.*, 2000), though this proposal has been challenged (Lander *et al.*, 2002).

Different levels of Dpp signaling activity lead to further, noncompartmental, subdivision along the AP axis in the *Drosophila* wing disc. The *spalt-major* (*salm*) and *spalt-related* (*slr*) genes are expressed in broad domains centered on the AP boundary in response to Dpp signaling (de Celis *et al.*, 1996a; Lecuit *et al.*, 1996), subsequently positioning specialized cells to form the wing veins (de Celis *et al.*, 1996a; Sturtevant *et al.*, 1997). *Salm* and *Slr* regulate the expression domains of *Caps* and *Tartan* and confer a medial-lateral affinity difference that contributes to regionalization along the AP axis (Milan *et al.*, 2001a). The *optomotor blind* gene is expressed in a broader domain under Dpp control and is required for cell survival in the center of the wing disc (Grimm and Pflugfelder, 1996; Lecuit *et al.*, 1996).

2.3.3. Wingless

Wingless (Wg) is a member of the Wnt family of secreted glycoproteins (Rijsewijk *et al.*, 1987) and fulfills a number of roles during development. Wg forms an extracellular gradient in the wing imaginal disc (Strigini and Cohen, 2000) and activates several target genes in a concentration-dependent manner to organize the DV axis of the wing (Zecca *et al.*, 1996; Neumann and Cohen, 1997) and leg (Struhl and Basler, 1993; Brook and Cohen, 1996; Jiang and Struhl, 1996) (Figures 4b, c, and 7) and the PD axis in the leg (Lecuit and Cohen, 1997) (Figure 6a). Hence it can be defined as a morphogen. Here, the aspects of its production, secretion, and travel in the imaginal discs will be discussed. The Wg signal transduction pathway has been reviewed elsewhere (Wodarz and Nusse, 1998).

2.3.3.1. Wg production Wg secretion requires the activity of the gene *porcupine* (*por*). Cells mutant for *por* show normal expression of the Wg protein but fail to secrete it. *por* encodes a multipass transmembrane protein with similarity to the acyltransferase *ski*, suggesting that Porcupine might acylate Wg (van den Heuvel *et al.*, 1993; Kadowaki *et al.*, 1996; Chamoun *et al.*, 2001; Lee and Treisman, 2001). In embryos, localized apical secretion of Wg has been shown to be important for its function (Simmonds *et al.*, 2001). It is not known

whether polarized secretion is important in the imaginal discs.

2.3.3.2. Wg movement Diffusion of extracellular Wg protein has been shown to be sufficient for gradient formation in the wing disc (Strigini and Cohen, 2000). Mechanisms involving endocytosis/resecretion and argosomes have also been proposed to explain Wg movement. Wg secretion requires dynamin activity; however, once secreted, Wg movement does not require dynamin-mediated endocytosis/exocytosis (Strigini and Cohen, 2000). Indeed, *dynammin* mutant cells accumulate higher than normal levels of extracellular Wg protein, suggesting that endocytosis plays an important role in shaping the gradient by removing Wg (Strigini and Cohen, 2000).

Evidence for vesicle-based transport of Wg has been presented for the embryo. Progeny of Wg-producing cells move away from the segment boundary in the embryo, allowing delayed secretion of Wg by cells at a distance from the compartment boundary where Wg mRNA is expressed (Pfeiffer *et al.*, 2000). For this reason, a membrane-tethered form of Wg is sufficient to confer a normal range of Wg signaling in the embryo. This mechanism might operate in conjunction with diffusion of secreted Wg. Endocytosis of Wg promotes its degradation in lysosomes (Dubois *et al.*, 2001). However, resecretion of Wg has also been shown to occur, raising the possibility that this mechanism may also contribute significantly to Wg movement in the embryo (Moline *et al.*, 1999; Pfeiffer *et al.*, 2002).

Another mechanism involving vesicle-mediated transport has been proposed based on the observation of Wg-containing exovesicles, called argosomes (Greco *et al.*, 2001). Argosomes were shown to move through the wing disc epithelium, showing a high degree of colocalization with Wg (review: Vincent and Magee, 2002). Despite the lack of direct evidence that these Wg-containing vesicles are required for gradient formation, it is certainly plausible that argosomes contribute to Wg movement. If Wg proves to be acylated, like Hh, argosomes may provide the means to move the lipid-modified ligand.

HSPGs also play an important role in Wg movement and signaling. HSPGs bind Wg at the cell surface and are required for cells to be Wg-responsive (Reichsman *et al.*, 1996). Genetic analysis of components of the biosynthetic pathway of proteoglycans such as Fringe connection (*Frc*) (Selva *et al.*, 2001), Sugarless (*Sgl*) (Binari *et al.*, 1997; Hacker *et al.*, 1997; Haerry *et al.*, 1997), Sulfateless (*Sfl*) (Lin *et al.*, 1999; Baeg *et al.*, 2001), Notum

(Gerlitz and Basler, 2002; Giraldez *et al.*, 2002), and the glypican core proteins Dally and Dally-like (Dlp) (Lin *et al.*, 1999; Tsuda *et al.*, 1999; Baeg *et al.*, 2001) revealed that they influence Wg signaling and/or movement (reviews: Bernfield *et al.*, 1999; Lin and Perrimon, 2000; Perrimon and Bernfield, 2000; Selleck, 2001). It is, therefore, possible that HSPGs act as low-affinity receptors to stabilize Wg or, perhaps, as coreceptors to facilitate interaction with the signaling receptors Frizzled (Fz) and *Drosophila* Frizzled 2 (DFz2). Interestingly, most mutations that affect proteoglycan biosynthesis also affect other signaling molecules. Mutations in *dally* genetically interact with *dpp* (Jackson *et al.*, 1997), while *sgl* and *sfl* also affect fibroblast growth factor (FGF) signaling (Lin *et al.*, 1999).

The *Drosophila* Wg receptor DFz2 is downregulated in response to Wg signaling (Cadigan *et al.*, 1998). This reduces the sensitivity of cells exposed to high levels of Wg and allows Wg to travel farther (Figure 7c). Response to Wg activity is also modulated by Wg-dependent induction of Naked (Nkd), an EF hand containing protein that serves as a signaling antagonist (Rousset *et al.*, 2001, 2002; Zeng *et al.*, 2000). Wg also regulates the expression of another secreted inhibitor, Notum (Giraldez *et al.*, 2002). These mechanisms collaborate to fine-tune different morphogen gradients. This allows the formation of a reproducible pattern of differentiation and a constant shape and size during animal development.

2.4. Growth Control in *Drosophila* Limbs

The size and shape of the adult appendages are determined by the rates of cell division, growth, and death in the imaginal discs (reviews: Bryant and Simpson, 1984; Day and Lawrence, 2000). How is the final and constant size of the discs controlled during development?

2.4.1. Extrinsic Regulation of Size

Growth of the imaginal discs is influenced by the nutritional state of the larva. This does not influence body pattern per se, only tissue size (Johnston *et al.*, 1999; Britton *et al.*, 2002). One mediator of nutrition-dependent size control is the Insulin/PI3-kinase pathway (Chen *et al.*, 1996; Leever *et al.*, 1996; Montagne *et al.*, 1999; Weinkove *et al.*, 1999; Britton *et al.*, 2002; Ikeya *et al.*, 2002; Oldham *et al.*, 2002; reviews: Edgar, 1999; Oldham *et al.*, 2000). In addition to insulin, PI3-kinase signaling can be activated by other growth factors and

cytokines. PI3-kinase signaling influences the protein biosynthetic capacity of cells by activation of S6-kinase (S6K, the kinase that phosphorylates 40S ribosomal protein S6) (Montagne *et al.*, 1999; Thomas, 2002). Activation of target of rapamycin (TOR) signaling is also dependent on nutritional conditions and also directly influences S6K through a parallel, insulin-independent pathway (reviews: Gingras *et al.*, 2001; Oldham and Hafen, 2003). TOR-dependent activation of S6K results in direct activation of translation initiation factors and upregulation of a subset of ribosomal RNAs (Jefferies *et al.*, 1997; Dennis *et al.*, 1999; Meyuhas and Hornstein, 2000).

Thus, nutrition-dependent growth control by different pathways seems to be integrated by the regulation of S6K. These two pathways seem to be linked through the action of the *tuberous sclerosis complex* (*tsc*) genes. Tsc1 and Tsc2 form a complex that inhibits TOR, preventing growth-dependent protein synthesis. In the presence of growth factors, PI3-kinase signaling results in the phosphorylation of Tsc1 and Tsc2. This relieves inhibition of TOR and stimulates protein synthesis (Inoki *et al.*, 2002; Manning *et al.*, 2002; review: Marygold and Leever, 2002).

2.4.2. Intrinsic Regulation of Size

During development, growth and patterning appear to be tightly linked. By and large patterning appears to determine growth and not vice versa (review: Day and Lawrence, 2000) but there are clear exceptions (Teleman and Cohen, 2000). One view is that size is specified by morphogen gradients emanating from the compartment boundaries (Lawrence and Struhl, 1996; Serrano and O'Farrell, 1997). Clearly, Wg and Dpp signaling are capable of inducing growth and cell cycle progression in a context-dependent manner (Nellen *et al.*, 1996; Neumann and Cohen, 1996b, 1997; Martin-Castellanos and Edgar, 2002). However, morphogens cannot simply act as mitogens because they persist within the limb primordium, even after the organ has reached its final size, and proliferation rates do not correlate simply with proximity to the signaling centers.

The polar coordinate model (French *et al.*, 1976; Bryant and Simpson, 1984) proposed an explanation by suggesting that growth stops once the set of positional values produced by the morphogen gradients is completed: juxtaposition of cells with disparate positional values triggering growth and intercalation. ("Intercalation" is a term used to indicate the insertion or introduction of anything among others; in this case introduction of structures of

intermediate positional value.) Experimentally induced discontinuities in positional values would be expected to result in intercalary growth. This was certainly the case when discontinuities were generated by surgical removal of a fragment of an imaginal disc leading to regeneration. However, when positional discontinuities were produced by genetic mosaics, no intercalary growth or patterning was observed. For example, clones of cells expressing activated components of morphogen signaling pathways (such as the activated form of the Dpp receptor Tkv) do not trigger intercalary cell proliferation or patterning whereas clones expressing the secreted signaling proteins do so (Lecuit *et al.*, 1996; Nellen *et al.*, 1996). Overexpression of Dpp in patterns expected to abolish the gradients also fail to block growth. Thus, there is compelling evidence against the idea that growth is driven by intercalation of missing positional values.

Detailed analyses of cell proliferation patterns and cell lineage analysis using X-ray induced clones during larval development have shown no correlation between growth, division, and the distance of a cell from the compartment boundaries (Gonzalez-Gaitan *et al.*, 1994; Milan *et al.*, 1996). Groups of neighboring cells divide synchronously with respect to their cell cycle stage; these groups are located at random positions throughout the wing anlage. The evidence currently available suggests that morphogen gradients are read out at quite low spatial resolution. Noncompartmental subdivisions of both wings and legs (e.g., wing interveins or leg segments) behave as semiautonomous units of growth control (review: Milan and Cohen, 2000a). Thus, elimination of a vein or a leg joint causes a reduction in size of the two adjacent intervein or leg segments territories to that of a single one (de Celis *et al.*, 1996a; de Celis, 1998). There is no strong effect on the size of surrounding territories. These observations are difficult to reconcile with a model in which the morphogen gradients directly control final wing or leg size. Rather, they suggest that continuous subdivision of the limb primordia into smaller territories may be an important intermediate step in the growth regulation process.

An alternative to the polar coordinate model, the Entelechia model, has been proposed by Antonio García-Bellido (García-Bellido and de Celis, 1992; García-Bellido and García-Bellido, 1998). According to this model, cell proliferation is driven by differences in the activity of certain genes (“Martial genes”). Although the nature of these genes is not defined, it is assumed that the level of their activity can be communicated to neighboring cells, so that differences in level can trigger division of nearby

cells. It is assumed in the model that the level of Martial genes is increased in the daughter cells, thus leading to a propagation of a wave of cell division across the tissue. An initial asymmetry in the activity levels of Martial genes is needed to trigger the process. This is envisaged to result from differences in cells on opposite sides of compartment boundaries. Cell proliferation stops when the differences between cells fall below detection thresholds, thus reaching the Entelechia condition (“Entelechia”: Aristotelian term for completion or perfection). This self-assembly model explains how short-range cell interactions control final organ size, independently of morphogen gradients. Although logically consistent, this model is quite abstract. It is not evident what sort of signals could propagate across the epithelium. The basic premise that proliferation in one group of cells should drive a wave of proliferation that travels across the tissue is, in principle, testable using genetic mosaics.

Yet there is evidence that molecules involved in patterning such as Notch, Wg, and Dpp can drive cell proliferation. Overactivation of Dpp signaling causes accelerated wing cell growth and cell cycle progression in a coordinated way (Martin-Castellanos and Edgar, 2002). Experimental removal of Dpp, alternatively, leads to undergrowth (Burke and Basler, 1996; Kim *et al.*, 1996). It has been suggested that Dpp acts as a survival factor and that its loss eventually results in removal of tissue from the epithelium by apoptosis (Moreno *et al.*, 2002). Thus, while patterning molecules have been shown to influence cell proliferation, their role is more likely to be of permissive than of instructive nature.

2.5. Evolutionary Conservation of Limb Development

Vertebrate limb development has also been extensively studied because it provides an excellent experimental model to study the cellular and molecular mechanisms that regulate pattern formation during embryogenesis. A combination of classical embryological studies, ectopic expression analyses in the chick embryo, and gene disruption in the mouse has contributed to understanding how growth and patterning are controlled in the vertebrate limb bud (reviews: Johnson and Tabin, 1997; Schwabe *et al.*, 1998; Capdevila and Izpisua Belmonte, 2001; Tickle and Munsterberg, 2001). There is ample evidence now that similar genes are involved in both vertebrate and invertebrate appendage formations. Does that mean that vertebrate and invertebrate limbs are homologous structures? Since molecular

tools have become available, a more rigorous examination of the evolutionary conservation of limb development is possible. In this section, a brief review is done on the mechanisms underlying vertebrate appendage development and compare them with invertebrate limb development, as exemplified by *Drosophila*.

The limb primordium (limb bud) is composed of mesenchymal cells derived from the somatic portion of the lateral plate mesoderm (LPM) and is covered by a layer of ectoderm (Figure 8). It appears at specific locations along the body axis of the developing embryo and will grow out of the body wall to form the limb. Interactions between the mesenchyme and the ectoderm coordinate growth and patterning of the primordium through the activity of organizing centers such as the apical ectodermal ridge (AER) and the zone of polarizing activity (ZPA).

2.5.1. Initiation of Limb Budding

The mechanisms that allocate the limb fields within the lateral plate mesoderm are still a matter of debate. It has been suggested that the establishment of overlapping domains of *Hox* gene expression prepatterns the embryonic flank at specific positions for limb induction. This proposal is based on *Hox* expression patterns; no functional studies have been performed to date (Oliver *et al.*, 1990; Rancourt *et al.*, 1995).

The onset of limb bud formation is marked by a bulge in the lateral wall of the embryo. It results from the continuous proliferation of cells in the prospective limb-forming region and a concomitant decrease in cell proliferation elsewhere along the length of the LPM (Searls and Janners, 1971). Signaling mechanisms that mediate directional transfer of information between different tissues such as the intermediate mesoderm (IM), the LPM, and the surface ectoderm are involved in initiation of the limb bud. It is not entirely clear, to date, which factors are responsible for induction of limb outgrowth. One mesenchymal signal that is essential for limb outgrowth is the product of the *Fgf10* gene (Figure 8a and b). *Fgf10* is a member of the FGF family of secreted proteins that signal by binding to high-affinity cell surface receptors (FGFRs), thereby activating intracellular signaling pathways including the RAS-MAPK pathway. *Fgf10* is initially widely expressed in the LPM but at the stage of limb initiation is restricted to the prospective limb regions. Both loss-of-function and gain-of-function analyses have shown that *Fgf10* is a key factor in limb bud initiation (Ohuchi *et al.*, 1997; Min *et al.*, 1998; Sekine *et al.*, 1999). What induces *Fgf10* in the

LPM is unclear, though. *Fgf8* expressed in the intermediate mesoderm was originally proposed to mediate limb outgrowth by inducing *Fgf10* (e.g., Cohn *et al.*, 1995). The juxtaposition of IM and LPM was considered to be essential for this process, since ablation of the mesonephritic mesoderm by laser microsurgery abolished limb development (Geduspan and Solursh, 1992). However, genetic evidence for a role of *Fgf8* in the IM for limb field formation is lacking. Recently it has been reported that *Wnt-2b* and *Wnt-8c* (like *Wg* members of the *Wnt* family of secreted signaling molecules) control *Fgf10* expression in the chick forelimb and hindlimb, respectively (Kawakami *et al.*, 2001; Ng *et al.*, 2002; review: Martin, 2001) (Figure 8a). Whether or not this signal emanates from the IM and if other axial structures like the embryonic organizer (Hensen's node) or the somites are required for limb field initiation remains unclear.

2.5.2. Induction of the AER and Limb Outgrowth

Shortly after the limb bud first becomes visible, changes in cell shape within the surface ectoderm result in the appearance of a ridge that runs along the distal margin of each limb bud (Figure 8c). Once this AER has formed, the limb bud elongates along its PD axis, flattens along its DV axis, and becomes asymmetric along its AP axis. AER formation is a critical event because it produces signals that organize limb development (review: Capdevila and Izpisua Belmonte, 2001). The integrity of the AER is essential for continued cell proliferation after initiation of limb budding (Todt and Fallon, 1984). Surgical removal of the AER results in limb truncation at any time during development.

Several genes expressed in the surface ectoderm already show a DV asymmetry prior to AER induction, e.g., *Radical fringe* (*Rfng*) and *En-1* (one of the vertebrate *engrailed* homologs). *Rfng* is expressed in the dorsal ectoderm (Laufer *et al.*, 1997; Rodriguez-Esteban *et al.*, 1997), whereas *En-1* is expressed exclusively in ventral cells (Davis and Joyner, 1988). The AER forms right at the interface between the *Rfng* expressing and nonexpressing cells, *En-1* ensuring repression of *Rfng* ventrally. Through this mechanism, a sharp boundary is maintained. Ectopic expression of *En-1* in dorsal cells was reported to repress *Rfng* in the chick embryo and to induce ectopic AERs and outgrowths, showing that the juxtaposition of *Rfng* positive and negative cells is able to set up the AER (Laufer *et al.*, 1997; Rodriguez-Esteban *et al.*, 1997). However, *Rfng*^{-/-} mice show normal limb development (Moran *et al.*, 1999b). Functional redundancy with

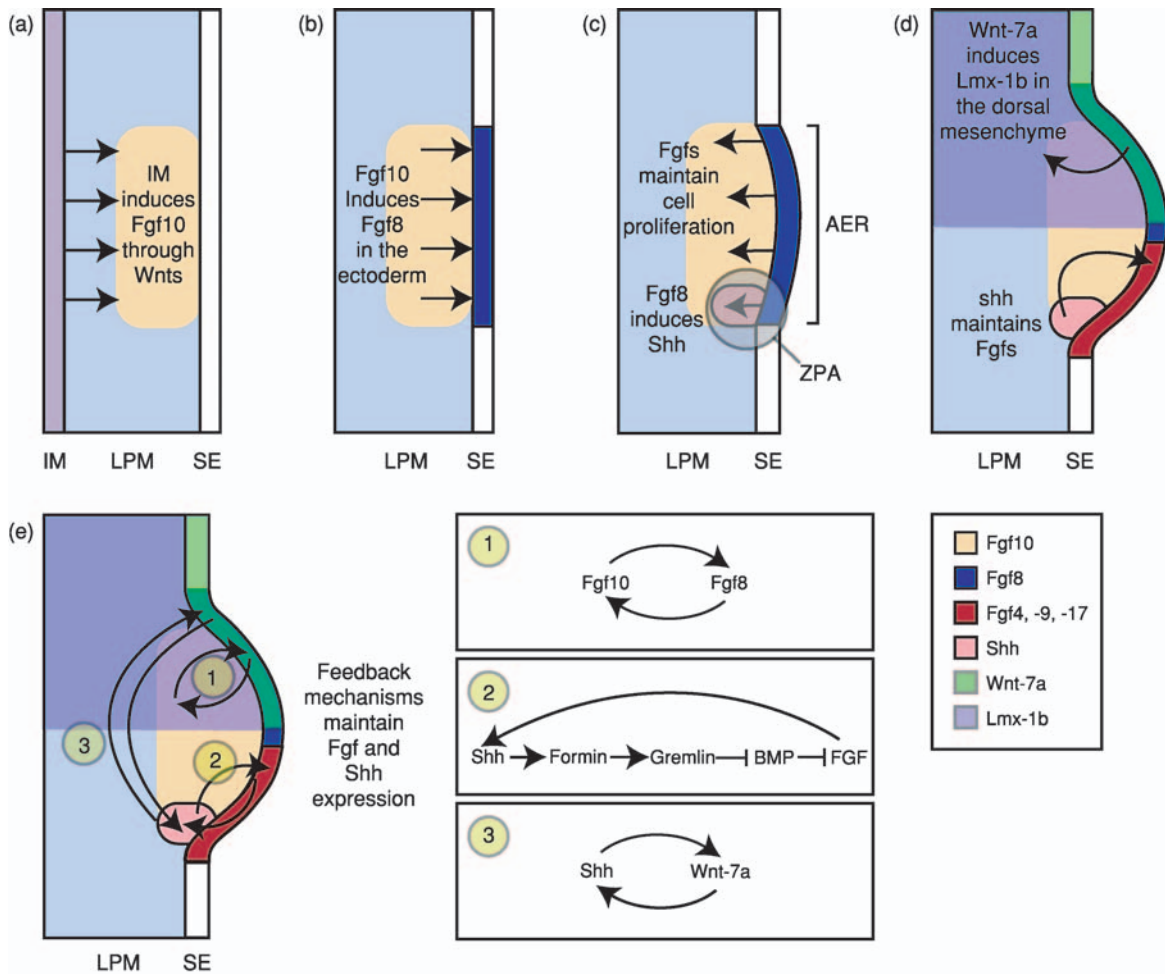


Figure 8 Vertebrate limb development. (a) Signaling from the intermediate mesoderm (IM) involving members of the Wnt family induces expression of Fgf10 in the lateral plate mesoderm (LPM). (b) Lateral plate Fgf10 induces Fgf8 expression in the surface ectoderm (SE), establishing the apical ectodermal ridge (AER). (c) Fgf signaling from the AER maintains cell proliferation in the underlying mesenchyme. Fgf8 signaling from the AER induces the expression of Sonic hedgehog (Shh) in the ventral mesenchyme, establishing the zone of polarizing activity (ZPA). (d) Shh signaling maintains Fgf signaling in the AER. Wnt-7a signaling induces the expression of Lmx-1b in the dorsal mesenchyme, where it is essential for dorsal specification. (e) Three feedback mechanisms maintain coordinate activity of the organization of three body axes. Fgf10 and Fgf8 maintain cell proliferation in the mesoderm, ensuring proper development of the proximal–distal axis. Fgf signaling from the AER maintains Shh in the ZPA. Wnt signaling from the dorsal ectoderm is required for Shh maintenance in the ZPA. The ZPA is the integration point for axis coordination in the vertebrate limb. Details of the autoregulatory loops 1–3 are shown at right.

other Rfng homologs (Lunatic Fringe and Manic Fringe) is unlikely, because expression of neither of them was detectable in the limb primordium (Johnston *et al.*, 1997; Moran *et al.*, 1999a). Whether these apparently contradictory results can be explained in terms of differences between the mouse and the chicken remains to be determined.

Fgf10 signaling from the LPM to the somatic ectoderm is essential for AER formation. It has been proposed that Fgf10 may be the mesenchymal mediator of the limb induction signal and thus function as the endogenous inducer of Fgf8 expression in the overlying ectoderm (Ohuchi *et al.*, 1997) (Figure 8b). Fgf8 signaling from the AER is, in turn,

essential for normal limb development (Lewandoski *et al.*, 2000; Moon and Capecchi, 2000). Maintenance of Fgf10 expression in the limb bud mesoderm and Fgf8 expression in the AER is most likely dependent on a mutual positive feedback mechanism (Figure 8c and d). Recently it has been demonstrated that Wnt-3a is required for this Fgf8/Fgf10 loop to be sustained (Kawakami *et al.*, 2001; Barrow *et al.*, 2003). Interestingly, ablation of the AER can be rescued by artificial expression of Fgf2, Fgf4, or Fgf8 (Niswander *et al.*, 1993). These factors are also capable of inducing an ectopic AER and any of them can sustain the AER's morphogenetic functions. Their redundancy can be explained by the fact

that they signal through the same FGFR (Fgf1c) in the mesoderm. Hindlimb buds of mice lacking both Fgf4 and Fgf8 do not develop a proper limb. However, since limb buds initially form, they are not required for AER formation but are essential for the expression of numerous genes in the underlying mesenchyme (e.g., Sonic hedgehog (Shh)) (Sun *et al.*, 2002). Taken together, Fgfs mediate the function of the AER in terms of limb outgrowth. They stimulate cell proliferation in the underlying mesoderm and are required to maintain Shh expression (see Section 2.5.4).

2.5.3. DV Patterning

The actual patterning of the outgrowing limb bud is tightly linked to the positioning of the AER itself (see Section 2.5.2). Many structures of the fully grown vertebrate limb show DV asymmetry, like tendons, bones, and muscles. Early transplantation experiments suggested that signaling from the dorsal non-ridge ectoderm is responsible for the establishment of DV asymmetry in the limb (MacCabe *et al.*, 1974). One of the signaling molecules responsible for dorsal patterning is Wnt-7a, which induces the LIM-homeodomain transcription factor Lmx-1b in the dorsal mesenchyme. Lmx-1b then specifies dorsal identities in the limb (Parr and McMahon, 1995; Riddle *et al.*, 1995; Vogel *et al.*, 1995; Chen *et al.*, 1998; review: Chen and Johnson, 1999) (Figure 8d). En-1 acts as a ventralizing factor and is required for repression of Wnt-7a in the ventral ectoderm (Loomis *et al.*, 1998). BMPs are necessary and sufficient to regulate the ventral expression of En-1 and are therefore required for DV patterning of the limb (Ahn *et al.*, 2001; Pizette *et al.*, 2001).

2.5.4. The ZPA as the Organizer of AP Polarity

In addition to DV polarity, the vertebrate limb obviously has an AP asymmetry axis as evident in muscles, bones, and tendons. Growth and patterning along the AP axis is tightly linked with development of the PD axis. This process is controlled by an interplay between the AP organizer (the ZPA) and the AER.

The ZPA is a group of cells in the posterior mesenchyme of the limb bud and its effects have been shown to be mediated by Shh (Riddle *et al.*, 1993; Chang *et al.*, 1994; Lopez-Martinez *et al.*, 1995; (Figure 8c and d). Shh is a homolog of the *Drosophila* secreted signaling molecule Hedgehog (see Section 2.3.1). While Shh is not required to establish initial AP polarity, it is absolutely essential for growth and patterning of intermediate and distal elements of the limb, and is the only factor known to

date to mediate the effects of the ZPA. Shh expression is dependent on retinoic acid (RA) signaling (Helms *et al.*, 1996) and also requires AER signals, which restrict it to distal regions (Niswander *et al.*, 1994). In turn, Shh is also required for AER maintenance. Continuous Fgf signaling in the AER (which positively feeds back on Shh maintenance) depends on the limitation of BMP signaling from the underlying mesoderm (Pizette and Niswander, 1999). This effect has been proposed to be mediated by Shh through the BMP antagonist Gremlin (Capdevila *et al.*, 1999; Merino *et al.*, 1999; Zuniga *et al.*, 1999). The specification and maintenance of the AP and PD axes are tightly linked through this mechanism.

Furthermore, Shh expression is dependent on Wnt-7a signaling from the dorsal ectoderm (Yang and Niswander, 1995). Since Wnt-7a is also required for the establishment of dorsal fate (Parr and McMahon, 1995) (see Section 2.5.3), dorsal signals contribute to the regulation of AP patterning. The formation of all three axes of the limb is therefore tightly connected (Figure 8).

2.5.5. Proximal Patterning of the Vertebrate Limb

Interestingly, proximal patterning in the vertebrate limb seems to be dependent on similar factors to those in *Drosophila*. The *Pbx1* and *Meis* genes (homologs of *Exd* and *Hth*, respectively) are restricted to proximal parts of the limb bud. Activity of the *Meis* genes is both necessary and sufficient to confer proximal fate, similar to the action of *Hth* in *Drosophila* (Capdevila *et al.*, 1999; Mercader *et al.*, 1999, 2000).

2.5.6. Compartments in the Vertebrate Limb

The vertebrate limb bud ectoderm is subdivided into compartments (review: Tickle and Munsterberg, 2001). This subdivision occurs between dorsal and ventral cells even though there is some debate about the exact position of the lineage restriction boundary. All studies agree that the boundary of the ventral compartment lies at the midpoint of the AER. Surprisingly, in some experiments, dorsal cells seem to be able to cross this boundary to fill the entire ridge in the chick (but do not move into ventral ectoderm) (Altabef *et al.*, 1997). How this result can be reconciled with the existence of the ventral lineage restriction boundary remains unclear. In the mouse, the compartment boundary within the AER is transient. Additionally, a second lineage boundary between dorsal ectoderm and the AER exists (Kimmel *et al.*, 2000).

The midpoint of the AER corresponds exactly to the En-1 expression domain. However, *En-1* is not a ventral selector gene because the lineage boundary remains intact in *En-1*^{-/-} mice (Loomis *et al.*, 1996). Also, misexpression of *En-1* does not perturb the boundary (Altabef *et al.*, 2000). The mechanism responsible for the establishment of compartmental subdivision in the limb bud ectoderm therefore remains to be identified. For this reason, it has not been possible to experimentally eliminate boundary formation to investigate whether the existence of the boundary is essential for AER formation.

2.5.7. Similarities and Differences between *Drosophila* and Vertebrate Limb Development

Both *Drosophila* and vertebrate limbs are outgrowths of the body wall. In principle, they serve the animal to fulfill similar tasks. It is obvious that some elements of the regulatory circuitry and some of the same signaling pathways are used for limb generation in different organisms. This observation raises the question of whether *Drosophila* and vertebrate limbs are truly homologous structures or whether they are merely constructed according to a different plan, but using the same toolbox. The consideration of a few examples leads us to favor the latter view.

One cause for concern is raised by the idea that a DV body axis inversion has taken place between vertebrates and invertebrates (Arendt and Nubler-Jung, 1994; 1997; Holley *et al.*, 1995; De Robertis and Sasai, 1996). Should this be the case, then dorsal and ventral should be reversed in the limbs as well. In *Drosophila*, Ap is required for establishment of the dorsal compartment. It has been suggested that two LIM-homeodomain proteins, Lhx2 and Lmx1, perform Ap-related functions in vertebrate development (Rodriguez-Esteban *et al.*, 1998; Rincon-Limas *et al.*, 1999). Lmx1 confers dorsal identity in the vertebrate limb mesenchyme but is not involved in limb outgrowth (Parr and McMahon, 1995; Riddle *et al.*, 1995; Vogel *et al.*, 1995; Chen *et al.*, 1998). If these were homologous processes, Lmx1 is expected to be ventral in the vertebrate limb. In fact, Lmx1 is not very closely related to Ap, and is much more similar to the *Drosophila* LIM-homeodomain factors Lim1 and Lim3, neither of which seems to be involved in wing development (Hobert and Westphal, 2000).

The function of Lhx2 in the vertebrate limb remains even more elusive. Lhx2 is expressed in distal mesoderm in the limb bud (Rincon-Limas

et al., 1999; Lu *et al.*, 2000) and has the capacity to induce Rfng in the interlimb but not in the limb field. However, no AER is induced and mice lacking functional Lhx2 do not show a limb phenotype. It was proposed that Lhx2 performs a growth function (Porter *et al.*, 1997; Rodriguez-Esteban *et al.*, 1998). One could speculate that functional redundancy with another very closely related LIM-homeodomain factor, Lhx9, could explain these observations. Lhx9 is expressed in the forelimb and hindlimb mesenchyme but no further functional data is available (Retaux *et al.*, 1999). It will, therefore, depend on careful loss-of-function analyses of Lhx2 and Lhx9 to determine whether the multiple functions of Ap in *Drosophila* wing development are performed by these proteins in the vertebrate limb.

The expression pattern of Rfng in the dorsal ectoderm (see Section 2.5.2) is reminiscent of the dorsal pattern of expression of Fng in the *Drosophila* wing imaginal disc. However, in contrast to *Drosophila*, there is no data that supports the role of Rfng in DV patterning of the vertebrate limb (Laufer *et al.*, 1997; Rodriguez-Esteban *et al.*, 1997). Moreover, Lhx2 and Rfng are not coexpressed under wild-type circumstances, with Lhx2 being expressed only in more distal tissues in the limb bud. Therefore, the inducibility of Rfng in the interlimb region by Lhx2 is not proof for a functional conservation between Lhx2/Rfng and Ap/Fng, respectively. Furthermore, the role of Rfng in the establishment of DV patterning and the AER remains unclear (see Section 2.5.2), and a functional conservation between Fng and Rfng, in terms of limb organization, appears unlikely.

Like in the *Drosophila* wing, *En-1* plays an important role in vertebrate development. However, while in the *Drosophila* wing it performs the role of a selector gene for posterior patterning, in the vertebrate limb bud it acts in ventral ectoderm and, therefore, performs quite a different role than in *Drosophila*. Nonetheless Hh and Shh are both implicated in sending signals from posterior cells.

It is not obvious how one could make an argument for a homologous developmental regulatory network. Nevertheless, the same “genetic toolbox” is used in *Drosophila* limb development and in the formation of the vertebrate limb. Therefore, it seems more plausible that limbs in different organisms are not homologous as appendage structures, but are likely to have a common origin in evolution as body wall outgrow (see Panganiban *et al.*, 1997).

Appendix Genes and proteins involved in limb development

Abbreviation	Gene or protein name	Function
<i>ac-sc</i>	<i>achaete-scute complex</i>	Proneural genes
<i>ap</i>	<i>apterous</i>	Dorsal selector gene
<i>ara</i>	<i>araucaan</i>	Part of the Iroquois complex; important for notum patterning
<i>argos</i>	<i>argos</i>	Component of the EGFR signal transduction pathway
<i>bs</i>	<i>blistered</i>	<i>Drosophila</i> homolog of the serum response factor (SRF)
BMP	Bone morphogenetic protein	Class of secreted factors important in development of fly (Dpp) and vertebrate development (e.g., BMP2, 4); belong to the transforming growth factor- β (TGF- β) family
<i>brk</i>	<i>brinker</i>	Antagonist of Dpp signaling
<i>Bx</i>	<i>Beadex</i> ; synonym for <i>dLMO</i>	Antagonist of Apterous activity
<i>caps</i>	<i>capricious</i>	Leucine-rich-repeat (LRR) transmembrane protein involved in dorsal-ventral affinity regulation
<i>caup</i>	<i>caupolican</i>	Part of the Iroquois Complex; important for notum patterning
<i>Chp</i>	<i>Chip (dLDB)</i>	Cofactor required for Apterous activity
<i>Ci</i>	<i>Cubitus interruptus</i>	Transcription factor essential for Hh signal transduction
<i>col (kn)</i>	<i>collier (knot)</i>	<i>hh</i> target gene, required for specification of veins three and four in the wing
<i>cos-2</i>	<i>costal-2</i>	Component of the Hedgehog signal transduction pathway
<i>dac</i>	<i>dachshund</i>	Target gene of Wg and Dpp in the <i>Drosophila</i> leg
<i>dad</i>	<i>daughters against dpp</i>	Inhibitory Smad
<i>dally</i>	<i>dally</i>	Glypican core protein, related to Dally-like
<i>dlp</i>	<i>dally-like</i>	Glypican core protein, related to Dally
<i>DFz2 (fz2)</i>	<i>Drosophila Frizzled 2</i>	Wg receptor for the canonical Wg pathway
<i>disp</i>	<i>dispatched</i>	Transmembrane protein of the Patched (Ptc) family; required for release of cholesterol-modified Hedgehog (Hh)
<i>DI</i>	<i>Delta</i>	Notch ligand
<i>dLDB</i>	<i>Drosophila LIM-domain binding</i>	Cofactor required for Apterous activity
<i>Dll</i>	<i>Distal-less</i>	Wg target gene required for distal specification of <i>Drosophila</i> legs
<i>dLMO</i>	<i>Drosophila LIM-domain only</i>	Antagonist of Apterous activity
<i>Dpp</i>	<i>Decapentaplegic</i>	TGF β homolog; important for anterior-posterior patterning in <i>Drosophila</i> limbs
<i>Ddyn</i>	<i>dynammin</i>	Protein involved in formation and release of endocytic vesicles
<i>E(spl)</i>	<i>Enhancer of split</i>	<i>Notch</i> target gene
EGF	Epidermal growth factor	Ligand for the EGF receptor
EGFR	Epidermal growth factor receptor	Receptor tyrosine kinase
<i>el</i>	<i>elbow</i>	Zinc-finger transcriptional repressor, required for distal specification of both wings and legs; binds to No ocelli (Noc)
<i>en</i>	<i>engrailed</i>	Posterior selector gene
En-1	Engrailed 1	Vertebrate homolog of the transcriptional repressor Engrailed
Eph		Transmembrane receptor; able to signal bidirectionally
Ephrin		Ligand for the Eph receptor; able to signal bidirectionally
<i>exd</i>	<i>extradenticle</i>	A binding partner of Hth
FGF	Fibroblast growth factor	Class of secreted factors important in many processes of vertebrate development (e.g., Fgf8, Fgf70)
FGFR	Fibroblast growth factor receptor	Class of receptor tyrosine kinases binding to the secreted proteins of the Fgf class (with different specificities)
<i>fng</i>	<i>fringe</i>	Glycosyltransferase required for Notch-Delta interaction
<i>formin</i>	<i>formio</i>	In the context of limb development, the <i>ld</i> mutants are gain-of-function mutants that cause misexpression of <i>gremlin</i> and inhibit BMP activity. Formins were misidentified as the <i>ld</i> gene product
<i>frc</i>	<i>fringe connection</i>	Enzyme required for heparan-sulfate proteoglycan biosynthesis
<i>fz</i>	<i>frizzled</i>	Wg receptor for the planar polarity pathway
<i>gbb</i>	<i>glass bottom boat</i>	BMP-like factor in <i>Drosophila</i>
Gremlin		BMP antagonist
<i>hh</i>	<i>hedgehog</i>	Morphogen important for anterior-posterior patterning in the <i>Drosophila</i> wing
<i>hindsight</i>	<i>hindsight</i>	Transcription factor, allelic to <i>pebble</i> (<i>peb</i>)
<i>hth</i>	<i>homothorax</i>	Homeodomain transcription factor; required for proximal specification of both wings and legs
<i>inv</i>	<i>invected</i>	Posterior selector gene, closely related to <i>engrailed</i>
<i>Iro-C</i>	<i>Iroquois Complex</i>	Gene complex consisting of <i>ara</i> , <i>caup</i> , and <i>mirr</i> ; important for notum patterning
Lfng	Lunatic fringe	Member of the Fringe family of glycosyltransferases in vertebrates
Lhx2		Vertebrate LIM homeodomain protein
Lhx9		Vertebrate LIM homeodomain protein
Lim1		Transcription factor
Lim3		Transcription factor
Lmx1		Vertebrate LIM homeodomain protein

Continued

Appendix Continued

Abbreviation	Gene or protein name	Function
<i>Mad</i>	<i>Mothers against Dpp</i>	<i>Drosophila</i> SMAD, signal transducer of the Dpp pathway
<i>med</i>	<i>medea</i>	<i>Drosophila</i> co-SMAD
<i>Meis</i>		Vertebrate homolog of <i>hth</i>
<i>Mfng</i>	Manic fringe	Member of the Fringe family of glycosyltransferases in vertebrates
<i>mirr</i>	<i>mirror</i>	Part of the Iroquois Complex; important for notum patterning
<i>msh</i>	<i>muscle segment homeobox</i>	Transcription factor mediating dorsal wing identity
<i>mtv</i>	<i>master of Thickveins</i>	Downregulates the Dpp receptor Thickveins
<i>N</i>	<i>Notch</i>	Transmembrane receptor
<i>nkd</i>	<i>naked</i>	EF-hand containing protein; Wingless-antagonist
<i>noc</i>	<i>no ocelli</i>	Zinc-finger transcriptional repressor, required for distal specification of both wings and legs; binds to Elbow (EI)
<i>notum</i>	<i>notum</i>	Negative regulator of <i>wingless</i>
<i>nub</i>	<i>nubbin</i>	POU-domain transcription factor; essential for wing and hinge development
<i>omb</i>	<i>optomotor blind</i>	Target gene of the Dpp pathway
<i>Pbx1</i>	postbithorax 1	Vertebrate homolog of <i>Exd</i>
<i>Pc</i>	<i>Polycomb</i>	Involved in chromatin based gene silencing
<i>PI3K</i>	phosphatidylinositol-3-kinase	Phosphorylates phosphatidylinositol at position 3; important mediator of insulin-dependent growth signals
<i>PKA</i>	Protein kinase A	Negative regulator of <i>hh</i> signaling; promotes Ci processing
<i>pnr</i>	<i>pannier</i>	Encoding transcription factor required for notum patterning
<i>pnt</i>	<i>punt</i>	Type II Dpp receptor in <i>Drosophila</i>
<i>por</i>	<i>porcupine</i>	Required for secretion of Wingless (Wg)
<i>PS1</i>	Position-specific antigen 1	Integrin-type adhesion molecule
<i>PS2</i>	Position-specific antigen 2	Integrin-type adhesion molecule
<i>ptc</i>	<i>patched</i>	Hh-receptor; transmembrane protein
<i>Rfng</i>	Radical fringe	Member of the Fringe family of glycosyltransferases in vertebrates
<i>rho</i>	<i>rhomboid</i>	Membrane protein promoting the cleavage of the membrane-anchored TGFalpha-like growth factor Spitz
<i>rn</i>	<i>rotund</i>	Zinc-finger transcription factor of the Krüppel family; important for hinge formation
<i>S6K</i>	S6 kinase saxophone	Phosphorylates the small ribosomal protein 6
<i>scw</i>	<i>screw</i>	BMP-like factor in <i>Drosophila</i>
<i>sd</i>	<i>scalloped</i>	Transcription factor required for wing development; Sd binds to Vestigial (Vg)
<i>Ser</i>	<i>Serrate</i>	Notch ligand
<i>sfl</i>	<i>sulfateless</i>	Enzyme required for heparan-sulfate proteoglycan biosynthesis
<i>sgl</i>	<i>sugarless</i>	Enzyme required for heparan-sulfate proteoglycan biosynthesis
<i>Shh</i>	Sonic hedgehog	Vertebrate homolog of the morphogen Hedgehog
<i>ski</i>	<i>skinny hedgehog</i>	Acyl transferase
<i>slmb</i>	<i>slimb</i>	Negative regulator of Hh signaling
<i>smo</i>	<i>smoothed</i>	Transmembrane protein; Hh pathway component
<i>salm</i>	<i>spalt-major</i>	Target gene of the Dpp pathway, required for vein formation in the <i>Drosophila</i> wing; highly related to and found in a complex with <i>spalt-related</i>
<i>slr</i>	<i>spalt-related</i>	Target gene of the Dpp pathway, required for vein formation in the wing
<i>TGF-β</i>	Transforming growth factor-β	Class of extracellular growth factors
<i>tkv</i>	<i>thickveins</i>	Type II Dpp receptor in <i>Drosophila</i>
<i>TOR</i>	<i>target of rapamycin</i>	Activity reflects nutritional conditions and leads to the activation of S6 kinase (S6K)
<i>trn</i>	<i>tartan</i>	Leucine-rich-repeat (LRR) protein involved in dorsal-ventral affinity regulation
<i>Tsc1</i>	<i>tuberous sclerosis complex 1</i>	Forms a complex with Tsc2; inhibits target of rapamycin (TOR) when dephosphorylated
<i>Tsc2 (gig)</i>	<i>tuberous sclerosis complex 2 (gigas)</i>	Forms a complex with Tsc1; inhibits target of rapamycin (TOR) when dephosphorylated
<i>tsh</i>	<i>teashirt</i>	Required for proximal specification of both wings and legs
<i>tvv</i>	<i>tout-velu</i>	Enzyme required for heparan-sulfate proteoglycan biosynthesis
<i>trx</i>	<i>trithorax</i>	Counteracts the silencing effects of <i>Pc</i> on chromatin to maintain gene activity
<i>ve</i>	<i>veinlet</i>	Same as <i>rhomboid</i>
<i>vg</i>	<i>vestigial</i>	Transcription factor required for wing development; Vg binds to Scalloped (Sd)
<i>vn</i>	<i>vein</i>	EGFR ligand
<i>vvl</i>	<i>ventral veins lacking</i>	Homeobox transcription factor
<i>wg</i>	<i>wingless</i>	Secreted signaling molecule
<i>Wnt</i>	Wingless-related MMTV integration site I	Secreted signaling molecules

References

- Abu-Shaar, M., Mann, R., 1998. Generation of multiple antagonistic domains along the proximodistal axis during *Drosophila* leg development. *Development* 125, 3821–3830.
- Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., *et al.*, 2000. The genome sequence of *Drosophila melanogaster*. *Science* 287, 2185–2195.
- Agulnick, A.D., Taira, M., Breen, J.J., Tanaka, T., Dawid, I.B., *et al.*, 1996. Interactions of the LIM-domain-binding factor Ldb1 with LIM homeodomain proteins. *Nature* 384, 270–272.
- Ahn, K., Mishina, Y., Hanks, M.C., Behringer, R.R., Crenshaw, E.B., III, 2001. BMPR-IA signaling is required for the formation of the apical ectodermal ridge and dorsal–ventral patterning of the limb. *Development* 128, 4449–4461.
- Altabef, M., Clarke, J.D., Tickle, C., 1997. Dorso–ventral ectodermal compartments and origin of apical ectodermal ridge in developing chick limb. *Development* 124, 4547–4556.
- Altabef, M., Logan, C., Tickle, C., Lumsden, A., 2000. Engrailed-1 misexpression in chick embryos prevents apical ridge formation but preserves segregation of dorsal and ventral ectodermal compartments. *Devel. Biol.* 222, 307–316.
- Arbeitman, M.N., Furlong, E.E., Imam, F., Johnson, E., Null, B.H., *et al.*, 2002. Gene expression during the life cycle of *Drosophila melanogaster*. *Science* 297, 2270–2275.
- Arendt, D., Nubler-Jung, K., 1994. Inversion of dorsoventral axis? *Nature* 371, 26.
- Arendt, D., Nubler-Jung, K., 1997. Dorsal or ventral: similarities in fate maps and gastrulation patterns in annelids, arthropods and chordates. *Mech. Devel.* 61, 7–21.
- Ashe, H.L., Levine, M., 1999. Local inhibition and long-range enhancement of Dpp signal transduction by Sog. *Nature* 398, 427–431.
- Averof, M., Cohen, S.M., 1997. The evolutionary origin of insect wings from ancient respiratory appendages. *Nature* 385, 627–630.
- Aza-Blanc, P., Ramírez-Weber, F.-A., Laget, M.-P., Schwartz, C., Kornberg, T.B., 1997. Proteolysis that is inhibited by Hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor. *Cell* 89, 1043–1053.
- Azpiazu, N., Morata, G., 2000. Function and regulation of homothorax in the wing imaginal disc of *Drosophila*. *Development* 127, 2685–2693.
- Azpiazu, N., Morata, G., 2002. Distinct functions of homothorax in leg development in *Drosophila*. *Mech. Devel.* 119, 55–67.
- Bach, I., 2000. The LIM domain: regulation by association. *Mech. Devel.* 91, 5–17.
- Baeg, G.H., Lin, X., Khare, N., Baumgartner, S., Perrimon, N., 2001. Heparan sulfate proteoglycans are critical for the organization of the extracellular distribution of Wingless. *Development* 128, 87–94.
- Baonza, A., Garcia-Bellido, A., 1999. Dual role of *extramacrochaetae* in cell proliferation and cell differentiation during wing morphogenesis in *Drosophila*. *Mech. Devel.* 80, 133–146.
- Barrow, J.R., Thomas, K.R., Boussadia-Zahui, O., Moore, R., Kemler, R., *et al.*, 2003. Ectodermal Wnt3/ beta-catenin signaling is required for the establishment and maintenance of the apical ectodermal ridge. *Genes Devel.* 17, 394–409.
- Basler, K., Struhl, G., 1994. Compartment boundaries and the control of *Drosophila* limb pattern by *hedgehog* protein. *Nature* 368, 208–214.
- Bate, M., Martinez-Arias, A., 1991. The embryonic origin of imaginal discs in *Drosophila*. *Development* 112, 755–761.
- Bellaiche, Y., The, I., Perrimon, N., 1998. *Tout-velu* is a *Drosophila* homologue of the putative tumour suppressor *EXT-1* and is needed for Hh diffusion. *Nature* 394, 85–88.
- Bellen, H.J., O’Kane, C.J., Wilson, C., Grossniklaus, U., Pearson, R., *et al.*, 1989. P-element-enhancer detection: a versatile method to study development in *Drosophila*. *Genes Devel.* 3, 1288–1300.
- Bernfield, M., Gotte, M., Park, P.W., Reizes, O., Fitzgerald, M.L., *et al.*, 1999. Functions of cell surface heparan sulfate proteoglycans. *Annu. Rev. Biochem.* 68, 729–777.
- Bier, E., 2000. Drawing lines in the *Drosophila* wing: initiation of wing vein development. *Curr. Opin. Genet. Devel.* 10, 393–398.
- Binari, R.C., Staveley, B.E., Johnson, W.A., Godavarti, R., Sasisekharan, R., *et al.*, 1997. Genetic evidence that heparin-like glycosaminoglycans are involved in wingless signaling. *Development* 124, 2623–2632.
- Bishop, S.A., Klein, T., Martinez-Arias, A., Couso, J.P., 1999. Composite signalling from Serrate and Delta establishes leg segments in *Drosophila* through Notch. *Development* 126, 2993–3003.
- Blair, S.S., 1992. *shaggy* (*zeste-white 3*) and the formation of supernumerary bristle precursors in the developing wing blade of *Drosophila*. *Devel. Biol.* 152, 263–278.
- Blair, S.S., 1993. Mechanisms of compartment formation: evidence that non-proliferating cells do not play a critical role in defining the D/V lineage restriction in the developing wing of *Drosophila*. *Development* 119, 339–351.
- Blair, S.S., 1995. Compartments and appendage development in *Drosophila*. *BioEssays* 17, 299–309.
- Blair, S.S., Brower, D.L., Thomas, J.B., Zavortink, M., 1994. The role of *apterous* in the control of dorsoventral compartmentalization and PS integrin gene expression in the developing wing of *Drosophila*. *Development* 120, 1805–1815.
- Blair, S.S., Ralston, A., 1997. Smoothed-mediated Hedgehog signalling is required for the maintenance of the anterior–posterior lineage restriction in the developing wing of *Drosophila*. *Development* 124, 4053–4063.
- Bouwmeester, T., Kim, S.-H., Sasai, Y., Lu, B., DeRobertis, E., 1996. Cereberus is a head-inducing

- secreted factor expressed in the anterior endoderm of Spemann's organizer. *Nature* 382, 595–601.
- Britton, J.S., Lockwood, W.K., Li, L., Cohen, S.M., Edgar, B.A., 2002. *Drosophila's* insulin/PI3-kinase pathway coordinates cellular metabolism with nutritional conditions. *Devel. Cell* 2, 239–249.
- Brook, W.J., Cohen, S.M., 1996. Antagonistic interactions between Wingless and Decapentaplegic responsible for dorsal–ventral pattern in the *Drosophila* leg. *Science* 273, 1373–1377.
- Brook, W.J., Diaz-Benjumea, F.J., Cohen, S.M., 1996. Organizing spatial pattern in limb development. *Annu. Rev. Cell Devel. Biol.* 12, 161–180.
- Brower, D.L., Jaffe, S.M., 1989. Requirements for integrins during *Drosophila* wing development. *Nature* 342, 285–287.
- Brückner, K., Klein, R., 1998. Signaling by Eph receptors and their ephrin ligands. *Curr. Opin. Neurobiol.* 8, 375–382.
- Brückner, K., Perez, L., Clausen, H., Cohen, S., 2000. Glycosyltransferase activity of Fringe modulates Notch–Delta interactions. *Nature* 406, 411–415.
- Brummel, T.J., Twombly, V., Marques, G., Wrana, J.L., Newfeld, S.J., et al., 1994. Characterization and relationship of Dpp receptors encoded by *saxophone* and *thick veins* genes in *Drosophila*. *Cell* 78, 251–261.
- Bryant, P.J., Simpson, P., 1984. Intrinsic and extrinsic control of growth in developing organs. *Q. Rev. Biol.* 59, 387–415.
- Burke, R., Basler, K., 1996. Dpp receptors are autonomously required for cell proliferation in the entire developing *Drosophila* wing. *Development* 122, 2261–2269.
- Burke, R., Nellen, D., Bellotto, M., Hafen, E., Senti, K.A., et al., 1999. Dispatched, a novel sterol-sensing domain protein dedicated to the release of cholesterol-modified hedgehog from signaling cells. *Cell* 99, 803–815.
- Busturia, A., Morata, G., 1988. Ectopic expression of homeotic genes caused by the elimination of the *Polycomb* gene in *Drosophila* imaginal epidermis. *Development* 104, 713–720.
- Butler, M.J., Jacobsen, T.L., Cain, D.M., Jarman, M.G., Hubank, M., et al., 2003. Discovery of genes with highly restricted expression patterns in the *Drosophila* wing disc using DNA oligonucleotide microarrays. *Development* 130, 659–670.
- Cadigan, K.M., Fish, M.P., Rulifson, E.J., Nusse, R., 1998. Wingless repression of *Drosophila* *frizzled 2* expression shapes the Wingless morphogen gradient in the wing. *Cell* 93, 767–777.
- Calleja, M., Moreno, E., Pelaz, S., Morata, G., 1996. Visualization of gene expression in living adult *Drosophila*. *Science* 274, 252–255.
- Campbell, G., 2002. Distalization of the *Drosophila* leg by graded EGF-receptor activity. *Nature* 418, 781–785.
- Campbell, G., Tomlinson, A., 1995. Initiation of the proximal-distal axis in insect legs. *Development* 121, 619–628.
- Campbell, G., Tomlinson, A., 1998. The roles of homeobox genes *aristalless* and *Distal-less* in patterning legs and wings of *Drosophila*. *Development* 125, 4483–4493.
- Campbell, G., Tomlinson, A., 1999. Transducing the Dpp morphogen gradient in the wing of *Drosophila*: regulation of Dpp targets by brinker. *Cell* 96, 553–562.
- Capdevila, J., Guerrero, I., 1994. Targeted expression of the signalling molecule decapentaplegic induces pattern duplications and growth alterations in *Drosophila* wings. *EMBO J.* 13, 4459–4468.
- Capdevila, J., Izpisua Belmonte, J.C., 2001. Patterning mechanisms controlling vertebrate limb development. *Annu. Rev. Cell Devel. Biol.* 17, 87–132.
- Capdevila, J., Tsukui, T., Rodriguez-Esteban, C., Zappavigna, V., Izpisua Belmonte, J.C., 1999. Control of vertebrate limb outgrowth by the proximal factor Meis2 and distal antagonism of BMPs by Gremlin. *Mol. Cell* 4, 839–849.
- Casares, F., Mann, R.S., 2000. A dual role for homothorax in inhibiting wing blade development and specifying proximal wing identities in *Drosophila*. *Development* 127, 1499–1508.
- Caspary, T., Garcia-Garcia, M.J., Huangfu, D., Eggenschwiler, J.T., Wyler, M.R., et al., 2002. Mouse dispatched homolog1 is required for long-range, but not juxtacrine, Hh signaling. *Curr. Biol.* 12, 1628–1632.
- Castelli-Gair, J., Akam, M., 1995. How the Hox gene *Ultrabithorax* specifies two different segments: the significance of spatial and temporal regulation within metameres. *Development* 121, 2973–2982.
- Cavodeassi, F., Modolell, J., Campuzano, S., 2000. The *Iroquois* homeobox genes function as dorsal selectors in the *Drosophila* head. *Development* 127, 1921–1929.
- Cavodeassi, F., Modolell, J., Gomez-Skarmeta, J.L., 2001. The *Iroquois* family of genes: from body building to neural patterning. *Development* 128, 2847–2855.
- Cavodeassi, F., Rodriguez, I., Modolell, J., 2002. Dpp signalling is a key effector of the wing–body wall subdivision of the *Drosophila* mesothorax. *Development* 129, 3815–3823.
- Chamoun, Z., Mann, R.K., Nellen, D., von Kessler, D.P., Bellotto, M., et al., 2001. Skinny hedgehog, an acyltransferase required for palmitoylation and activity of the hedgehog signal. *Science* 293, 2080–2084.
- Chang, D.T., Lopez, A., von Kessler, D.P., Chiang, C., Simandl, B.K., et al., 1994. Products, genetic linkage and limb patterning activity of a murine hedgehog gene. *Development* 120, 3339–3353.
- Chen, C., Jack, J., Garofalo, R.S., 1996. The *Drosophila* insulin receptor is required for normal growth. *Endocrinology* 137, 846–856.
- Chen, H., Johnson, R.L., 1999. Dorsoventral patterning of the vertebrate limb: a process governed by multiple events. *Cell Tissue Res.* 296, 67–73.
- Chen, H., Lun, Y., Ovchinnikov, D., Kokubo, H., Oberg, K.C., et al., 1998. Limb and kidney defects in *Lmx1b*

- mutant mice suggest an involvement of *LMX1B* in human nail-patella syndrome. *Nature Genet.* 19, 51–55.
- Chen, Y., Struhl, G., 1996. Dual roles for Patched in sequestering and transducing Hedgehog. *Cell* 87, 553–563.
- Cho, K.O., Chern, J., Izaddoost, S., Choi, K.W., 2000. Novel signaling from the peripodial membrane is essential for eye disc patterning in *Drosophila*. *Cell* 103, 331–342.
- Chuang, P.T., Kornberg, T.B., 2000. On the range of hedgehog signaling. *Curr. Opin. Genet. Devel.* 10, 515–522.
- Cohen, B., McGuffin, M.E., Pfeifle, C., Segal, D., Cohen, S.M., 1992. *apterous*: a gene required for imaginal disc development in *Drosophila* encodes a member of the LIM family of developmental regulatory proteins. *Genes Devel.* 6, 715–729.
- Cohen, B., Simcox, A.A., Cohen, S.M., 1993. Allocation of the thoracic imaginal disc primordia in the *Drosophila* embryo. *Development* 117, 597–608.
- Cohen, B., Wimmer, E.A., Cohen, S.M., 1991. Early development of leg and wing primordia in the *Drosophila* embryo. *Mech. Devel.* 33, 229–240.
- Cohen, S.M., 1990. Specification of limb development in the *Drosophila* embryo by positional cues from segmentation genes. *Nature* 343, 173–177.
- Cohen, S.M., 1993. Imaginal disc development. In: Martinez-Arias, A., Bate, M. (Eds.), *Drosophila Development*, vol. 2. Cold Spring Harbor Press, Cold Spring Harbor, NY, pp. 747–841.
- Cohen, S.M., Brönnner, G., Küttner, F., Jürgens, G., Jäckle, H., 1989. *Distal-less* encodes a homoeodomain protein required for limb development in *Drosophila*. *Nature* 338, 432–434.
- Cohn, M.J., Izpisua-Belmonte, J.C., Abud, H., Heath, J.K., Tickle, C., 1995. Fibroblast growth factors induce additional limb development from the flank of chick embryos. *Cell* 80, 739–746.
- Couso, J.P., Bishop, S., Martinez-Arias, A., 1994. The wingless signalling pathway and the patterning of the wing margin in *Drosophila*. *Development* 120, 621–636.
- Couso, J.P., Knust, E., Martinez-Arias, A., 1995. *Serrate* and *wingless* cooperate to induce vestigial gene expression and wing formation in *Drosophila*. *Curr. Biol.* 5, 1437–1448.
- Crick, F.H., Lawrence, P.A., 1975. Compartments and polyclones in insect development. *Science* 189, 340–347.
- Dahmann, C., Basler, K., 1999. Compartment boundaries: at the edge of development. *Trends Genet.* 15, 320–326.
- Dahmann, C., Basler, K., 2000. Opposing transcriptional outputs of Hedgehog signaling and engrailed control compartmental cell sorting at the *Drosophila* A/P boundary. *Cell* 100, 411–422.
- D'Alessio, M., Frasch, M., 1996. *msh* may play a conserved role in dorsoventral patterning of the neuroectoderm and mesoderm. *Mech. Devel.* 58, 217–231.
- Das, P., Maduzia, L.L., Wang, H., Finelli, A.L., Cho, S.H., et al., 1998. The *Drosophila* gene *Medea* demonstrates the requirement for different classes of Smads in dpp signaling. *Development* 125, 1519–1528.
- Davis, C.A., Joyner, A.L., 1988. Expression patterns of the homeobox-containing genes *En-1* and *En-2* and the proto-oncogene *int-1* diverge during mouse development. *Genes Devel.* 2, 1736–1744.
- Day, S.J., Lawrence, P.A., 2000. Measuring dimensions: the regulation of size and shape. *Development* 127, 2977–2987.
- de Celis, J.F., 1998. Positioning and differentiation of veins in the *Drosophila* wing. *Int. J. Devel. Biol.* 42, 335–343.
- de Celis, J.F., Barrio, R., 2000. Function of the *spalt/spalt*-related gene complex in positioning the veins in the *Drosophila* wing. *Mech. Devel.* 91, 31–41.
- de Celis, J.F., Barrio, R., Kafatos, F.C., 1996a. A gene complex acting downstream of *dpp* in *Drosophila* wing morphogenesis. *Nature* 381, 421–424.
- de Celis, J.F., García-Bellido, A., Bray, S.J., 1996b. Activation and function of *Notch* at the dorsal-ventral boundary of the wing imaginal disc. *Development* 122, 359–369.
- de Celis, J.F., Llimargas, M., Casanova, J., 1995. *Ventral veinless*, the gene encoding the Cf1a transcription factor, links positional information and cell differentiation during embryonic and imaginal development in *Drosophila melanogaster*. *Development* 121, 3405–3416.
- de Celis, J.F., Tyler, D.M., de Celis, J., Bray, S., 1998. Notch signaling mediates segmentation of the *Drosophila* leg. *Development* 125, 4617–4626.
- Del Alamo Rodriguez, D., Terriente, J., Galindo, M.I., Couso, J.P., Diaz-Benjumea, F.J., 2002. Different mechanisms initiate and maintain wingless expression in the *Drosophila* wing hinge. *Development* 129, 3995–4004.
- Denef, N., Neubueser, D., Perez, L., Cohen, S.M., 2000. Hedgehog induces opposite changes in turnover and subcellular localization of Patched and Smoothed. *Cell* 102, 521–531.
- Dennis, P.B., Fumagalli, S., Thomas, G., 1999. Target of rapamycin (TOR): balancing the opposing forces of protein synthesis and degradation. *Curr. Opin. Genet. Devel.* 9, 49–54.
- De Robertis, E.M., Sasai, Y., 1996. A common plan for dorsoventral patterning in Bilateria. *Nature* 380, 37–40.
- Diaz-Benjumea, F.J., Cohen, S.M., 1993. Interaction between dorsal and ventral cells in the imaginal disc directs wing development in *Drosophila*. *Cell* 75, 741–752.
- Diaz-Benjumea, F.J., Cohen, S.M., 1995. *Serrate* signals through *Notch* to establish a *Wingless*-dependent organizer at the dorsal/ventral compartment boundary of the *Drosophila* wing. *Development* 121, 4215–4225.
- Diaz-Benjumea, F.J., Cohen, B., Cohen, S.M., 1994. Cell interactions between compartments establishes the

- proximal–distal axis of *Drosophila* legs. *Nature* 372, 175–179.
- Diez del Corral, R., Aroca, P., Gomez-Skarmeta, J.L., Cavodeassi, F., Modolell, J., 1999. The Iroquois homeodomain proteins are required to specify body wall identity in *Drosophila*. *Genes Dev.* 13, 1754–1761.
- Doherty, D., Fenger, G., Younger-Shepherd, S., Jan, L.-Y., Jan, Y.-N., 1996. Dorsal and ventral cells respond differently to the *Notch* ligands *Delta* and *Serrate* during *Drosophila* wing development. *Genes Dev.* 10, 421–434.
- Dominguez, M., de Celis, J.F., 1998. A dorsal/ventral boundary established by *Notch* controls growth and polarity in the *Drosophila* eye. *Nature* 396, 276–278.
- Dubois, L., Lecourtois, M., Alexandre, C., Hirst, E., Vincent, J.P., 2001. Regulated endocytic routing modulates wingless signaling in *Drosophila* embryos. *Cell* 105, 613–624.
- Duman-Scheel, M., Weng, L., Xin, S., Du, W., 2002. Hedgehog regulates cell growth and proliferation by inducing Cyclin D and Cyclin E. *Nature* 417, 299–304.
- Eaton, S., Kornberg, T., 1990. Repression of *ci-D* in posterior compartments of *Drosophila* by *engrailed*. *Genes Dev.* 4, 1068–1077.
- Edgar, B.A., 1999. From small flies come big discoveries about size control. *Nature Cell Biol.* 1, E191–E193.
- Entchev, E.V., Gonzalez-Gaitan, M.A., 2002. Morphogen gradient formation and vesicular trafficking. *Traffic* 3, 98–109.
- Entchev, E.V., Schwabedissen, A., Gonzalez-Gaitan, M., 2000. Gradient formation of the TGF-beta homolog Dpp. *Cell* 103, 981–991.
- Fernandez-Funez, P., Lu, C.H., Rincon-Limas, D.E., García-Bellido, A., Botas, J., 1998. The relative expression amounts of *apterous* and its co-factor *dLdb/Chip* are critical for dorso-ventral compartmentalization in the *Drosophila* wing. *EMBO J.* 17, 6846–6853.
- Fleming, R.J., Gu, Y., Hukriede, N.A., 1997. *Serrate*-mediated activation of *Notch* is specifically blocked by the product of the gene *fringe* in the dorsal compartment of the *Drosophila* wing imaginal disc. *Development* 124, 2973–2981.
- Fleming, R.J., Scottgale, T.N., Diederich, R.J., Artavanis-Tsakonas, S., 1990. The gene *Serrate* encodes a putative EGF-like transmembrane protein essential for proper ectodermal development in *Drosophila melanogaster*. *Genes Dev.* 4, 2188–2201.
- Freeman, M., Gurdon, J.B., 2002. Regulatory principles of developmental signaling. *Annu. Rev. Cell Dev. Biol.* 18, 515–539.
- French, V., Bryant, P.J., Bryant, S.V., 1976. Pattern regulation in epimorphic fields. *Science* 193, 969–981.
- Fristrom, D., Gotwals, P., Eaton, S., Kornberg, T.B., Sturtevant, M., et al., 1994. *Blistered*: a gene required for vein/intervein formation in wings of *Drosophila*. *Development* 120, 2661–2671.
- Funakoshi, Y., Minami, M., Tabata, T., 2001. *mtv* shapes the activity gradient of the Dpp morphogen through regulation of *thickveins*. *Development* 128, 67–74.
- Furlong, E.E., Andersen, E.C., Null, B., White, K.P., Scott, M.P., 2001. Patterns of gene expression during *Drosophila* mesoderm development. *Science* 293, 1629–1633.
- Gabay, L., Seger, R., Shilo, B.Z., 1997. *In situ* activation pattern of *Drosophila* EGF receptor pathway during development. *Science* 277, 1103–1106.
- Galindo, M.I., Bishop, S.A., Greig, S., Couso, J.P., 2002. Leg patterning driven by proximal–distal interactions and EGFR signaling. *Science* 297, 256–259.
- García-Bellido, A., 1975. Genetic control of wing disc development in *Drosophila*. *Ciba Found. Symp.* 161–182.
- García-Bellido, A., Cortes, F., Milan, M., 1994. Cell interactions in the control of size in *Drosophila* wings. *Proc. Natl Acad. Sci. USA* 91, 10222–10226.
- García-Bellido, A., de Celis, J.F., 1992. Developmental genetics of the venation pattern of *Drosophila*. *Annu. Rev. Genet.* 26, 277–304.
- García-Bellido, A., Merriam, J.R., 1971a. Parameters of the wing imaginal disc development of *Drosophila melanogaster*. *Devel. Biol.* 24, 61–87.
- García-Bellido, A., Ripoll, P., Morata, G., 1973. Developmental compartmentalisation of the wing disc of *Drosophila*. *Nature New Biol.* 245, 251–253.
- García-Bellido, A., Ripoll, P., Morata, G., 1976. Developmental compartmentalization in the dorsal mesothoracic disc of *Drosophila*. *Devel. Biol.* 48, 132–147.
- García-Bellido, A., Santamaria, P., 1972. Developmental analysis of the wing disc in the mutant *engrailed* of *Drosophila melanogaster*. *Genetics* 72, 87–104.
- García-Bellido, A.C., García-Bellido, A., 1998. Cell proliferation in the attainment of constant sizes and shapes: the Entelechia model. *Int. J. Devel. Biol.* 42, 353–362.
- Geduspan, J.S., Solursh, M., 1992. A growth-promoting influence from the mesonephros during limb outgrowth. *Devel. Biol.* 151, 242–250.
- Gerlitz, O., Basler, K., 2002. Wingful, an extracellular feedback inhibitor of Wingless. *Genes Dev.* 16, 1055–1059.
- Gibson, M.C., Schubiger, G., 2000. Peripodial cells regulate proliferation and patterning of *Drosophila* imaginal discs. *Cell* 103, 343–350.
- Gibson, M.C., Schubiger, G., 2001. *Drosophila* peripodial cells, more than meets the eye? *BioEssays* 23, 691–697.
- Gingras, A.C., Raught, B., Sonenberg, N., 2001. Regulation of translation initiation by FRAP/mTOR. *Genes Dev.* 15, 807–826.
- Giraldez, A.J., Copley, R.R., Cohen, S.M., 2002. HSPG modification by the secreted enzyme Notum shapes the Wingless morphogen gradient. *Devel. Cell* 2, 667–676.
- Godt, D., Tepass, U., 1998. *Drosophila* oocyte localization is mediated by differential cadherin-based adhesion. *Nature* 395, 387–391.

- Gomez-Skarmeta, J.L., Modolell, J., 1996. *arauca* and *caupolican* provide a link between compartment subdivisions and patterning of sensory organs and veins in the *Drosophila* wing. *Genes Devel.* 10, 2935–2945.
- Gonzalez-Crespo, S., Abu-Shaar, M., Torres, M., Martinez, A.C., Mann, R.S., *et al.*, 1998. Antagonism between extradenticle function and Hedgehog signaling in the developing limb. *Nature* 394, 196–200.
- Gonzalez-Gaitan, M., Capdevila, M.P., Garcia-Bellido, A., 1994. Cell proliferation patterns in the wing imaginal disc of *Drosophila*. *Mech. Devel.* 46, 183–200.
- Gonzalez-Reyes, A., St Johnston, D., 1998. The *Drosophila* AP axis is polarised by the cadherin-mediated positioning of the oocyte. *Development* 125, 3635–3644.
- Greco, V., Hannus, M., Eaton, S., 2001. Argosomes: a potential vehicle for the spread of morphogens through epithelia. *Cell* 106, 633–645.
- Grimm, S., Pflugfelder, G., 1996. Control of the gene *optomotor-blind* in *Drosophila* wing development by *decapentaplegic* and *wingless*. *Science* 271, 1601–1604.
- Gurdon, J.B., Bourillot, P.Y., 2001. Morphogen gradient interpretation. *Nature* 413, 797–803.
- Hacker, U., Lin, X., Perrimon, N., 1997. The *Drosophila* *sugarless* gene modulates Wingless signaling and encodes an enzyme involved in polysaccharide biosynthesis. *Development* 124, 3565–3573.
- Haerry, T.E., Heslip, T.R., Marsh, J.L., O'Connor, M.B., 1997. Defects in glucuronate biosynthesis disrupt Wingless signaling in *Drosophila*. *Development* 124, 3055–3064.
- Halder, G., Polaczyk, P., Kraus, M.E., Hudson, A., Kim, J., *et al.*, 1998. The Vestigial and Scalloped proteins act together to directly regulate wing-specific gene expression in *Drosophila*. *Genes Devel.* 12, 3900–3909.
- Han, K., Levine, M.S., Manley, J.L., 1989. Synergistic activation and repression of transcription by *Drosophila* homeobox proteins. *Cell* 56, 573–583.
- Han, K., Manley, J.L., 1993. Functional domains of the *Drosophila* Engrailed protein. *EMBO J.* 12, 2723–2733.
- Helms, J.A., Kim, C.H., Eichele, G., Thaller, C., 1996. Retinoic acid signaling is required during early chick limb development. *Development* 122, 1385–1394.
- Hobert, O., Westphal, H., 2000. Functions of LIM-homeobox genes. *Trends Genet.* 16, 75–83.
- Hoffmann, F.M., Goodman, W., 1987. Identification in transgenic animals of the *Drosophila* decapentaplegic sequences required for embryonic dorsal pattern formation. *Genes Devel.* 1, 615–625.
- Holley, S.A., Jackson, P.D., Sasai, Y., Lu, B., De Robertis, E.M., *et al.*, 1995. A conserved system for dorsal-ventral patterning in insects and vertebrates involving sog and chordin. *Nature* 376, 249–253.
- Hudson, J.B., Podos, S.D., Keith, K., Simpson, S.L., Ferguson, E.L., 1998. The *Drosophila* *Medea* gene is required downstream of *dpp* and encodes a functional homolog of human Smad4. *Development* 125, 1407–1420.
- Hynes, R.O., 1999. Cell adhesion: old and new questions. *Trends Cell Biol.* 9, M33–M37.
- Ikeya, T., Galic, M., Belawat, P., Nairz, K., Hafen, E., 2002. Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in *Drosophila*. *Curr. Biol.* 12, 1293–1300.
- Incardona, J.P., Gruenberg, J., Roelink, H., 2002. Sonic hedgehog induces the segregation of patched and smoothed in endosomes. *Curr. Biol.* 12, 983–995.
- Incardona, J.P., Lee, J.H., Robertson, C.P., Enga, K., Kapur, R.P., *et al.*, 2000. Receptor-mediated endocytosis of soluble and membrane-tethered Sonic hedgehog by Patched-1. *Proc. Natl Acad. Sci. USA* 97, 12044–12049.
- Ingham, P.W., 1981. *Trithorax*: a new homeotic mutation of *Drosophila melanogaster*. II. The role of *Trx*⁺ after embryogenesis. *Roux's Arch. Dev. Biol.* 190, 365–369.
- Ingham, P.W., 2000. How cholesterol modulates the signal. *Curr. Biol.* 10, R180–R183.
- Ingham, P.W., 2001. Hedgehog signaling: a tale of two lipids. *Science* 294, 1879–1881.
- Inoki, K., Li, Y., Zhu, T., Wu, J., Guan, K.L., 2002. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nature Cell Biol.* 4, 648–657.
- Irish, V.F., Gelbart, W.M., 1987. The *decapentaplegic* gene is required for dorsal-ventral patterning of the *Drosophila* embryo. *Genes Devel.* 1, 868–879.
- Irvine, K.D., Rauskolb, C., 2001. Boundaries in development: formation and function. *Annu. Rev. Cell Dev. Biol.* 17, 189–214.
- Irvine, K.D., Wieschaus, E., 1994. *fringe*, a boundary specific signalling molecule, mediates interactions between dorsal and ventral cells during *Drosophila* wing development. *Cell* 79, 595–606.
- Isshiki, T., Takeichi, M., Nose, A., 1997. The role of the *msb* homeobox gene during *Drosophila* neurogenesis: implication for the dorsoventral specification of the neuroectoderm. *Development* 124, 3099–3109.
- Jackson, S.M., Nakato, H., Sugiura, M., Jannuzzi, A., Oakes, R., *et al.*, 1997. *dally*, a *Drosophila* glypican, controls cellular responses to the TGF- β -related morphogen, Dpp. *Development* 124, 4113–4120.
- Jasper, H., Benes, V., Schwager, C., Sauer, S., Clauder-Munster, S., *et al.*, 2001. The genomic response of the *Drosophila* embryo to JNK signaling. *Devel. Cell* 1, 579–586.
- Jaynes, J.B., O'Farrell, P.H., 1988. Activation and repression of transcription by homeodomain-containing proteins that bind a common site. *Nature* 336, 744–749.
- Jaynes, J.B., O'Farrell, P.H., 1991. Active repression of transcription by the engrailed homeodomain protein. *EMBO J.* 10, 1427–1433.
- Jazwinska, A., Kirov, N., Wieschaus, E., Roth, S., Rushlow, C., 1999. The *Drosophila* gene *brinker* reveals a novel mechanism of Dpp target gene regulation. *Cell* 96, 563–573.
- Jefferies, H.B., Fumagalli, S., Dennis, P.B., Reinhard, C., Pearson, R.B., *et al.*, 1997. Rapamycin suppresses

- 5'TOP mRNA translation through inhibition of p70s6k. *EMBO J.* 16, 3693–3704.
- Jiang, J., Struhl, G., 1995. Protein kinase A and Hedgehog signaling in *Drosophila* limb development. *Cell* 80, 563–572.
- Jiang, J., Struhl, G., 1996. Complementary and mutually exclusive activities of Decapentaplegic and Wingless organize axial pattern during *Drosophila* limb development. *Cell* 86, 401–409.
- Jiang, J., Struhl, G., 1998. Regulation of the Hedgehog and Wingless signalling pathways by the F-box/WD40-repeat protein Slimb. *Nature* 391, 493–496.
- Johnson, R.L., Tabin, C.J., 1997. Molecular models for vertebrate limb development. *Cell* 90, 979–990.
- Johnston, L.A., Prober, D.A., Edgar, B.A., Eisenman, R.N., Gallant, P., 1999. *Drosophila myc* regulates cellular growth during development. *Cell* 98, 779–790.
- Johnston, S.H., Rauskolb, C., Wilson, R., Prabhakaran, B., Irvine, K.D., et al., 1997. A family of mammalian *Fringe* genes implicated in boundary determination and the Notch pathway. *Development* 124, 2245–2254.
- Joyner, A.L., Liu, A., Millet, S., 2000. Otx2, Gbx2 and Fgf8 interact to position and maintain a mid-hindbrain organizer. *Curr. Opin. Cell Biol.* 12, 736–741.
- Jungbluth, S., Larsen, C., Wizenmann, A., Lumsden, A., 2001. Cell mixing between the embryonic midbrain and hindbrain. *Curr. Biol.* 11, 204–207.
- Jurata, L.W., Kenny, D.A., Gill, G.N., 1996. Nuclear LIM interactor, a rhombotin and LIM homeodomain interacting protein, is expressed early in neuronal development. *Proc. Natl Acad. Sci. USA* 93, 11693–11698.
- Jurata, L.W., Pfaff, S.L., Gill, G.N., 1998. The nuclear LIM domain interactor NLI mediates homo- and heterodimerization of LIM domain transcription factors. *J. Biol. Chem.* 273, 3152–3157.
- Kadowaki, T., Wilder, E., Klingensmith, J., Zachary, K., Perrimon, N., 1996. The segment polarity gene porcupine encodes a putative multitransmembrane protein involved in Wingless processing. *Genes Devel.* 10, 3116–3128.
- Kawakami, Y., Capdevila, J., Buscher, D., Itoh, T., Rodriguez-Esteban, C., et al., 2001. WNT signals control FGF-dependent limb initiation and AER induction in the chick embryo. *Cell* 104, 891–900.
- Kennerdell, J.R., Carthew, R.W., 1998. Use of dsRNA-mediated genetic interference to demonstrate that *frizzled* and *frizzled 2* act in the Wingless pathway. *Cell* 95, 1017–1026.
- Kerber, B., Monge, I., Mueller, M., Mitchell, P.J., Cohen, S.M., 2001. The AP-2 transcription factor is required for joint formation and cell survival in *Drosophila* leg development. *Development* 128, 1231–1238.
- Kim, J., Irvine, K.D., Carroll, S.B., 1995. Cell recognition, signal induction and symmetrical gene activation at the dorsal/ventral boundary of the developing *Drosophila* wing. *Cell* 82, 795–802.
- Kim, J., Johnson, K., Chen, H.J., Carroll, S., Laughon, A., 1997a. *Drosophila* Mad binds to DNA and directly mediates activation of *vestigial* by Decapentaplegic. *Nature* 388, 304–308.
- Kim, J., Magee, J., Carroll, S.B., 1997b. Intercompartmental signaling and the regulation of *vestigial* expression at the dorsoventral boundary of the developing *Drosophila* wing. *Cold Spring Harb. Symp. Quant. Biol.* 62, 283–291.
- Kim, J., Sebring, A., Esch, J.J., Kraus, M.E., Vorwerk, K., et al., 1996. Integration of positional signals and regulation of wing formation by *Drosophila vestigial* gene. *Nature* 382, 133–138.
- Kimmel, R.A., Turnbull, D.H., Blanquet, V., Wurst, W., Loomis, C.A., et al., 2000. Two lineage boundaries coordinate vertebrate apical ectodermal ridge formation. *Genes Devel.* 14, 1377–1389.
- Klebes, A., Biehs, B., Cifuentes, F., Kornberg, T.B., 2002. Expression profiling of *Drosophila* imaginal discs. *Genome Biol.* 3, RESEARCH0038.
- Klein, T., Couso, J.P., Martinez-Arias, A., 1998. Wing development and specification of dorsal cell fates in the absence of apterous in *Drosophila*. *Curr. Biol.* 8, 417–420.
- Klein, T., Martinez-Arias, A., 1998. Different spatial and temporal interactions between *Notch*, *wingless*, and *vestigial* specify proximal and distal pattern elements of the wing in *Drosophila*. *Devel. Biol.* 194, 196–212.
- Kornberg, T., 1981. Compartments in the abdomen of *Drosophila* and the role of the *engrailed* locus. *Devel. Biol.* 86, 363–372.
- Kornberg, T., Siden, I., O'Farrell, P., Simon, M., 1985. The *engrailed* locus of *Drosophila*: *in situ* localization of transcripts reveals compartment-specific expression. *Cell* 40, 45–53.
- Lander, A.D., Nie, Q., Wan, F.Y., 2002. Do morphogen gradients arise by diffusion? *Devel. Cell* 2, 785–796.
- Laufer, E., Dahn, R., Orozco, O.E., Yeo, C.Y., Pisenti, J., et al., 1997. Expression of Radical fringe in limb-bud ectoderm regulates apical ectodermal ridge formation. *Nature* 386, 366–373.
- Lawrence, P.A., Casal, J., Struhl, G., 1999a. *hedgehog* and *engrailed*: pattern formation and polarity in the *Drosophila* abdomen. *Development* 126, 2431–2439.
- Lawrence, P.A., Casal, J., Struhl, G., 1999b. The Hedgehog morphogen and gradients of cell affinity in the abdomen of *Drosophila*. *Development* 126, 2441–2449.
- Lawrence, P.A., Struhl, G., 1982. Further studies on the *engrailed* phenotype in *Drosophila*. *EMBO J.* 7, 827–833.
- Lawrence, P.A., Struhl, G., 1996. Morphogens, compartments and pattern: lessons from *Drosophila*? *Cell* 85, 951–961.
- Lecuit, T., Brook, W.J., Ng, M., Calleja, M., Sun, H., et al., 1996. Two distinct mechanisms for long-range patterning by Decapentaplegic in the *Drosophila* wing. *Nature* 381, 387–393.

- Lecuit, T., Cohen, S.M., 1997. Proximal–distal axis formation in the *Drosophila* leg. *Nature* 388, 139–145.
- Lecuit, T., Cohen, S.M., 1998. Dpp receptor levels contribute to shaping the Dpp morphogen gradient in the *Drosophila* wing imaginal disc. *Development* 125, 4901–4907.
- Lee, J.D., Treisman, J.E., 2001. Sightless has homology to transmembrane acyltransferases and is required to generate active Hedgehog protein. *Curr. Biol.* 11, 1147–1152.
- Lee, J.J., Ekker, S.C., von Kessler, D.P., Porter, J.A., Sun, B.I., et al., 1994. Autoproteolysis in *hedgehog* protein biogenesis. *Science* 266, 1528–1537.
- Lee, J.J., von Kessler, D.P., Parks, S., Beachy, P.A., 1992. Secretion and localized transcript suggest a role in positional signalling for products of the segmentation gene *hedgehog*. *Cell* 71, 33–50.
- Leevers, S.J., Weinkove, D., MacDougall, L.K., Hafen, E., Waterfield, M.D., 1996. The *Drosophila* phosphoinositide 3-kinase Dp110 promotes cell growth. *EMBO J.* 15, 6584–6594.
- Lepage, T., Cohen, S.M., Diaz-Benjumea, F.J., Parkhurst, S.M., 1995. Signal transduction by cAMP-dependent protein kinase A in *Drosophila* limb patterning. *Nature* 373, 711–715.
- Letsou, A., Arora, K., Wrana, J.L., Simin, K., Twombly, V., et al., 1995. *Drosophila* Dpp signaling is mediated by the *punt* gene product: a dual ligand-binding type II receptor of the TGF- β receptor family. *Cell* 80, 899–908.
- Lewandoski, M., Sun, X., Martin, G.R., 2000. Fgf8 signalling from the AER is essential for normal limb development. *Nat. Genet.* 26, 460–463.
- Leyns, L., Bouwmeester, T., Kim, S.-H., Piccolo, S., De Robertis, E.M., 1997. Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer. *Cell* 88, 747–756.
- Li, W., Ohlmeyer, J.T., Lane, M.E., Kalderon, D., 1995. Function of protein kinase A in Hedgehog signal transduction and *Drosophila* imaginal disc development. *Cell* 80, 553–562.
- Lin, X., Buff, E.M., Perrimon, N., Michelson, A.M., 1999. Heparan sulfate proteoglycans are essential for FGF receptor signaling during *Drosophila* embryonic development. *Development* 126, 3715–3723.
- Lin, X., Perrimon, N., 2000. Role of heparan sulfate proteoglycans in cell–cell signaling in *Drosophila*. *Matrix Biol.* 19, 303–307.
- Liu, X., Grammont, M., Irvine, K.D., 2000. Roles for *scalloped* and *vestigial* in regulating cell affinity and interactions between the wing blade and the wing hinge. *Devel. Biol.* 228, 287–303.
- Loomis, C.A., Harris, E., Michaud, J., Wurst, W., Hanks, M., et al., 1996. The mouse *Engrailed-1* gene and ventral limb patterning. *Nature* 382, 360–363.
- Loomis, C.A., Kimmel, R.A., Tong, C.X., Michaud, J., Joyner, A.L., 1998. Analysis of the genetic pathway leading to formation of ectopic apical ectodermal ridges in mouse *Engrailed-1* mutant limbs. *Development* 125, 1137–1148.
- Lopez-Martinez, A., Chang, D.T., Chiang, C., Porter, J.A., Ros, M.A., et al., 1995. Limb-patterning activity and restricted posterior localization of the amino-terminal product of Sonic hedgehog cleavage. *Curr. Biol.* 5, 791–796.
- Lu, C.H., Rincon-Limas, D.E., Botas, J., 2000. Conserved overlapping and reciprocal expression of *msh/Msx1* and *apterous/Lhx2* in *Drosophila* and mice. *Mech. Devel.* 99, 177–181.
- Ma, Y., Erkner, A., Gong, R., Yao, S., Taipale, J., et al., 2002. Hedgehog-mediated patterning of the mammalian embryo requires transporter-like function of dispatched. *Cell* 111, 63–75.
- MacCabe, J.A., Errick, J., Saunders, J.W., Jr., 1974. Ectodermal control of the dorsoventral axis in the leg bud of the chick embryo. *Devel. Biol.* 39, 69–82.
- Madhavan, M.M., Schneiderman, H.A., 1977. Histological analysis of the dynamics of growth of imaginal discs and histoblast nests during the larval development of *Drosophila melanogaster*. *Roux's Arch. Devel. Biol.* 183, 269–305.
- Mann, R.S., Carroll, S.B., 2002. Molecular mechanisms of selector gene function and evolution. *Curr. Opin. Genet. Devel.* 12, 592–600.
- Mann, R.S., Morata, G., 2000. The developmental and molecular biology of genes that subdivide the body of *Drosophila*. *Annu. Rev. Cell Devel. Biol.* 16, 243–271.
- Manning, B.D., Tee, A.R., Logsdon, M.N., Blenis, J., Cantley, L.C., 2002. Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberlin as a target of the phosphoinositide 3-kinase/akt pathway. *Mol. Cell* 10, 151–162.
- Martin, G., 2001. Making a vertebrate limb: new players enter from the wings. *BioEssays* 23, 865–868.
- Martin-Castellanos, C., Edgar, B.A., 2002. A characterization of the effects of Dpp signaling on cell growth and proliferation in the *Drosophila* wing. *Development* 129, 1003–1013.
- Marygold, S.J., Leevers, S.J., 2002. Growth signaling: TSC takes its place. *Curr. Biol.* 12, R785–R787.
- Massague, J., Blain, S.W., Lo, R.S., 2000. TGF β signaling in growth control, cancer, and heritable disorders. *Cell* 103, 295–309.
- Massague, J., Chen, Y.G., 2000. Controlling TGF- β signaling. *Genes Devel.* 14, 627–644.
- McDowell, N., Zorn, A.M., Crease, D.J., Gurdon, J.B., 1997. Activin has direct long-range signalling activity and can form a concentration gradient by diffusion. *Curr. Biol.* 7, 671–681.
- McNeill, H., 2000. Sticking together and sorting things out: adhesion as a force in development. *Nature Rev. Genet.* 1, 100–108.
- McNeill, H., Yang, C.H., Brodsky, M., Ungos, J., Simon, M.A., 1997. *mirror* encodes a novel PBX-class homeoprotein that functions in the definition of the

- dorsal-ventral border in the *Drosophila* eye. *Genes Devel.* 11, 1073-1082.
- Meinhardt, H., 1983. Cell determination boundaries as organizing regions for secondary embryonic fields. *Devel. Biol.* 96, 375-385.
- Mellitzer, G., Xu, Q., Wilkinson, D.G., 1999. Eph receptors and ephrins restrict cell intermingling and communication. *Nature* 400, 77-81.
- Mercader, N., Leonardo, E., Azpiazu, N., Serrano, A., Morata, G., *et al.*, 1999. Conserved regulation of proximodistal limb axis development by Meis1/Hth. *Nature* 402, 425-429.
- Mercader, N., Leonardo, E., Piedra, M.E., Martinez, A.C., Ros, M.A., *et al.*, 2000. Opposing RA and FGF signals control proximodistal vertebrate limb development through regulation of Meis genes. *Development* 127, 3961-3970.
- Merino, R., Rodriguez-Leon, J., Macias, D., Ganan, Y., Economides, A. N., *et al.*, 1999. The BMP antagonist Gremlin regulates outgrowth, chondrogenesis and programmed cell death in the developing limb. *Development* 126, 5515-5522.
- Méthot, N., Basler, K., 1999. Hedgehog controls limb development by regulating the activities of distinct transcriptional activator and repressor forms of Cubitus interruptus. *Cell* 96, 819-831.
- Meyuhas, O., Hornstein, E., 2000. Translational Control of TOP mRNAs. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Micchelli, C.A., Blair, S.S., 1999. Dorsoventral lineage restriction in wing imaginal discs requires Notch. *Nature* 401, 473-476.
- Milan, M., Campuzano, S., García-Bellido, A., 1996. Cell-cycling and patterned cell proliferation in the wing primordium of *Drosophila*. *Proc. Natl Acad. Sci. USA* 93, 640-645.
- Milan, M., Cohen, S.M., 1999a. Notch signaling is not sufficient to define the affinity boundary between dorsal and ventral compartments. *Mol. Cell* 4, 1073-1078.
- Milan, M., Cohen, S.M., 1999b. Regulation of LIM homeodomain activity *in vivo*: a tetramer of dLDB and apterous confers activity and capacity for regulation by dLMO. *Mol. Cell* 4, 267-273.
- Milan, M., Cohen, S.M., 2000a. Subdividing cell populations in the developing limbs of *Drosophila*: do wing veins and leg segments define units of growth control? *Devel. Biol.* 217, 1-9.
- Milan, M., Cohen, S.M., 2000b. Temporal regulation of apterous activity during development of the *Drosophila* wing. *Development* 127, 3069-3078.
- Milan, M., Cohen, S.M., 2003. A re-evaluation of the contributions of Apterous and Notch to the dorsoventral lineage restriction boundary in the *Drosophila* wing. *Development* 130, 553-562.
- Milan, M., Diaz-Benjumea, F.J., Cohen, S.M., 1998. *Beadex* encodes an LMO protein that regulates Apterous LIM-homeodomain activity in *Drosophila* wing development: a model for LMO oncogene function. *Genes Devel.* 12, 2912-2920.
- Milan, M., Weihe, U., Perez, L., Cohen, S.M., 2001a. The LRR proteins capricious and Tartan mediate cell interactions during DV boundary formation in the *Drosophila* wing. *Cell* 106, 785-794.
- Milan, M., Weihe, U., Tiong, S., Bender, W., Cohen, S.M., 2001b. *msh* specifies dorsal cell fate in the *Drosophila* wing. *Development* 128, 3263-3268.
- Min, H., Danilenko, D.M., Scully, S.A., Bolon, B., Ring, B.D., *et al.*, 1998. Fgf-10 is required for both limb and lung development and exhibits striking functional similarity to *Drosophila branchless*. *Genes Devel.* 12, 3156-3161.
- Minami, M., Kinoshita, N., Kamoshida, Y., Tanimoto, H., Tabata, T., 1999. *brinker* is a target of Dpp in *Drosophila* that negatively regulates Dpp-dependent genes. *Nature* 398, 242-246.
- Mohler, J., Vani, K., 1992. Molecular organisation and embryonic expression of the *hedgehog* gene involved in cell-cell communication in segmental patterning of *Drosophila*. *Development* 115, 957-971.
- Moline, M.M., Southern, C., Bejsovec, A., 1999. Directionality of Wingless protein transport influences epidermal patterning in the *Drosophila* embryo. *Development* 126, 4375-4384.
- Moloney, D.J., Panin, V.M., Johnston, S.H., Chen, J., Shao, L., *et al.*, 2000. Fringe is a glycosyltransferase that modifies Notch. *Nature* 406, 369-375.
- Montagne, J., Stewart, M.J., Stocker, H., Hafen, E., Kozma, S.C., *et al.*, 1999. *Drosophila* S6 kinase: a regulator of cell size. *Science* 285, 2126-2129.
- Moon, A.M., Capecchi, M.R., 2000. Fgf8 is required for outgrowth and patterning of the limbs. *Nature Genet.* 26, 455-459.
- Moran, J.L., Johnston, S.H., Rauskolb, C., Bhalerao, J., Bowcock, A.M., *et al.*, 1999a. Genomic structure, mapping, and expression analysis of the mammalian *Lunatic*, *Manic*, and *Radical fringe* genes. *Mamm. Genome.* 10, 535-541.
- Moran, J.L., Levorse, J.M., Vogt, T.F., 1999b. Limbs move beyond the radical fringe. *Nature* 399, 742-743.
- Morata, G., Lawrence, P.A., 1975. Control of compartment development by the engrailed gene in *Drosophila*. *Nature* 255, 614-617.
- Morata, G., Ripoll, P., 1975. Minutes: mutants of *Drosophila* autonomously affecting cell division rate. *Devel. Biol.* 42, 211-221.
- Morcillo, P., Rosen, C., Baylies, M.K., Dorsett, D., 1997. Chip, a widely expressed chromosomal protein required for segmentation and activity of a remote wing margin enhancer in *Drosophila*. *Genes Devel.* 11, 2729-2740.
- Moreno, E., Basler, K., Morata, G., 2002. Cells compete for decapentaplegic survival factor to prevent apoptosis in *Drosophila* wing development. *Nature* 416, 755-759.
- Mullor, J.L., Calleja, M., Capdevila, J., Guerrero, I., 1997. Hedgehog activity, independent of Decapentaplegic,

- participates in wing disc patterning. *Development* 124, 1227–1237.
- Nellen, D., Burke, R., Struhl, G., Basler, K., 1996. Direct and long-range action of a Dpp morphogen gradient. *Cell* 85, 357–368.
- Nestoras, K., Lee, H., Mohler, J., 1997. Role of *knot* (*kn*) in wing patterning in *Drosophila*. *Genetics* 147, 1203–1212.
- Neumann, C.J., Cohen, S.M., 1996a. Distinct mitogenic and cell fate specification functions of wingless in different regions of the wing development. *Development* 122, 1781–1789.
- Neumann, C.J., Cohen, S.M., 1996b. A hierarchy of cross-regulation involving *Notch*, *wingless*, *vestigial* and *cut* organizes the dorsal/ventral axis of the *Drosophila* wing. *Development* 122, 3477–3485.
- Neumann, C.J., Cohen, S.M., 1997. Long-range action of Wingless organizes the dorsal–ventral axis of the *Drosophila* wing. *Development* 124, 871–880.
- Newfeld, S.J., Chartoff, E.H., Graff, J.M., Melton, D.A., Gelbart, W.M., 1996. *Mothers against dpp* encodes a conserved cytoplasmic protein required in DPP/TGF- β responsive cells. *Development* 122, 2099–2108.
- Ng, J.K., Kawakami, Y., Buscher, D., Raya, A., Itoh, T., et al., 2002. The limb identity gene *Tbx5* promotes limb initiation by interacting with Wnt2b and Fgf10. *Development* 129, 5161–5170.
- Ng, M., Diaz-Benjumea, F.J., Cohen, S.M., 1995. *nubbin* encodes a POU-domain protein required for proximal–distal patterning in the *Drosophila* wing. *Development* 121, 589–599.
- Ng, M., Diaz-Benjumea, F.J., Vincent, J.P., Wu, J., Cohen, S.M., 1996. Specification of the wing by localized expression of wingless protein. *Nature* 381, 316–318.
- Niswander, L., Jeffrey, S., Martin, G.R., Tickle, C., 1994. A positive feedback loop coordinates growth and patterning in the vertebrate limb. *Nature* 371, 609–612.
- Niswander, L., Tickle, C., Vogel, A., Booth, I., Martin, G.R., 1993. FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. *Cell* 75, 579–587.
- Nüsslein-Volhard, C., Wieschaus, E., 1980. Mutations affecting segment number and polarity in *Drosophila*. *Nature* 287, 795–801.
- Nybakken, K., Perrimon, N., 2002. Heparan sulfate proteoglycan modulation of developmental signaling in *Drosophila*. *Biochim. Biophys. Acta* 1573, 280–291.
- O’Brochta, D.A., Bryant, P.J., 1985. A zone of non-proliferating cells at a lineage restriction boundary in *Drosophila*. *Nature* 313, 138–141.
- Ohkuma, Y., Horikoshi, M., Roeder, R.G., Desplan, C., 1990. Binding site dependent direct activation and repression of *in vitro* transcription by *Drosophila* homeodomain proteins. *Cell* 61, 475–484.
- Ohuchi, H., Nakagawa, T., Yamamoto, A., Araga, A., Ohata, T., et al., 1997. The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor. *Development* 124, 2235–2244.
- O’Keefe, D.D., Thomas, J.B., 2001. *Drosophila* wing development in the absence of dorsal identity. *Development* 128, 703–710.
- Oldham, S., Bohni, R., Stocker, H., Brogiolo, W., Hafen, E., 2000. Genetic control of size in *Drosophila*. *Phil. Trans. Roy. Soc. B* 355, 945–952.
- Oldham, S., Hafen, E., 2003. Insulin/IGF and target of rapamycin signaling: a TOR de force in growth control. *Trends Cell Biol.* 13, 79–85.
- Oldham, S., Stocker, H., Laffargue, M., Wittwer, F., Wymann, M., et al., 2002. The *Drosophila* insulin/IGF receptor controls growth and size by modulating PtdInsP(3) levels. *Development* 129, 4103–4109.
- Oliver, G., De Robertis, E.M., Wolpert, L., Tickle, C., 1990. Expression of a homeobox gene in the chick wing bud following application of retinoic acid and grafts of polarizing region tissue. *EMBO J.* 9, 3093–3099.
- Padgett, R.W., Das, P., Krishna, S., 1998. TGF-beta signaling, Smads, and tumor suppressors. *BioEssays* 20, 382–390.
- Padgett, R.W., St. Johnston, R.D., Gelbart, W.M., 1987. A transcript from a *Drosophila* pattern gene predicts a protein homologous to the transforming growth factor- β family. *Nature* 325, 81–84.
- Pan, D.J., Rubin, G., 1995. Protein kinase and hedgehog act antagonistically in regulating *decapentaplegic* transcription in *Drosophila* imaginal discs. *Cell* 80, 543–552.
- Panganiban, G., Irvine, S.M., Lowe, C., Roehl, H., Corley, L.S., et al., 1997. The origin and evolution of animal-appendages. *Proc. Natl Acad. Sci. USA* 94, 5162–5166.
- Panin, V.M., Papayannopoulos, V., Wilson, R., Irvine, K.D., 1997. Fringe modulates Notch–ligand interactions. *Nature* 387, 908–913.
- Panin, V.M., Shao, L., Lei, L., Moloney, D.J., Irvine, K.D., et al., 2002. Notch ligands are substrates for protein O-fucosyltransferase-1 and Fringe. *J. Biol. Chem.* 277, 29945–29952.
- Parr, B.A., McMahon, A.P., 1995. Dorsalizing signal Wnt-7a required for normal polarity of D–V and A–P axes of mouse limb. *Nature* 374, 350–353.
- Patel, N.H., Martin-Blanco, E., Coleman, K.G., Poole, S.J., Ellis, M. C., et al., 1989. Expression of *engrailed* proteins in arthropods, annelids, and chordates. *Cell* 58, 955–968.
- Perrimon, N., Bernfield, M., 2000. Specificities of heparan sulphate proteoglycans in developmental processes. *Nature* 404, 725–728.
- Pfeiffer, S., Alexandre, C., Calleja, M., Vincent, J.P., 2000. The progeny of wingless-expressing cells deliver the signal at a distance in *Drosophila* embryos. *Curr. Biol.* 10, 321–324.
- Pfeiffer, S., Ricardo, S., Manneville, J.B., Alexandre, C., Vincent, J.P., 2002. Producing cells retain and recycle Wingless in *Drosophila* embryos. *Curr. Biol.* 12, 957–962.

- Phillips, R., Whittle, J.R.S., 1993. *wingless* expression mediates determination of peripheral nervous system elements in late stages of *Drosophila* wing disc development. *Development* 118, 427–438.
- Pichaud, F., Casares, F., 2000. *homothorax* and *iroquois-C* genes are required for the establishment of territories within the developing eye disc. *Mech. Devel.* 96, 15–25.
- Pizette, S., Abate-Shen, C., Niswander, L., 2001. BMP controls proximodistal outgrowth, via induction of the apical ectodermal ridge, and dorsoventral patterning in the vertebrate limb. *Development* 128, 4463–4474.
- Pizette, S., Niswander, L., 1999. BMPs negatively regulate structure and function of the limb apical ectodermal ridge. *Development* 126, 883–894.
- Placzek, M., Tessier-Lavigne, M., Yamada, T., Jessell, T.M., Dodd, J., 1990. Mesodermal control of neural cell identity: floorplate induction by the notochord. *Science* 250, 985–988.
- Porter, F.D., Drago, J., Xu, Y., Cheema, S.S., Wassif, C., et al., 1997. *Lhx2*, a LIM homeobox gene, is required for eye, forebrain, and definitive erythrocyte development. *Development* 124, 2935–2944.
- Porter, J.A., von Kessler, D.P., Ekker, S.C., Young, K.E., Lee, J.J., et al., 1995. The product of *hedgehog* autoproteolytic cleavage active in local and long-range signalling. *Nature* 374, 363–366.
- Porter, J.A., Young, K.E., Beachy, P.A., 1996. Cholesterol modification of hedgehog signaling proteins in animal development. *Science* 274, 255–259.
- Rafferty, L.A., Sutherland, D.J., 1999. TGF-beta family signal transduction in *Drosophila* development: from Mad to Smads. *Devel. Biol.* 210, 251–268.
- Ramain, P., Khechumian, R., Khechumian, K., Arbogast, N., Ackermann, C., et al., 2000. Interactions between chip and the achaete/scute-daughterless heterodimers are required for pannier-driven proneural patterning. *Mol. Cell* 6, 781–790.
- Ramirez-Weber, F.A., Kornberg, T.B., 1999. Cytonemes: cellular processes that project to the principal signaling center in *Drosophila* imaginal discs. *Cell* 97, 599–607.
- Ramirez-Weber, F.A., Kornberg, T.B., 2000. Signaling reaches to new dimensions in *Drosophila* imaginal discs. *Cell* 103, 189–192.
- Rancourt, D.E., Tsuzuki, T., Capecchi, M.R., 1995. Genetic interaction between *hoxb-5* and *hoxb-6* is revealed by nonallelic noncomplementation. *Genes Devel.* 9, 108–122.
- Rauskolb, C., Irvine, K.D., 1999. Notch-mediated segmentation and growth control of the *Drosophila* leg. *Devel. Biol.* 210, 339–350.
- Rebay, I., Fleming, R.J., Fehon, R.G., Cherbas, L., Cherbas, P., et al., 1991. Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. *Cell* 67, 687–699.
- Reichsman, F., Smith, L., Cumberledge, S., 1996. Glycosaminoglycans can modulate extracellular localization of the wingless protein and promote signal transduction. *J. Cell Biol.* 135, 819–827.
- Retaux, S., Rogard, M., Bach, I., Failli, V., Besson, M.J., 1999. *Lhx9*: a novel LIM-homeodomain gene expressed in the developing forebrain. *J. Neurosci.* 19, 783–793.
- Riddle, R.D., Ensini, M., Nelson, C., Tsuchida, T., Jessell, T.M., et al., 1995. Induction of the LIM homeobox gene *Lmx1* by WNT7a establishes dorsoventral pattern in the vertebrate limb. *Cell* 83, 631–640.
- Riddle, R.D., Johnson, R.L., Laufer, E., Tabin, C., 1993. Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* 75, 1401–1416.
- Rijsewijk, F., Schuermann, M., Wagenaar, E., Parren, P., Weigel, D., et al., 1987. The *Drosophila* homologue of the mouse mammary oncogene *int-1* is identical to the segment polarity gene *wingless*. *Cell* 50, 649–657.
- Rincon-Limas, D.E., Lu, C.H., Canal, I., Botas, J., 2000. The level of DLDB/CHIP controls the activity of the LIM homeodomain protein Apterous: evidence for a functional tetramer complex *in vivo*. *EMBO J.* 19, 2602–2614.
- Rincon-Limas, D.E., Lu, C.H., Canal, I., Calleja, M., Rodriguez-Esteban, C., et al., 1999. Conservation of the expression and function of *apterous* orthologs in *Drosophila* and mammals. *Proc. Natl Acad. Sci. USA* 96, 2165–2170.
- Rodriguez, I., Basler, K., 1997. Control of compartmental affinity boundaries by hedgehog. *Nature* 389, 614–618.
- Rodriguez-Esteban, C., Schwabe, J.W., De La Pena, J., Foy, B., Eshelman, B., et al., 1997. Radical fringe positions the apical ectodermal ridge at the dorsoventral boundary of the vertebrate limb. *Nature* 386, 360–366.
- Rodriguez-Esteban, C., Schwabe, J.W., De La Pena, J., Rincon-Limas, D.E., Magallon, J., et al., 1998. *Lhx2*, a vertebrate homologue of *apterous*, regulates vertebrate limb outgrowth. *Development* 125, 3925–3934.
- Rong, Y.S., Golic, K.G., 2000. Gene targeting by homologous recombination in *Drosophila*. *Science* 288, 2013–2018.
- Rong, Y.S., Titen, S.W., Xie, H.B., Golic, M.M., Bastiani, M., et al., 2002. Targeted mutagenesis by homologous recombination in *D. melanogaster*. *Genes Devel.* 16, 1568–1581.
- Rorth, P., 1996. A modular misexpression screen in *Drosophila* detecting tissue specific phenotypes. *Proc. Natl Acad. Sci. USA* 93, 12418–12422.
- Rorth, P., Szabo, K., Bailey, A., Laverty, T., Rehm, J., et al., 1998. Systematic gain-of-function genetics in *Drosophila*. *Development* 125, 1049–1057.
- Ross, J.J., Shimmi, O., Vilmos, P., Petryk, A., Kim, H., et al., 2001. Twisted gastrulation is a conserved extracellular BMP antagonist. *Nature* 410, 479–483.
- Rousset, R., Mack, J.A., Wharton, K.A., Jr., Axelrod, J.D., Cadigan, K.M., et al., 2001. Naked cuticle targets dishevelled to antagonize Wnt signal transduction. *Genes Devel.* 15, 658–671.

- Rousset, R., Wharton, K.A., Jr., Zimmermann, G., Scott, M.P., 2002. Zinc-dependent interaction between *dishevelled* and the *Drosophila* Wnt antagonist *naked cuticle*. *J. Biol. Chem.* 277, 49019–49026.
- Ruberte, E., Marty, T., Nellen, D., Affolter, M., Basler, K., 1995. An absolute requirement for both the type II and type I receptors, *punt* and *thickveins*, for Dpp signaling *in vivo*. *Cell* 80, 889–897.
- Rulifson, E.J., Blair, S.S., 1995. *Notch* regulates *wingless* expression and is not required for reception of the paracrine *wingless* signal during wing margin neurogenesis in *Drosophila*. *Development* 121, 2813–2824.
- Schwabe, J.W., Rodriguez-Esteban, C., Izpisua Belmonte, J.-C., 1998. Limbs are moving: where are they going? *Trends Genet.* 14, 229–235.
- Schwartz, C., Locke, J., Nishida, C., Kornberg, T.B., 1995. Analysis of *cubitus interruptus* regulation in *Drosophila* embryos and imaginal discs. *Development* 121, 1625–1635.
- Searls, R.L., Janners, M.Y., 1971. The initiation of limb bud outgrowth in the embryonic chick. *Devel. Biol.* 24, 198–213.
- Sekine, K., Ohuchi, H., Fujiwara, M., Yamasaki, M., Yoshizawa, T., *et al.*, 1999. Fgf10 is essential for limb and lung formation. *Nature Genet.* 21, 138–141.
- Selleck, S.B., 2000. Proteoglycans and pattern formation: sugar biochemistry meets developmental genetics. *Trends Genet.* 16, 206–212.
- Selleck, S.B., 2001. Genetic dissection of proteoglycan function in *Drosophila* and *C. elegans*. *Semin. Cell Devel. Biol.* 12, 127–134.
- Selva, E.M., Hong, K., Baeg, G.H., Beverley, S.M., Turco, S.J., *et al.*, 2001. Dual role of the fringe connection gene in both heparan sulphate and fringe-dependent signalling events. *Nature Cell Biol.* 3, 809–815.
- Serrano, N., O'Farrell, P., 1997. Limb morphogenesis: connections between patterning and growth. *Curr. Biol.* 7, 186–195.
- Shoresch, M., Orgad, S., Schmueli, O., Werczbergrer, R., Gelbaum, D., *et al.*, 1998. Overexpression *Beadex* mutations and loss of function *heldup-a* mutations in *Drosophila* affect the 3' regulatory and coding components, respectively, of the *Dlmo* gene. *Genetics* 150, 283–299.
- Simeone, A., 2000. Positioning the isthmic organizer where *Otx2* and *Gbx2* meet. *Trends Genet.* 16, 237–240.
- Simmonds, A.J., Brook, W.J., Cohen, S.M., Bell, J.B., 1995. Distinguishable functions for *engrailed* and *invected* in anterior–posterior patterning in the *Drosophila* wing. *Nature* 376, 424–427.
- Simmonds, A.J., dosSantos, G., Livne-Bar, I., Krause, H.M., 2001. Apical localization of *wingless* transcripts is required for wingless signaling. *Cell* 105, 197–207.
- Simmonds, A.J., Liu, X., Soanes, K.H., Krause, H.M., Irvine, K.D., *et al.*, 1998. Molecular interactions between *Vestigial* and *Scalloped* promote wing formation in *Drosophila*. *Genes Devel.* 12, 3815–3820.
- Simpson, P., Schneiderman, H.A., 1975. Isolation of temperature sensitive mutations blocking clone development in *Drosophila melanogaster*, and the effects of a temperature sensitive cell lethal mutation on pattern formation in imaginal discs. *Roux's Arch. Devel. Biol.* 178, 247–275.
- Sisson, J.C., Ho, K.S., Suyama, K., Scott, M.P., 1997. Costal2, a novel kinesin-related protein in the Hedgehog signaling pathway. *Cell* 90, 235–245.
- Smith, S.T., Jaynes, J.B., 1996. A conserved region of *Engrailed*, shared among all *en-*, *gsc-*, *NK1-*, *NK2-* and *msh-* class homeoproteins, mediates active transcriptional repression *in vivo*. *Development* 122, 3141–3150.
- Snodgrass, R.E., 1935. Principles of Insect Morphology. McGraw Hill, New York.
- Speicher, S.A., Thomas, U., Hinz, U., Knust, E., 1994. The *Serrate* locus of *Drosophila* and its role in morphogenesis of the wing imaginal discs: control of cell proliferation. *Development* 120, 535–544.
- Spemann, H., Mangold, H., 1924. Über Induktion von Embryonenanlagen durch Implantation artfremder Organismen. *Arch. Mikrosk. Anat. Entw. Mech.* 100, 599–638.
- Steinberg, M.S., Takeichi, M., 1994. Experimental specification of cell sorting, tissue spreading, and specific spatial patterning by quantitative differences in cadherin expression. *Proc. Natl Acad. Sci USA* 91, 206–209.
- Steiner, E., 1976. Establishment of compartments in the developing leg imaginal discs of *Drosophila melanogaster*. *Roux's Arch. Devel. Biol.* 180, 9–30.
- St. Pierre, S.E., Galindo, M.I., Couso, J.P., Thor, S., 2002. Control of *Drosophila* imaginal disc development by rotund and roughened eye: differentially expressed transcripts of the same gene encoding functionally distinct zinc finger proteins. *Development* 129, 1273–1281.
- Strigini, M., Cohen, S.M., 1997. A Hedgehog activity gradient contributes to AP axial patterning of the *Drosophila* wing. *Development* 124, 4697–4705.
- Strigini, M., Cohen, S.M., 1999. Formation of morphogen gradients in the *Drosophila* wing. *Semin. Cell Devel. Biol.* 10, 335–344.
- Strigini, M., Cohen, S.M., 2000. Wingless gradient formation in the *Drosophila* wing. *Curr. Biol.* 10, 293–300.
- Struhl, G., Basler, K., 1993. Organizing activity of *wingless* protein in *Drosophila*. *Cell* 72, 527–540.
- Sturtevant, M.A., Biehs, B., Marin, E., Bier, E.A., 1997. The *spalt* gene links the A/P compartment boundary to a linear adult structure in the *Drosophila* wing. *Development* 124, 21–32.
- Sturtevant, M.A., Bier, E., 1995. Analysis of the genetic hierarchy guiding wing vein development in *Drosophila*. *Development* 121, 785–801.
- Sun, X., Mariani, F.V., Martin, G.R., 2002. Functions of FGF signalling from the apical ectodermal ridge in limb development. *Nature* 418, 501–508.
- Tabata, T., Eaton, S., Kornberg, T.B., 1992. The *Drosophila hedgehog* gene is expressed specifically in posterior

- compartment cells and is a target of *engrailed* regulation. *Genes Devel.* 6, 2635–2645.
- Tabata, T., Kornberg, T., 1994. Hedgehog is a signalling protein with a key role in patterning *Drosophila* imaginal discs. *Cell* 76, 89–102.
- Tabata, T., Schwartz, C., Gustavson, E., Ali, Z., Kornberg, T.B., 1995. Creating a *Drosophila* wing *de novo*: the role of *engrailed* and the compartment border hypothesis. *Development* 121, 3359–3369.
- Tanimoto, H., Itoh, S., ten Dijke, P., Tabata, T., 2000. Hedgehog creates a gradient of Dpp activity in *Drosophila* wing imaginal discs. *Mol. Cell* 5, 59–71.
- Teleman, A.A., Cohen, S.M., 2000. Dpp gradient formation in the *Drosophila* wing imaginal disc. *Cell* 103, 971–980.
- Teleman, A.A., Strigini, M., Cohen, S.M., 2001. Shaping morphogen gradients. *Cell* 105, 559–562.
- The, I., Bellaiche, Y., Perrimon, N., 1999. Hedgehog movement is regulated through *tout velu* dependent synthesis of a heparan sulfate proteoglycan. *Mol. Cell* 4, 633–639.
- Theisen, H., Haerry, T.E., O'Connor, M.B., Marsh, J.L., 1996. Developmental territories created by mutual antagonism between Wingless and Decapentaplegic. *Development* 122, 3939–3948.
- Theodosiou, N.A., Zahng, S., Wang, W.Y., Xu, T., 1998. *slimb* coordinates *wg* and *dpp* expression in the dorsal-ventral and anterior-posterior axes during limb development. *Development* 125, 3411–3416.
- Thomas, G., 2002. The S6 kinase signaling pathway in the control of development and growth. *Biol. Res.* 35, 305–313.
- Tickle, C., Munsterberg, A., 2001. Vertebrate limb development: the early stages in chick and mouse. *Curr. Opin. Genet. Devel.* 11, 476–481.
- Todt, W.L., Fallon, J.F., 1984. Development of the apical ectodermal ridge in the chick wing bud. *J. Embryol. Exp. Morphol.* 80, 21–41.
- Toyoda, H., Kinoshita-Toyoda, A., Selleck, S.B., 2000. Structural analysis of glycosaminoglycans in *Drosophila* and *Caenorhabditis elegans* and demonstration that *tout-velu*, a *Drosophila* gene related to EXT tumor suppressors, affects heparan sulfate *in vivo*. *J. Biol. Chem.* 275, 2269–2275.
- Tsuda, M., Kamimura, K., Nakato, H., Archer, M., Staatz, W., *et al.*, 1999. The cell-surface proteoglycan Dally regulates Wingless signalling in *Drosophila*. *Nature* 400, 276–280.
- Tsuneizumi, K., Nakayama, T., Kamoshida, Y., Kornberg, T.B., Christian, J.L., *et al.*, 1997. *Daughters against dpp* modulates *dpp* organizing activity in *Drosophila* wing development. *Nature* 389, 627–631.
- Turing, A., 1952. The chemical basis of morphogenesis. *Phil. Trans. Roy. Soc.* 237, 37–72.
- van den Heuvel, M., Harryman-Samos, C., Klingensmith, J., Perrimon, N., Nusse, R., 1993. Mutations in the segment polarity genes *wingless* and *porcupine* impair secretion of the wingless protein. *EMBO J.* 12, 5293–5302.
- van den Heuvel, M., Nusse, R., Johnston, P., Lawrence, P.A., 1989. Distribution of the wingless gene product in *Drosophila* embryos: a protein involved in cell-cell communication. *Cell* 59, 739–749.
- van Meyel, D.J., O'Keefe, D.D., Jurata, L.W., Thor, S., Gill, G.N., *et al.*, 1999. Chip and apterous physically interact to form a functional complex during *Drosophila* development. *Mol. Cell* 4, 259–265.
- Vervoort, M., Crozatier, M., Valle, D., Vincent, A., 1999. The COE transcription factor Collier is a mediator of short-range Hedgehog-induced patterning of the *Drosophila* wing. *Curr. Biol.* 17, 632–639.
- Vincent, J.P., Magee, T., 2002. Argosomes: membrane fragments on the run. *Trends Cell Biol.* 12, 57–60.
- Vleminckx, K., Kemler, R., 1999. Cadherins and tissue formation: integrating adhesion and signaling. *Bio-Essays* 21, 211–220.
- Vogel, A., Rodriguez, C., Warnken, W., Izpisua Belmonte, J.-C., 1995. Dorsal cell fate specified by chick Lmx1 during vertebrate limb development. *Nature* 378, 716–720.
- Wang, S., Krinks, M., Lin, K., Luyten, F.P., Moos, M.J., 1997. Frzb, a secreted protein expressed in the Spemann organizer, binds and inhibits Wnt-8. *Cell* 88, 757–766.
- Wang, S.H., Simcox, A., Campbell, G., 2000. Dual role for *Drosophila* epidermal growth factor receptor signaling in early wing disc development. *Genes Devel.* 14, 2271–2276.
- Weigmann, K., Cohen, S.M., 1999. Lineage tracing cells born in different domains along the PD axis of the developing *Drosophila* leg. *Development* 126, 3823–3830.
- Weihe, U., Dorfman, R., Wernet, M.F., Cohen, S.M., Milan, M., 2004. Proximodistal subdivision of *Drosophila* legs and wings: the *elbow-no ocelli* gene complex. *Development* 131 (4), 761–774.
- Weihe, U., Milan, M., Cohen, S.M., 2001. Regulation of Apterous activity in *Drosophila* wing development. *Development* 128, 4615–4622.
- Weinkove, D., Neufeld, T.P., Twardzik, T., Waterfield, M.D., Leever, S.J., 1999. Regulation of imaginal disc cell size, cell number and organ size by *Drosophila* class I(A) phosphoinositide 3-kinase and its adaptor. *Curr. Biol.* 9, 1019–1029. (Erratum: *Curr. Biol.* 9, R867.)
- Wiersdorff, V., Lecuit, T., Cohen, S.M., Mlodzik, M., 1996. *Mad* acts downstream of the Dpp receptors and reveals differential requirement for *dpp* signaling between initiation and propagation of morphogenesis in the *Drosophila* eye. *Development* 122, 2153–2162.
- Wieschaus, E., Gehring, W., 1976. Clonal analysis of primordial disc cells in the early embryo of *Drosophila melanogaster*. *Devel. Biol.* 50, 249–263.
- Williams, J.A., Paddock, S.W., Carroll, S.B., 1993. Pattern formation in a secondary field: a hierarchy of regulatory genes subdivides the developing *Drosophila* wing disc into discrete subregions. *Development* 117, 571–584.
- Williams, J.A., Paddock, S.W., Vorwerk, K., Carroll, S.B., 1994. Organization of wing formation and induction

- of a wing-patterning gene at the dorsal/ventral compartment boundary. *Nature* 368, 299–305.
- Wisotzkey, R.G., Mehra, A., Sutherland, D.J., Dobens, L.L., Liu, X., *et al.*, 1998. *Medea* is a *Drosophila Smad4* homolog that is differentially required to potentiate Dpp responses. *Development* 125, 1433–1445.
- Wodarz, A., Nusse, R., 1998. Mechanisms of Wnt signaling in development. *Annu. Rev. Cell Devel. Biol.* 14, 59–88.
- Wolpert, L., 1969. Positional information and the spatial pattern of cellular differentiation. *J. Theoret. Biol.* 25, 1–47.
- Wolpert, L., 1989. Positional information revisited. *Development* 107, 3–12.
- Wolpert, L., 1996. One hundred years of positional information. *Trends Genet.* 12, 359–364.
- Wu, J., Cohen, S.M., 1999. Proximodistal axis formation in the *Drosophila* leg: subdivision into proximal and distal domains by Homothorax and Distal-less. *Development* 126, 109–117.
- Wu, J., Cohen, S.M., 2000. Proximal distal axis formation in the *Drosophila* leg: distinct functions of *teashirt* and *homothorax* in the proximal leg. *Mech. Devel.* 94, 47–56.
- Xu, Q., Mellitzer, G., Robinson, V., Wilkinson, D.G., 1999. *In vivo* cell sorting in complementary segmental domains mediated by Eph receptors and ephrins. *Nature* 399, 267–271.
- Xu, T., Rubin, G.M., 1993. Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* 117, 1223–1237.
- Yamada, T., Placzek, M., Tanaka, H., Dodd, J., Jessell, T.M., 1991. Control of cell pattern in the developing nervous system: polarizing activity of the floor plate and notochord. *Cell* 64, 635–647.
- Yang, Y., Niswander, L., 1995. Interaction between the signalling molecules WNT7a and SHH during vertebrate limb development: dorsal signals regulate anteroposterior patterning. *Cell* 80, 939–947.
- Zecca, M., Basler, K., Struhl, G., 1995. Sequential organizing activities of *engrailed*, *hedgehog* and *decapentaplegic* in the *Drosophila* wing. *Development* 121, 2265–2278.
- Zecca, M., Basler, K., Struhl, G., 1996. Direct and long-range action of a Wingless morphogen gradient. *Cell* 87, 833–844.
- Zecca, M., Struhl, G., 2002a. Control of growth and patterning of the *Drosophila* wing imaginal disc by EGFR-mediated signaling. *Development* 129, 1369–1376.
- Zecca, M., Struhl, G., 2002b. Subdivision of the *Drosophila* wing imaginal disc by EGFR-mediated signaling. *Development* 129, 1357–1368.
- Zeng, C., Justice, N.J., Abdelilah, S., Chan, Y.M., Jan, L.Y., *et al.*, 1998. The *Drosophila* LIM-only gene, *dLMO*, is mutated in *Beadex* alleles and might represent an evolutionarily conserved function in appendage development. *Proc. Natl Acad. Sci. USA* 95, 10637–10642.
- Zeng, W., Wharton, K.A., Jr., Mack, J.A., Wang, K., Gadabaw, M., *et al.*, 2000. *naked cuticle* encodes an inducible antagonist of Wnt signalling. *Nature* 403, 789–795.
- Zuniga, A., Haramis, A.P., McMahon, A.P., Zeller, R., 1999. Signal relay by BMP antagonism controls the SHH/FGF4 feedback loop in vertebrate limb buds. *Nature* 401, 598–602.

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3 Early Embryonic Development: Neurogenesis (CNS)

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3.1. Introduction

The relatively simple organization of insect embryos provides a useful model for investigating the mechanisms that generate and pattern complex nervous systems. Certain insects, such as the grasshopper, have very large embryos and easily accessible cells. This has allowed the analysis of cellular events as well as the manipulation of their nervous systems during development (Zinn and Condron, 1994; Sanchez *et al.*, 1995). For a number of years, *Drosophila* has lagged behind because of its small size, compactness, and rapid development. The generation of sophisticated genetic tools, however, has made it possible to overcome these limitations. Neural precursors and their progeny have been identified in fixed and living animals, through the use of monoclonal antibodies, transgenic lines, and cellular dyes (see below). Analyzing the phenotype of gain-of-function and loss-of-function mutations of genes affecting this process has resulted in the identification of the molecular pathways that lead to nervous system

differentiation. Finally, the evolutionary conservation amongst insects has made it possible to use the knowledge gained in the grasshopper in order to understand fly neural development (Anderson *et al.*, 1980; Stent and Weisblat, 1985; Boyan and Ball, 1993). The combined use of these approaches has shed light on the basic cellular and molecular mechanisms underlying insect neurogenesis.

Flies display a central nervous system (CNS) that includes the ventral nerve cord (VNC) and the brain, as well as a peripheral nervous system (PNS) (reviews: Campos-Ortega, 1993; Goodman and Doe, 1993) (see **Chapters 4 and 5**). PNS and CNS share most cellular and molecular developmental pathways. For the sake of simplicity, this chapter will mainly focus on the development of the VNC.

During embryogenesis, distinct neuronal cell types including motorneurons, sensory neurons, interneurons, and different types of glia are produced. The generation of neural diversity is a multistep process that requires different types of signals

(reviews: McConnell, 1995; Matsuzaki, 2000). On the one hand, neuralizing and inhibitory factors control the neural competence of large territories and the differentiation of neural precursors. On the other hand, positional cues control the type of neural precursors and, thereby, the type of neurons and glia that populate the nervous system.

3.2. Neural Differentiation

3.2.1. Neural Tissue Formation

The first step in nervous system development involves the acquisition of the neural competence. During embryonic development, two ventral territories acquire such competence, a wide one called the neuroectoderm, and a discrete one, the mesectoderm (reviews: Campos-Ortega, 1993; Goodman and Doe, 1993). The fly neuroectoderm is a heterogeneous tissue, in the sense that it gives rise to neurons, glia, and epidermis. Such heterogeneity, which is achieved via delamination of neural precursors, is a peculiarity of insect neurogenesis, since in vertebrates, the entire neural anlage invaginates, forms the neural tube, and produces neurons and glia (Doe and Smouse, 1990; Fraser, 1991; Salzberg and Bellen, 1996; Arendt and Nubler-Jung, 1999).

The mesectoderm separates the neuroectoderm from the mesoderm and is composed of two rows of VNC cells, one on each side of the embryo. Upon gastrulation, mesectodermal cells come together and align to form a single row of cells called the midline cells (review: Jacobs, 2000). The fly midline corresponds to the vertebrate floor plate and plays a role in axonal guidance.

3.2.1.1. The neuroectoderm In wild-type *Drosophila* embryos, the neuroectoderm becomes morphologically manifest during the initial phase of germ-band elongation. Positional information provided solely by the mother is sufficient to subdivide the embryo into three domains (ventral, lateral, and dorsal) corresponding to distinct fates (mesoderm, neuroectoderm, and nonneural ectoderm) (reviews: Rusch and Levine, 1996; Bier, 1997; Stathopoulos and Levine, 2002).

The Dorsal (Dl; NF- κ B related) protein is distributed throughout the cytoplasm of the growing oocytes and unfertilized eggs (Steward, 1987). After fertilization, it is released from the cytoplasm and enters the nuclei in the ventral region, whereas it stays in the cytoplasm in the dorsal region (Roth *et al.*, 1989; Steward, 1989; Gay and Keith, 1990). Nuclear Dorsal is present at high levels in the ventral presumptive mesodermal cells, at intermediate levels in lateral cells comprising the neuroectoderm, and absent from cells giving rise to nonneural ectoderm (Figure 1) (Roth *et al.*, 1989; Steward, 1989; Govind and Steward, 1991; review: Bier, 1997). Once in the nucleus, Dorsal controls gene expression in a concentration dependent fashion. It functions as an activator of genes expressed in ventral and lateral regions of the embryo, but it also acts as a repressor of genes expressed in the dorsal region.

In ventral cells, peak levels of Dorsal activate the mesoderm-determining genes *twist (twi)* and *snail (sna)* (Jiang *et al.*, 1991; Kosman *et al.*, 1991; Rao *et al.*, 1991). Intermediate levels of Dorsal in the lateral embryonic regions activate a set of genes that initiate the differentiation of neuroectoderm (Figure 1). At least seven genes appear to be activated in the neuroectoderm including *rhomboid (rho)*

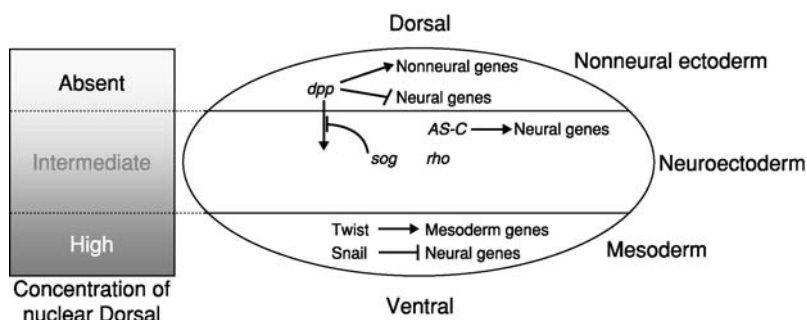


Figure 1 Dorsoventral patterning in the *Drosophila* embryo. Schematic representation of an embryo at blastoderm stage, lateral view. The molecular pathways induced by the gradient of nuclear Dorsal protein subdivide the embryo into three regions along the dorsoventral axis. High levels of nuclear Dorsal activate the expression of *twist (twi)* and *snail (sna)*, which specify mesoderm identity, while intermediate levels induce *short of gastrulation (sog)*, the *Achaete–Scute Complex (AS-C)*, and *rhomboid (rho)* expression, which specify neuroectoderm identity. Absence of nuclear Dorsal activates *decapentaplegic (dpp)* expression. Sog prevents Dpp from signaling to the neuroectoderm and permits the expression of neural genes. (Modified from Bier, E., 1997. Anti-neural-inhibition: a conserved mechanism for neural induction. *Cell* 89, 681–684.)

(Bier *et al.*, 1990), *short gastrulation (sog)* (François *et al.*, 1994), *lethal of scute [l(1)SC]* (a proneural gene belonging to the *achaete–scute* complex or AS-C) (Martin-Bermudo *et al.*, 1995), the *m7* and *m8* genes of the *enhancer of split complex [E(spl)]* (Knust *et al.*, 1987a), and *single-minded (sim)* (Kasai *et al.*, 1992). Cells of the dorsal nonneural ectoderm, which contain no nuclear Dorsal, express several genes including *decapentaplegic (dpp)*, *zerknüllt (zen)*, and *tolloid (tld)* (Ip *et al.*, 1991; Ray *et al.*, 1991; Shimell *et al.*, 1991; Huang *et al.*, 1993) (Figure 1). In ventral and lateral cells, Dorsal represses the expression of these genes (Ray *et al.*, 1991).

In vertebrates, neural tissue formation is controlled by the interplay of two types of molecules that display antagonistic functions: bone morphogenetic proteins (BMPs) and Chordin (reviews: Bier, 1997; Mehler *et al.*, 1997). While BMP4 blocks the ability of ectoderm to adopt the neural fate (review: Hemmati-Brivanlou and Melton, 1997), Chordin inhibits BMP4 signaling, thereby allowing neural differentiation (Sasai *et al.*, 1995; Hama and Weinstein, 2001). In *Drosophila*, *short gastrulation* (a Chordin ortholog) and *decapentaplegic* (a BMP4 ortholog) play similar roles in defining the domain of the ectoderm that will become the neuroectoderm (François and Bier, 1995; Holley *et al.*, 1995; Biehs *et al.*, 1996; reviews: Ferguson, 1996; Bier, 1997). Dpp is expressed in dorsal nonneural cells (Irish and Gelbart, 1987; St Johnston and Gelbart, 1987), whereas Sog is an extracellular protein expressed in the presumptive neuroectoderm (François *et al.*, 1994). Both Dpp and Sog likely diffuse from their site of production into adjacent territories. In the early blastoderm embryo, Sog prevents Dpp signaling from invading the neuroectoderm (François *et al.*, 1994; Biehs *et al.*, 1996) (Figure 1). Dpp signaling functions both to maintain expression of dorsally acting genes (e.g., *zen*) and to suppress expression of genes, the so-called proneural genes, which induce the differentiation of neural precursors (Ray *et al.*, 1991; Skeath *et al.*, 1992; Biehs *et al.*, 1996) (Figure 1). Complete loss of Dpp activity results in total transformation of dorsal ectoderm into neuroectoderm: both the amnioserosa (the dorsalmost region of the embryo) and the dorsal epidermis are lost in these mutant embryos (St Johnston and Gelbart, 1987). Genetic evidence supports the view that Sog opposes Dpp activity, as reducing the gene dose of *sog* rescues lethality resulting from weak mutations in the Dpp pathway (Biehs *et al.*, 1996).

3.2.1.2. The mesectoderm In *Drosophila* embryos, the mesectoderm corresponds to a single row of cells abutting the mesoderm. Mesectoderm identity

is induced by Sim, since midline cells fail to differentiate in its absence (Crews *et al.*, 1988; Thomas *et al.*, 1988; Nambu *et al.*, 1990). The *sim* gene acts as a master gene for midline identity, as shown by the observation that its ectopic expression leads to the transformation of most lateral CNS cells into midline cells (Nambu *et al.*, 1991). The *sim* gene encodes a bHLH-PAS (basic helix–loop–helix–Per–Arnt–Sim) transcription factor that heterodimerizes with the Tango (Tgo) protein (another bHLH-PAS transcription factor) to activate the expression of midline-specific genes (Sonnenfeld *et al.*, 1997; Ward *et al.*, 1998).

Genetic and molecular evidence indicate that *sim* expression is repressed by Snail and Suppressor of Hairless (Su(H)) in the mesoderm and the neuroectoderm, respectively (Kasai *et al.*, 1992; Morel and Schweisguth, 2000). On the other hand, *sim* expression is activated by Dorsal and Twist in the cells that are located between these two territories (Kosman *et al.*, 1991; Leptin, 1991; Kasai *et al.*, 1992) (Figure 2).

The molecular mechanisms defining the mesectoderm as a single row of *sim*-expressing cells along the dorsoventral axis start being elucidated. Results from transplantation experiments indicate that cell–cell signaling between mesodermal and nonmesodermal cells is required for the early expression of *sim* (Leptin and Roth, 1994). Moreover, mesectoderm specification also involves Notch (N) activity, confirming a role of cell signaling in the regulation of *sim* expression (Menne and Klambt, 1994; Martin-Bermudo *et al.*, 1995).

The current view of the Notch pathway calls for intracellular processing of Notch and formation of a complex between its intracellular domain and the Su(H) transcription factor (reviews: Jarriault *et al.*, 1995; Artavanis-Tsakonas *et al.*, 1999). The role of this complex is to activate the transcription of Notch responsive genes (Kidd *et al.*, 1998a; Lecourtois and Schweisguth, 1998; Schroeter *et al.*, 1998; Struhl and Adachi, 1998). However, recent data show that Su(H) can also act alone (Bray and Furriols, 2001). Indeed, Su(H) plays two roles: with the help of Notch, it upregulates *sim* expression in the mesectoderm, whereas it prevents *sim* expression in the neuroectoderm independently of Notch (Morel and Schweisguth, 2000).

3.2.2. Neural Precursor Formation

Within the neuroectoderm, also called ventral neurogenic region, nervous system development requires the activity of two classes of genes that control neural versus epidermal fate (Figure 3). Proneural genes promote whereas neurogenic genes inhibit neural

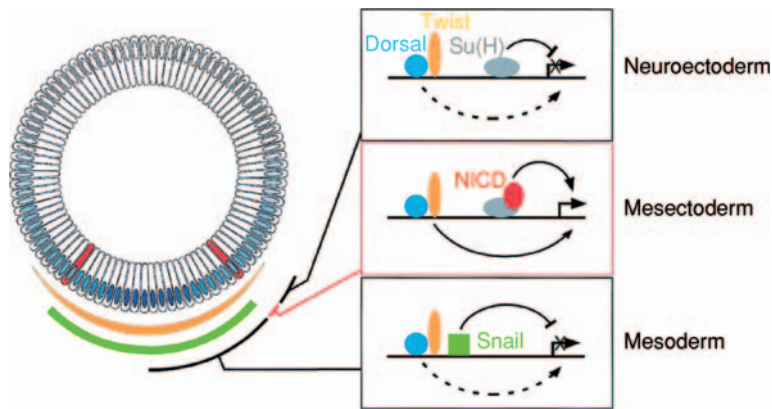


Figure 2 Molecular model for mesectoderm specification. Schematic representation of a cross-section of a fly embryo at blastoderm stage (left). In ventral nuclei, the combination of nuclear Dorsal (blue), Twist (orange), and Snail (green) transcription factors results in repression of *single-minded* (arrow at lower right). At more dorsal positions, lack of the Snail repressor as well as activation of the Notch pathway (the Notch intracellular domain (NICD) is shown in red) via the Suppressor of Hairless (Su(H)) partner and transcription factor (gray), activates mesectoderm specific genes (arrow at middle right). More dorsally, the combination of low/absent nuclear Dorsal and Su(H) dependent repression, in the absence of Notch signaling, leads to repression of *single-minded* (arrow at upper right). (Modified from Morel, V., Schweisguth, F., 2000. Repression by suppressor of hairless and activation by Notch are required to define a single row of single-minded expressing cells in the *Drosophila* embryo. *Genes Devel.* 14, 377–388.)

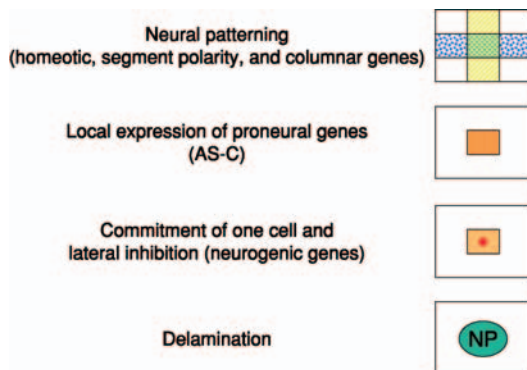


Figure 3 The neural pathway. The action of homeotic and segment polarity genes (yellow color in the first square) as well as columnar genes (blue color in the first square) subdivides the neuroectoderm in different regions along the AP and DV axis, respectively, and defines the position at which proneural clusters will be formed (green color in the first square). The expression of the AS-C in a cell cluster (orange rectangle) endows each cell of the cluster with the potential to become neural precursor. Progressively, due to differences in Delta expression within the cluster, the expression of the AS-C becomes restricted to a single cell at a stereotyped position. In all the other cells, AS-C expression progressively fades away (pale orange). The cell expressing AS-C at high levels corresponds to the presumptive neural precursor (red circle), while the adjacent ones are submitted to lateral inhibition and adopt the epidermal fate. Finally, the neural precursor (NP) emerges and delaminates from the ectoderm at a specific position, which defines the progeny of the lineage.

precursor formation, respectively (reviews: Skeath *et al.*, 1994; Hassan and Vaessin, 1996; Modolell and Campuzano, 1998; Rooke and Xu, 1998). These two gene classes act at the level of neural

precursor singling out cells for delamination through a cell communication process called lateral inhibition (review: Rooke and Xu, 1998). Three steps can be recognized in this process. First, groups of cells within the neurogenic region acquire a neural competence; second, single cells within these groups become neural precursors; and third, their singling out prevents adjacent cells from taking the same fate, and leads such cells to become epidermis (review: Skeath and Carroll, 1994) (Figures 3 and 4).

3.2.2.1. Proneural genes promote neural precursor formation The classical example of proneural genes is provided by the *achaete-scute* complex (reviews: Campuzano *et al.*, 1985; Alonso and Cabrera, 1988; Campos-Ortega, 1998). Genes belonging to this complex, such as *achaete* (*ac*), *scute* (*sc*), *lethal of scute*, *asense* (*ase*), encode bHLH transcription factors that are initially expressed in groups of equivalent cells (the proneural clusters) (Cabrera *et al.*, 1987; Jimenez and Campos-Ortega, 1990; Martin-Bermudo *et al.*, 1991; review: Skeath and Carroll, 1994). The expression of the AS-C endows each cell of the cluster with the potential to become neural precursor. Progressively, the expression of AS-C genes becomes restricted to a single cell at a stereotyped position within the cluster. This cell corresponds to the presumptive neural precursor that eventually delaminates from the neuroectoderm (Cabrera, 1990; Cubas *et al.*, 1991; Martin-Bermudo *et al.*, 1991; Skeath and Carroll, 1992) (Figures 3 and 4).

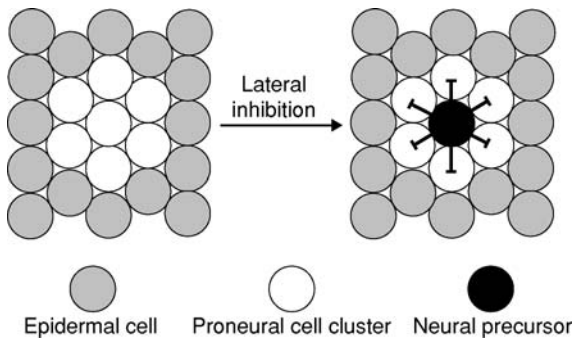


Figure 4 Lateral inhibition. Lateral inhibition is required for the selection of a single neural precursor cell during development. From a homogeneous population of epidermal cells (gray circles at left), a group of cells (the proneural cluster; white circles at left) acquires neural competence via the expression of proneural genes. Within the proneural cluster, one cell adopts the neural fate (black circle at right) due to high levels of Delta expression and thereby AS-C. This activates Notch signaling in the adjacent cells of the cluster, which inhibits AS-C expression in these cells (white circles at right). As a consequence, such cells are prevented from adopting the neural fate (black lines).

In *Drosophila*, loss-of-function analyses show that *achaete* and *scute* are required for the generation of most embryonic and adult external sense organs (Ghysen and Richelle, 1979; Jimenez and Campos-Ortega, 1990; Goriely *et al.*, 1991). Similarly, gain-of-function analyses show that the AS-C genes are sufficient to induce the development of ectopic sensory organs at the expense of epidermis (Romani *et al.*, 1989; Rodriguez *et al.*, 1990; Dominguez and Campuzano, 1993). In the CNS, mutations in the AS-C lead to neural hypoplasia due to a strong reduction of the proneural cluster and the neural precursor number (Cabrera *et al.*, 1987; Dambly-Chaudière and Ghysen, 1987; Jimenez and Campos-Ortega, 1990; Cubas *et al.*, 1991; Martin-Bermudo *et al.*, 1991). As for the PNS, additional copies of AS-C genes cause neural hyperplasia, indicating that these genes play an instructive role in the induction of the neural fate (Jimenez and Campos-Ortega, 1990; Huang *et al.*, 1994).

Neural determination is also controlled by bHLH genes during vertebrate CNS development (reviews: Brunet and Ghysen, 1999; Bertrand *et al.*, 2002). Two gene families have been identified as being necessary for neural development: the MASH family, closely related to the *achaete-scute* genes, as well as a broad family that includes Math, Neurogenin, and NeuroD members, which are related to other bHLH *Drosophila* genes, such as *target of Poxn* (*tap*) and *atonal* (*ato*) (Jarman *et al.*, 1993; Gautier *et al.*, 1997; Kageyama *et al.*, 1997). Together with ectopic expression experiments in the ectoderm of

Xenopus embryos, these data show that bHLH genes are necessary and sufficient to promote neurogenesis at the expense of the ectodermal fate, confirming their proneural potential (Lee *et al.*, 1995; Kim *et al.*, 1997; review: Lee, 1997).

It is interesting to note that members of the AS-C and *atonal* families account for all proneural activity in the fly PNS, but not in the CNS, where generation of some neural precursors does not require any of the known proneural genes. Potential bHLH candidates identified in the *Drosophila* genome are not expressed as expected for proneural factors, indicating that another family of proteins likely controls proneural activity in the fly CNS, and likely in vertebrates (Jimenez and Campos-Ortega, 1990; Moore *et al.*, 2000; Peyrefitte *et al.*, 2001). A future challenge will be to identify such proneural factors.

3.2.2.2. Neurogenic genes antagonize neural precursor formation Within the proneural cluster, selection of the neural precursor cell is mediated by the neurogenic genes (Figures 3–5). Loss-of-function mutations for such genes are characterized by nervous system hyperplasia indicating that they repress the neural fate. Neurogenic genes include those encoding the Notch receptor and its ligand Delta, as well as other genes that work in the Notch–Delta pathway (see below) (reviews: Artavanis-Tsakonas *et al.*, 1999; Baron *et al.*, 2002; Bertrand *et al.*, 2002). This pathway is involved in the process called lateral inhibition: the activation of Notch by Delta leads to receptor proteolysis (review: Chan and Jan, 1998), releasing the intracellular domain of Notch (NICD) into the cytoplasm. Then, NICD enters the nucleus and forms a complex with the Suppressor of Hairless transcription factor (Figure 5). Together, NICD and Su(H) activate specific targets (Bailey and Posakony, 1995) including genes of the *Enhancer of split Complex* (*E(spl)-C*), which encode transcription factors that inhibit the expression of proneural genes (Tata and Hartley, 1995; review: Bray, 1997). Since proneural genes activate Delta expression, cells in which the Notch pathway is activated eventually switch Delta expression off and adopt the epidermal fate (Kunisch *et al.*, 1994) (Figure 5).

The current view on the singling out of a single precursor proposes that, within the proneural cluster, all cells express proneural and neurogenic genes at similar levels. Subsequently, via mechanisms that are not understood yet, one cell of the cluster expresses more Delta than the adjacent cells. This effect is then amplified by lateral inhibition: activation of the Notch pathway in cells surrounding the presumptive neural precursor inhibits

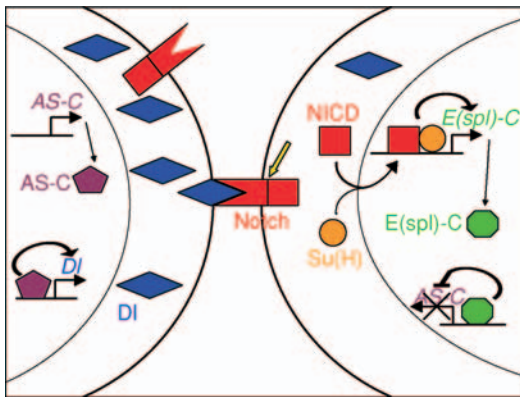


Figure 5 The Notch pathway in the proneural cluster. Thick and thin black lines indicate the cell and the nucleus membranes, respectively. Thick arrows and the T-bar indicate transcription activation and repression, respectively. Proneural genes of the AS-C (purple) activate Delta (DI, blue) expression in the proneural cluster. Subsequently, one cell of the cluster (to the left) expresses more Delta than the adjacent cells (one of them is represented to the right), as simplified in the schematic drawing. Lateral inhibition entails a positive feedback loop that reinforces and maintains the initial differences between neural precursor (high Delta, cell to the left) and the other cells of the proneural cluster (low Delta, to the right). The activation of Notch (red) by Delta leads to receptor proteolysis (yellow arrow), releasing the intracellular domain of Notch (NICD, red square) into the cytoplasm. Then, NICD is transferred to the nucleus, where, together with the Suppressor of Hairless protein (Su(H), orange), it activates target genes, mainly represented by the *Enhancer of split Complex* (*E(spl)-C*, green). These target genes encode transcription factors that inhibit the expression of proneural genes (AS-C).

the differentiation of other neural precursors (Figures 4 and 5). Thus, a slight unbalance of Delta expression within the proneural cluster is sufficient to trigger a signaling pathway that allows the increase and the maintenance of AS-C and Delta expression in one cell, the neural precursor.

Mutations associated with nervous system hyperplasia permitted the identification of several other neurogenic genes, including *Enhancer of split*, *neuralized* (*neur*), *mastermind* (*mam*), *big brain* (*bib*), *almondex* (*amx*), and *neurotic* (*O*-fucosyltransferase (*O*-*fut1*)) (Shannon, 1972; Knust *et al.*, 1987b; Yedvobnick *et al.*, 1988; Rao *et al.*, 1990; Boulianne *et al.*, 1991; Sasamura *et al.*, 2003). All these genes modulate the Notch pathway at different steps. For example, *neurotic* encodes an *O*-fucosyltransferase protein that modulates the Notch–Delta interaction, and *neuralized* encodes a ubiquitin ligase that upregulates endocytosis of Delta (Boulianne *et al.*, 1991; Sasamura *et al.*, 2003). Therefore, complex gene interactions modulating the Notch pathway are involved in neurogenesis, indicating that cell–cell interactions are absolutely required to promote neural differentiation within the neuroectoderm.

3.2.3. Fly Neural Precursors and Lineages

3.2.3.1. Neuroblast lineage analysis The *Drosophila* VNC consists of a sequence of repeated units called neuromeres, each divided into two hemineuromeres separated by the midline. The singling out of the neural precursor through the interaction of neurogenic and proneural genes leads to cell delamination into a subectodermal proliferative zone. This process is achieved in five spatiotemporally distinct waves (S1–S5) to form an invariant and roughly orthogonal pattern of 30 neural precursors per hemineuromere during germ band extension (Doe, 1992) (Figure 6).

Three classes of neural precursors can be distinguished by the type of progeny (Bossing *et al.*, 1996; Schmidt *et al.*, 1997, 1999) (Figure 7). Neural precursors that give rise only to neurons or glia are called neuroblasts (NBs) or glioblasts (GBs), respectively. A third type of precursor, called neuroglioblasts (NGB), produces neurons as well as glia. The expression profile of homeotic, segment polarity and columnar genes provides a panel of specific markers for each type of neural precursor cells (Doe, 1992) (Figure 6). The combination of stereotyped position and gene expression is sufficient to specifically identify most neural lineages at each stage of neurogenesis. Not only is the pattern of neural precursors in each neuromere invariant, but each precursor also produces an invariant population of neurons and glia. This last feature has been revealed using lineage tracers such as 1,1'-dioctaldecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) and horseradish peroxidase (HRP) that allow single precursors to be followed during development (see below) (Bossing and Technau, 1994; Bossing *et al.*, 1996; Schmidt *et al.*, 1997, 1999). The simple organization as well as the stereotyped behaviors of the neural lineages have made it possible to draw a precise map of the fly embryonic nervous system

3.2.3.2. Types of neural precursors

3.2.3.2.1. The neuroblast Each hemineuromere of the ventral cord contains 23 distinct NBs, some of them specific to abdominal or thoracic segments (Bossing and Technau, 1994; Bossing *et al.*, 1996; Schmidt *et al.*, 1997, 1999; review: Van De Bor and Giangrande, 2002). After delamination, neural precursors initiate a series of asymmetric self-renewing divisions, each mitotic event generating another NB and a small cell, the ganglion mother cell (GMC), which divides once and produces two neurons (Figure 7a). Normarski microscopy was used to examine the morphological differentiation of a single NB in *Drosophila*, and *camera lucida*

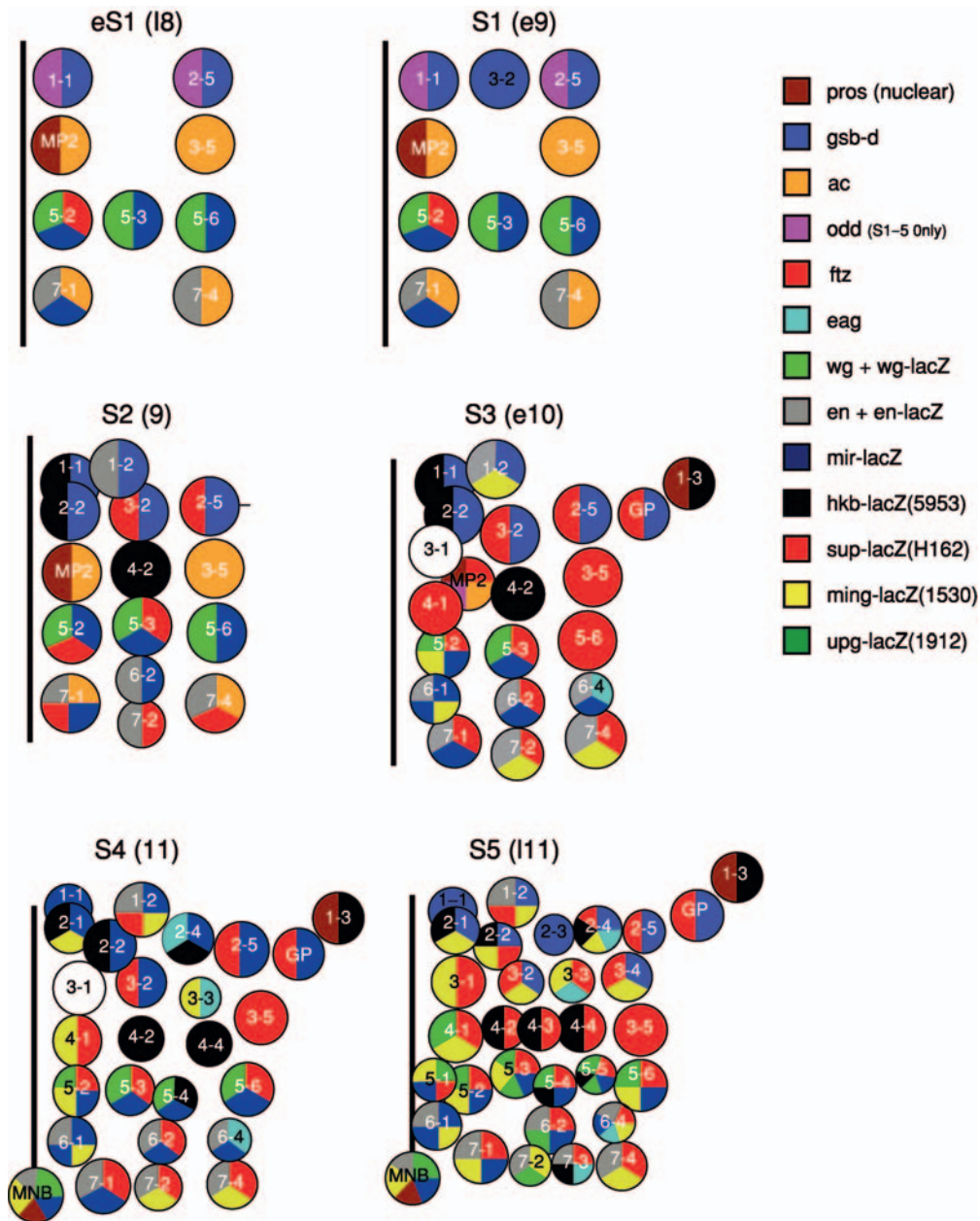


Figure 6 Identification of neuroblasts by the expression of specific molecular markers. Panels S1 to S5 indicate the five spatiotemporally distinct waves (stages are according to Campos-Ortega and Hartenstein, 1985). Numbers in brackets indicate embryonic stages, and e and l stand for early and late, respectively. In each panel, the vertical line indicates the midline. Neural precursors are distributed in seven rows (indicated by the first number in each circle) along the anteroposterior axis and five columns (indicated by the second number in each circle) along the dorsoventral axis. Each precursor is named according to its position along the axes and expresses a specific combination of molecular markers. The color coding illustrates the specific expression of the markers described at right; ac, en, wg, gsb-d, and odd represent protein markers; pros represents nuclear protein localization; eag corresponds to a mRNA pattern; wg-lacZ, en-lacZ, mir-lacZ, hkb-lacZ, svp-lacZ, ming-lacZ and upg-lacZ represent β -galactosidase staining patterns of enhancer trap lines inserted into these genes. GP, MNB, and MP2 indicate the glial precursor, median neuroblast, and midline precursor 2, respectively. The 3-1 precursor (white circle) shown in S3 and S4 does not express any of these markers. Gene abbreviations: pros (nuclear), *prospero*; gsb-d, *gooseberry-distal*; ac, *achaete*; odd, *odd-skipped*; ftz, *fushi-tarazu*; eag, *eagle*; wg and wg-lacZ, *wingless* and *wingless-lacZ*; en and en-lacZ, *engrailed* and *engrailed-lacZ*; mir-lacZ, *mirror* and *mirror-lacZ*; hkb-lacZ, *huckebein* and *huckebein-lacZ*; svp-lacZ, *seven-up* and *seven-up-lacZ*; ming-lacZ, *ming-lacZ* = *castor-lacZ*; upg-lacZ, *unplugged-lacZ*. (Modified from Hyper Neuroblast Map at <http://www.neuro.uoregon.edu>.)

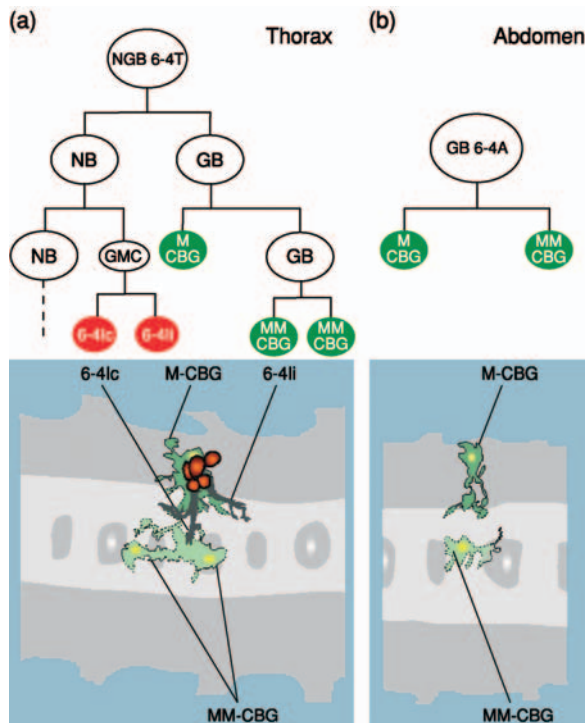


Figure 8 Drawings represent the 6-4 lineage as identified by Dil injection. The 6-4 cell produces different progenies in the thorax (a) and in the abdomen (b). (a) In the thorax, the 6-4 lineage (NGB 6-4T) gives rise to neurons (red circles) and glia (green circles). The dashed line indicates that the NB issued from the first division continues to proliferate and produces neurons, as shown by the presence of five neurons in the lower panel. (b) In the abdomen, the 6-4 lineage only produces glia (GB 6-4A). In the lower panels, cell lineages derived from 6-4 are shown in a dorsal view with anterior to the left. The cortex region and the neuropil of the ventral nerve cord are shown in gray and white, respectively. The medial dorsoventral channels, which mark the neuromere boundaries, are shown in light ovals. Clusters of neuronal cell bodies are red and their fiber projections dark gray. Glial cells are shown in green (dark and light green indicate a mediadorsal and a ventral position, respectively) and glial cell nuclei are shown in yellow. GB, glioblast; GMC, ganglion mother cell; NB, neuroblast; NGB, neuroglioblast; M-CBG, medial cell body glia; MM-CBG, medialmost cell body glia. Ic and II indicate interneurons (symbols are according to Bossing *et al.*, 1996).

1994; Bossing *et al.*, 1996; Schmidt *et al.*, 1997, 1999; review: Van De Bor and Giangrande, 2002). One of them is the abdominal counterpart of the thoracic 6-4 lineage (see below) and is called GB6-4A, while the other corresponds to the longitudinal glioblast. These precursors only generate glial cells (Figure 7c).

3.2.3.2.4. Midline Through the use of genetic approaches, including enhancer trap lines, in which a *LacZ* reporter gene is expressed in a lineage restricted manner, it was shown that midline neural

precursors occupy a stereotyped position along the anteroposterior axis within a segment. The differentiated progeny of each precursor was then determined by morphological criteria by placing Dil on early progenitors (Bossing and Technau, 1994).

Four NB lineages arise from the posterior part of the neuromere including the midline precursors (MP), the unpaired median intermediate (UMI) precursor, the precursors of ventral unpaired median (VUM) neurons, and the median neuroblast (MNB). The precise lineage tree of all these precursors is not yet fully understood (review: Jacobs, 2000).

A pool of 10–12 midline glia (MG) precursors is localized in the anterior part of the neuromere. From this pool of precursors, only three survive by the end of embryogenesis – the anterior, the medial, and the posterior MG precursor – the others undergoing apoptosis (see below) (Klamt and Goodman, 1991; Sonnenfeld and Jacobs, 1995; Dong and Jacobs, 1997; Zhou *et al.*, 1997b).

So far, no NGB have been found in the *Drosophila* mesectoderm. Interestingly, in other insect species such as *Schistocerca americana*, a NGB differentiates at the position normally occupied by the MNB in *Drosophila* (Condrón *et al.*, 1994; Condrón and Zinn, 1994).

3.3. Neural Patterning

A total of 16 abdominal, six thoracic, and six gnathal hemineuromeres make up the VNC. At the end of neurogenesis, each neuromere contains around 400 neurons and 60 glial cells (Bossing and Technau, 1994; Bossing *et al.*, 1996; Schmidt *et al.*, 1997, 1999). The type of neurons and glial cells in these neuromeres depends on the identity of neural precursors, which we will refer to as NBs for the sake of simplicity, irrespective of whether they generate neurons or glia. Thus, it is critical to dissect the genetic regulatory mechanisms that specify the identity of individual NBs.

Studies carried out in the last 10 years indicate that many of the genes that pattern the embryo along the main axes (anteroposterior or AP, dorsoventral or DV; see Chapters 1 and 2, also function in neurogenesis (review: Skeath, 1999). The activities of these genes create a Cartesian coordinate system that gives a unique fate to individual precursors depending on their position (review: Skeath and Thor, 2003). The invariant pattern of gene expression and progeny suggests that autonomous cues control the identity of each precursor. This is further confirmed by the observation that precursor cells keep their identity even when cultured as isolated

cells (Huff *et al.*, 1989; Broadus and Doe, 1997). One piece of evidence indicates, however, that neural precursors also display some plastic features: when late-born NBs are transplanted into early stage embryos, they acquire an early identity (Udolph *et al.*, 1995). Thus, heterochronic transplantation experiments show that neuroblast specification occurs under the control of stage-specific inductive signals that act in the neuroectoderm. Therefore, autonomous as well as nonautonomous processes appear to control precursor identity during nervous system development.

3.3.1. Spatial Cues Control Neuroblast Differentiation

Positional cues along the AP and DV axes allocate proper identities to different neuromeres as well as to different regions within each neuromere (Figure 3). Different classes of genes control the establishment of these coordinates: homeotic transcription factors define the AP identity of the different neuromeres (reviews: McGinnis and Krumlauf, 1992; Gehring, 1993; Chapter 1), whereas segment polarity and columnar genes, respectively, define the AP and the DV identities within the neuromere (review: Skeath, 1999).

3.3.1.1. Homeotic genes specify neuroblast identity along the AP axis The AP and DV axes of the early *Drosophila* embryo are established by two key maternal morphogens: Bicoid (Bcd) and Dorsal (Dl), respectively (Steward, 1987; Berleth *et al.*, 1988). Bicoid is expressed in a broad concentration gradient along the AP axis, with peak levels present at the anterior pole, while Dorsal is expressed in a gradient along the DV axis with peak levels along the ventral surface (Ip *et al.*, 1992). Both morphogens establish overlapping patterns of transcriptional activators and repressors in the early embryo, leading to expression of specific genes, including homeotic genes, which define territories along AP and DV axes (Ip *et al.*, 1992; Chapter 1).

The different behaviors of neural lineages between thoracic and abdominal neuromeres are explained by the expression profile of homeotic genes, which are expressed in specific domains along the rostrocaudal axis of the developing nervous system. In the fly CNS, the 1-1 lineage produces both neurons and glia in the abdomen, whereas it only produces neurons in the thorax (Udolph *et al.*, 1993; Prokop and Technau, 1994). Ultrabithorax (Ubx) and Abdominal-A (Abd-A) are not expressed in the two first thoracic segments (Figure 9a). It has been shown that Ubx or Abd-A misexpression in this region is sufficient to induce abdominal development of the complete 1-1 lineage and to override thoracic commitment, showing that

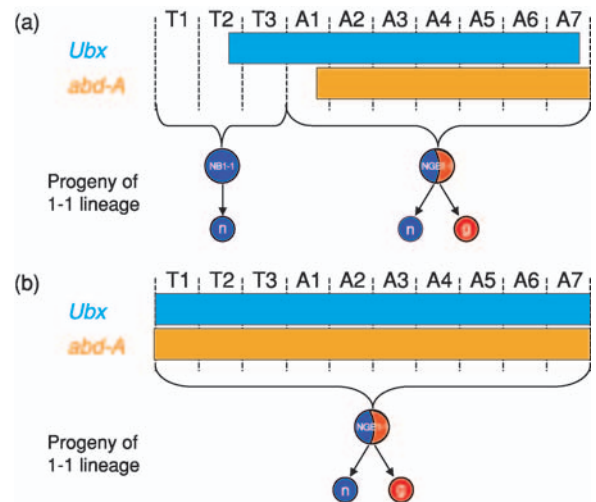


Figure 9 Homeotic genes control anteroposterior NB identity. Schematic representation of *Ultrabithorax* (*Ubx*) and *abdominal-A* (*abd-A*) gene expression domains in thoracic (T1 to T3) and abdominal (A1 to A7) neuromeres. Light blue and orange rectangles correspond to *Ubx* and *abd-A* expression domains, respectively. Anterior is to the left. Dashed vertical lines represent the boundaries of each neuromere. Blue and red indicate neuronal and glial potential/identity, respectively. (a) In wild-type embryos, *Ubx* (light blue) is expressed from T2 to A7 neuromeres whereas *abd-A* (orange) is expressed from A1 to A7. The 1-1 lineage produces both neurons (n) and glia (g) in the abdomen (NGB1-1, blue/red circle), whereas it produces only neurons in the thorax (NB1-1, blue circle). (b) When *Ubx* or *abd-A* are overexpressed in all neuromeres, the thoracic 1-1 lineage gives rise to neurons and glial cells, like in the abdomen.

activity of the homeotic genes *Ubx* and *abd-A* is required for abdominal specification (Prokop and Technau, 1994; Chapter 1) (Figure 9b).

Homeotic genes are conserved throughout evolution in their DNA binding domain (reviews: McGinnis and Krumlauf, 1992; Gehring, 1993; Chapter 1). The relative AP order of expression of *Hox* genes (the vertebrate orthologs of insect homeotic genes) in the developing vertebrate CNS is similar to the AP order of expression of homeotic genes in the *Drosophila* CNS, indicating that the role of these genes in patterning the nervous system is conserved during evolution (reviews: Boncinelli *et al.*, 1993; Krumlauf *et al.*, 1993). This is confirmed by cross-phylum gene exchange experiments showing that *Drosophila* homeotic genes can replace their vertebrate orthologs and vice versa.

While it is not clear whether *Hox* genes also control DV patterning in *Drosophila*, it has been recently shown that they do play a role in this process during vertebrate CNS development. In the mouse hindbrain, *Hoxa2* and *Hoxb2* are expressed in a pattern that defines complementary distribution along the DV axis, suggesting that these genes play a

role in DV patterning and neurogenesis (Davenne *et al.*, 1999). *Hoxa2* and double-mutant mice show DV differences in the distribution and the differentiation of early neurons, indicating a change in precursor cell identity (Davenne *et al.*, 1999). Given that nervous system development and definition of distinct neural patterns require a crosstalk between AP and DV patterning, these data suggest that, at least in vertebrates, homeotic genes integrate AP and DV positional information.

3.3.1.2. Segment polarity genes specify AP neuroblast differentiation and identity within the neuromere Most segment polarity genes were originally identified in the genetic screen carried out by Nüsslein-Volhard and Wieschaus (1980). The role of these genes is to subdivide each segment into an identical pattern of parallel rows. Segment polarity gene activity dissects the neuroectoderm into five transverse rows per hemineuromere and ensures that NBs that develop in different rows acquire different identities (Bhat, 1999).

Segment polarity genes include those encoding the secreted proteins Wingless (Wg) and Hedgehog (Hh), the transmembrane receptor Patched (Ptc) as well as the transcription factors Gooseberry (Gsb), Engrailed (En), and Invested (Inv) (Nüsslein-Volhard and Wieschaus, 1980; Baumgartner *et al.*, 1987; Baker, 1988; Ingham *et al.*, 1991; Mohler and Vani, 1992; Gustavson *et al.*, 1996). The expression profile of these genes subdivides the neuromere along the AP axis and spatially regulates the expression of the proneural genes *achaete* and *scute* (reviews: Skeath and Carroll, 1992, 1994). In the absence of one or more of these gene activities, a subset of proneural clusters and NBs fails to form. For example, *wg* is expressed in neuroectodermal cells that will give rise to row 5 proneural clusters, and its secreted product is also detected in adjacent rows 4 and 6 (Figure 10). Absence of *wg* results in a decrease of proneural cluster number in rows 4 and 6 indicating a failure in the formation of such clusters, whereas row 5 proneural clusters develop normally (Chu-LaGraff and Doe, 1993). Moreover, proneural clusters and NBs that develop in rows 4 and 6 acquire identity incorrect for their position. This indicates that *wg* promotes proneural cluster formation in rows 4 and 6 and permits them to acquire the proper identity (Chu-LaGraff and Doe, 1993) (Figure 10). These genes continue to be expressed in NBs, and often in their progeny, GMCs and neurons, suggesting that they play a role at later developmental stages.

Positional cues defining cell identity within the nervous system have also been identified in

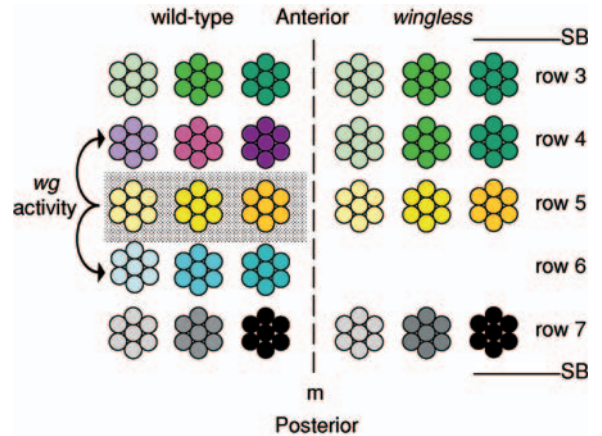


Figure 10 Segment polarity genes control NB differentiation and anteroposterior identity within the neuromere. Schematic representation of proneural clusters present within a hemineuromere of wild-type (left) or *wingless* (*wg*) mutant embryos. The two hemineuromeres are separated by the midline (m, dashed line). Anterior is to the top. At this stage, five rows of clusters are present along the AP axis, called rows 3 to 7. The segment boundaries (SB) are shown by two horizontal lines. Color coding identifies specific proneural clusters along the AP and DV axes. In the wild-type embryo, Wg is expressed in row 5 (dotted pattern) and acts on adjacent rows 4 and 6 (black arrows). In the *wg* mutant embryo, proneural clusters in row 5 are still present whereas the adjacent ones are missing (row 6, absent circles) or misspecified (row 4, green circles). Row 4 switching to row 3 identity was assessed by the expression of cell specific markers in NB and GMC progeny.

mesectodermal cells. In each neuromere, a single row of cells constitutes the midline cell population (review: Jacobs, 2000). The anterior midline cells produce midline glia, and the posterior ones midline neurons. Hedgehog (Hh) signaling to mesectodermal cells in the posterior domain of the neuromere is important for the determination of posterior neuronal identities. Loss of Hh results in extra midline glia (MG), because anterior lineages are duplicated when posterior identities are not specified (Hummel *et al.*, 1999a, 1999b). Conversely, expansion of Hh signaling reduces the MG number (review: Jacobs, 2000). Therefore, the Hh signaling pathway functions to determine neurons in the posterior neuromere, and is antagonistic to signals that contribute to MG identity in the anterior neuromere. Embryos mutants for *wg* and *ptc* have no anterior midline cells (including MG), indicating that *wg* and *ptc* contribute to the determination of the anterior midline identity (Zhou *et al.*, 1997a, 1997b).

3.3.1.3. Columnar genes specify DV neuroblast differentiation and identity within the neuromere Segment polarity gene action does not explain how NBs of the same row but different columns acquire different DV identities within the same segment.

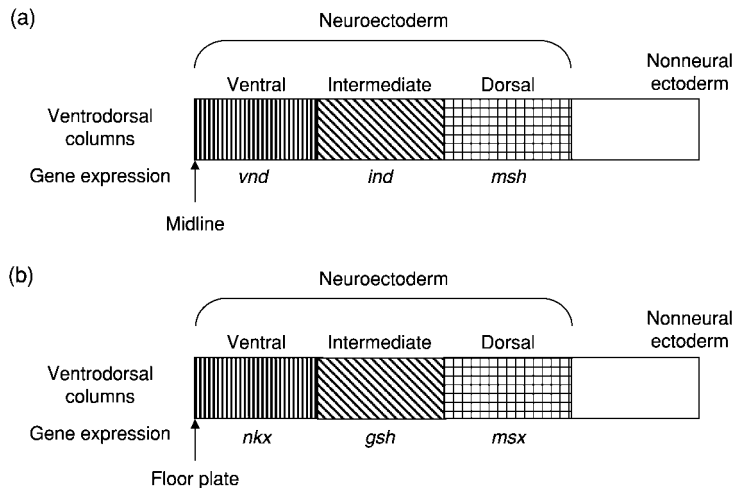


Figure 11 Evolutionary conservation of dorsoventral patterning within the neuroectoderm. Schematic representation of columnar gene expression domains in a hemineuromere of the (a) fly and (b) vertebrate neuroectoderm. Profiles of gene expression subdivide neuroectoderm into three ventrodorsal columns: *vnd/nkx*, *ind/gsh*, and *msh/msx* genes are expressed and control cell identity within ventral (vertical lines), intermediate (oblique lines), and dorsal (square motif) domains. Non neural ectoderm is indicated in white. The ventralmost position corresponds to the midline in flies and floor plate in vertebrates. (Modified from Cornell, R.A., Ohlen, T.V., 2000. *Vnd/nkx*, *ind/gsh*, and *msh/msx*: conserved regulators of dorsoventral neural patterning? *Curr. Opin. Neurobiol.* 10, 63–71.)

Four factors control this process: the three transcription factors ventral nerve cord defective (*Vnd*) (Chu *et al.*, 1998; McDonald *et al.*, 1998), intermediate nerve cord defective (*Ind*) (Weiss *et al.*, 1998), and muscle segment homeobox (*Msh*) (Isshiki *et al.*, 1997), as well as the *Drosophila* epidermal growth factor (EGF) receptor (*DER*), a receptor tyrosine kinase (Livneh *et al.*, 1985; Skeath, 1998). These factors play a decisive role in dividing the neuroectoderm into three separate longitudinal regions: *vnd* is expressed in the ventral (medial) column (Mellerick and Nirenberg, 1995), *ind* is expressed in the intermediate column (Weiss *et al.*, 1998), and *msh* is expressed in the dorsal (lateral) column (D'Alessio and Frasch, 1996) (Figure 11). *DER* is expressed and required in the intermediate column to promote NB formation, and in the ventral column to specify NB identities (Yagi *et al.*, 1998). The NBs born in the dorsal column develop normally in *DER* mutant embryos.

Absence of columnar genes leads to defects in NB formation and to misspecification of NB identity (review: Skeath, 1999). For example, *vnd* functions in the ventral column to promote the formation of ventral identity precursors and to repress those with intermediate identity. Absence of *vnd* induces ventral cells to acquire intermediate cell identity, and *ind* expression expands into the ventral and intermediate columns (Chu *et al.*, 1998; McDonald *et al.*, 1998; Weiss *et al.*, 1998). Conversely, ubiquitous overexpression of *vnd* induces intermediate

and some dorsal cells to acquire ventral identity (McDonald *et al.*, 1998). These results show that *vnd* specifies ventral column identity and prevents ventral precursors from adopting an intermediate column identity by repressing *ind*.

Elements of DV neural patterning have been evolutionarily conserved. Indeed, ortholog genes play the same role in vertebrates: the *nkx/vnd*, *Gsh-1,2/ind*, and *msx/msh* genes are expressed and control cell fates within ventral, intermediate, and dorsal domains, respectively, in the neural tube (review: Cornell and Ohlen, 2000) (Figure 11). It is interesting to note that upstream or downstream pathways to this “three column expression pattern” are not conserved during evolution, suggesting that this specific process plays a pivotal role in neuronal specification (review: Cornell and Ohlen, 2000).

It is also worth noting that, apart from mesectoderm-derived glia precursors, most glial precursors are localized in the dorsal part of the neuroectoderm, suggesting a role of columnar genes in the establishment of the gliogenic potential (Bossing *et al.*, 1996; Schmidt *et al.*, 1997, 1999).

A number of transcription factors such as Pannier (*Pnr*) and the products of the Iroquois Complex (*Iro-C*) have been shown to link positional cues and proneural cluster formation in the fly adult PNS (Ramain *et al.*, 1993; Leyns *et al.*, 1996; Garcia-Garcia *et al.*, 1999; Calleja *et al.*, 2000; Cavodeassi *et al.*, 2000). These transcription factors control the expression of the proneural genes in response to

extracellular molecules that assign spatial identity. Although it will be interesting to determine the role of these genes in CNS patterning, the embryonic profile of expression of some of them suggests that distinct molecular pathways may be used in adult PNS and embryonic CNS patterning.

3.3.2. Temporal Cues Control Neuroblast Differentiation

CNS development requires precise spatial as well as temporal cues. Indeed, fly neural precursors divide a fixed number of times and produce cells of distinct type at each division. For example, the first division of the 1-1 lineage always produces a GMC that gives rise to the aCC motorneuron and the pCC interneuron (in thorax and abdomen), whereas later divisions produce other types of motorneurons and interneurons in the thorax, and other types of interneurons as well as glia in the abdomen (Udolph *et al.*, 1993; Prokop and Technau, 1994). Thus, a temporal genetic program controls neural precursor differentiation.

Cell lineage, ablation, transplantation, *in vitro* culture, and genetic studies in *Drosophila* and other insects indicate that GMC birth order is the primary determinant of temporal identity (Doe and

Goodman, 1985a, 1985b; Furst and Mahowald, 1985; Prokop and Technau, 1994; Weigmann and Lehner, 1995; Schmidt *et al.*, 1999). Recent studies have indeed shown that *Drosophila* NBs undergo temporally ordered changes in gene expression (review: Zhong, 2003). This suggests that, as NBs divide, they become temporally restricted in the type of cells that they can generate.

Good candidates to regulate this process are the transcription factors encoded by the *hunchback* (*hb*), *Krüppel* (*Kr*), *POU domain protein* (*pdm*), *castor* (*cas*), and *grainyhead* (*grh*) genes (Brody and Odenwald, 2000, 2002) (Figure 12). The *hunchback* and *Krüppel* genes are expressed in early-born neurons, *pdm* is expressed in middle-born neurons, while *castor* and *grainyhead* are expressed in late-born neurons. The *hb*, *Kr*, *pdm*, *cas* and *grh* genes are sequentially expressed in NBs, with the GMC/progeny maintaining this transcription factor profile at their birth.

This creates a layered structure, with each layer differing from the adjacent one by the expression of a specific transcription factor (Figure 12a). Expression of *hb* and *Kr* is necessary and sufficient to induce an early-born cell fate in multiple lineages (Figure 12c) (Isshiki *et al.*, 2001). Thus, *hb* and *Kr*

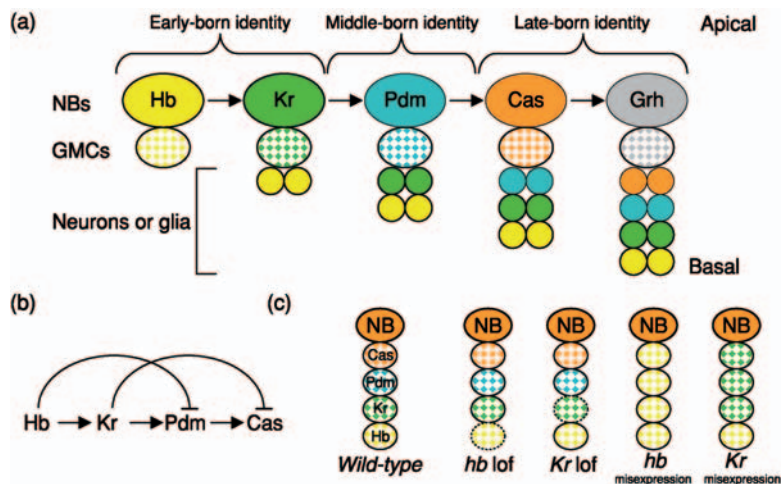


Figure 12 Temporal identity in NB lineages. (a) Schematic representation of a NB lineage: the neuroblast (NB), the ganglion mother cell (GMC), and its neuronal/glial progeny are represented from apical to basal. Color coding indicates specific gene expression and subdivides the lineage into adjacent layers according to birth date. During each temporal window of gene expression, GMCs are marked by the continuous presence of the temporal factor that is expressed in the NB at its birth. These transcription factors are also detected in postmitotic cells (neurons or glia). (b) Cross-regulation amongst “temporal” transcription factors. Loss-of-function and gain-of-function studies indicate cross-regulatory interactions between “temporal” transcription factors. Arrows and T-bars indicate activation and repression, respectively. These interactions ensure the sequential progression of temporal states during lineage development. (c) Schematic representation showing the temporal sequence of gene expression in wild-type and mutant GMCs. In wild-type, “temporal” genes are expressed in the NBs (large ovals) as well as in their progeny, the GMCs (lozenge-patterned small ovals). In loss-of-function (*lof*) *Hb* or *Kr* mutant embryos, the first or the second born identities are lost in the NB progeny, respectively. Conversely, forced continuous expression of *Hb* or *Kr* leads to the transformation of late-born identity to first or second born identity in the GMCs, respectively. Dashed circles represent abnormal GMC development (GMC transformed to a later born identity or dying GMC).

specify “early” temporal identity in *Drosophila* neural precursors.

These data indicate that loss of a temporal factor results in the alteration of neural identity in the layer in which this factor is usually expressed, and may lead to supernumerary cells expressing the transcription factor of the adjacent layer. These transcription factors, therefore, regulate the temporal identity (early versus late) of the NB and its progeny rather than cell fate (neuronal versus glial).

The molecular mechanisms underlying this temporal regulation are based on the fact that the transcription factor expressed in state 1 activates transcription factor in state 2 and represses transcription factor in state 3 (**Figure 12b**). For example, overexpression of Hb activates Kr and represses Pdm and Cas, whereas overexpression of Kr activates Pdm and represses Cas but has no effect on Hb expression leading to the model that each gene can activate the next gene in the pathway and repress the “next +1” gene (Brody and Odenwald, 2000; Ishiki *et al.*, 2001) (**Figure 12b**). Thus, temporal factors are responsible for upregulation of the next state, but also insulate the genetic program of adjacent temporal windows.

3.3.3. Molecular Control of Neuroblast Apoptosis, Quiescence, and “Reactivation”

The finding that temporal cues specify the identity of NB progeny raises an important question concerning the proliferative potential of neural stem cells. Fly NBs are submitted to a sharp proliferative control throughout development. Indeed, the number of cell divisions in the embryonic CNS depends on the type of NB. In most cases, the number of the progeny depends on the time of delamination, early-born NBs producing more neurons than late-born NBs (Bossing *et al.*, 1996, Schmidt *et al.*, 1997, 1999).

Moreover, the fly adult nervous system develops through extensive reorganization of the embryonic nervous system (review: Truman *et al.*, 1993) (see **Chapters 4** and **5**). A crucial pathway controlling this process is cell death by apoptosis in the sense that, once the embryonic nervous system has differentiated, some NBs undergo programmed cell death (Truman *et al.*, 1992) (see **Chapter 5**). For example, although each embryonic hemineuromere contains 30 NBs, their number decreases at later developmental stages. In the thorax of the larva, each neuromere retains about 23 NBs, while in the abdomen only three remain (Truman and Bate, 1988). Cell death genes of the *reaper* family account for this proapoptotic activity (Peterson *et al.*, 2002) (see **Chapter 5**).

Together with cell death, cell proliferation constitutes the other important pathway that determines the number of progeny and thereby shapes the adult CNS (Truman and Bate, 1988). While some NBs die, others stay quiescent and later resume proliferation (“NB reactivation”) to build up the adult nervous system. The NBs that are reactivated at larval stages cease to proliferate completely after a specific number of divisions, and eventually die by apoptosis. The homeotic gene *abd-A* determines the final number of progeny that each precursor generates in the larval VNC (Bello *et al.*, 2003). Indeed, thoracic and abdominal NBs show extensive differences in the extent of proliferation. Larval thoracic NBs resume division earlier and divide for longer periods of time than abdominal NBs. A burst of expression of *abd-A* regulates the time at which cell death occurs in the larval life and, therefore, schedules the end of neural proliferation (Bello *et al.*, 2003). This shows that homeotic genes link positional information to neural proliferation. The cell-autonomous requirement of Abd-A implies that an intracellular signaling cascade controls this process. It would be interesting to determine whether the “temporal” factors play a role in controlling such death programs.

Recent studies have also shed some light on a molecular mechanism controlling neural stem cell proliferation in the brain. The Even-skipped (Eve) homeodomain containing transcription repressor acts nonautonomously to stimulate cell division of quiescent optic lobe NBs (Park *et al.*, 2001). Genetic studies have shown that Eve interacts with Terribly reduced optic lobes (Troll) and, indeed, mutations in this gene arrest NB proliferation (Voigt *et al.*, 2002). The vertebrate ortholog of *troll* encodes an extracellular matrix molecule that acts as a coreceptor for fibroblast growth factor 2 (FGF2) signaling, calling for a role of cell–cell interactions in the control of neural stem cell proliferation. Interestingly, mutations in these genes do not affect the proliferation potential of NBs that give rise to the embryonic CNS and are not programmed to die.

Building up the nervous system relies on the sequential activation of specific molecular and cellular pathways. While the maternal contribution defines the neurogenic territory, zygotic neuralizing factors and patterning genes allow the differentiation of distinct neural precursors at the right place and time. In addition, most genes work in a combinatorial fashion. The superimposition of homeotic, columnar, and segment polarity gene expression creates, within each neuromere, a Cartesian coordinate system, in which each precursor displays a unique combination of gene activity. Finally, each step must integrate with the

others, as shown by the observation that patterning genes activate proneural genes and that temporal factors control each other's activity. This intricate network of pathways guarantees the fine-tuning that is necessary for the establishment of a very complex array of neural precursors.

3.4. Neuroblasts and Asymmetric Divisions

3.4.1. Neuroblast Lineages and Asymmetric Divisions

Positional and proneural cues induce NB patterning and formation, respectively, and endow cells with specific identities in the absence of cell division. NB delamination triggers a second phase of cell specification, in which cells of the nervous system acquire distinct identities upon divisions that are asymmetric.

Asymmetric cell divisions play a crucial role in the establishment of cell diversity during development (Matsuzaki, 2000; Doe and Bowerman, 2001; Knoblich, 2001; Segal and Bloom, 2001). Asymmetry may be directed by extrinsic cues via cell-cell interaction between the progeny of a precursor cell. Alternatively, it may originate through the unequal segregation of intracellular determinants that direct distinct identities in each of the daughter cells. Although these represent extreme situations, in many instances both processes are required in the same division.

Asymmetric divisions of NBs and GMCs play crucial roles in specifying neural cell types and neuronal/glia identities, respectively (review: Fuerstenberg *et al.*, 1998). NB division is asymmetric in both size and identity of daughter cells (review: Doe *et al.*, 1998). Typically, NBs divide perpendicularly to the neuroepithelial layer and produce a GMC, from the basal side of the parent NB. While NBs undergo this type of asymmetric division repeatedly, GMCs divide only once along the same axis as the NB and produce two distinct cells that do not divide any more (review: Fuerstenberg *et al.*, 1998). NB and GMC divisions are characterized by stereotyped localization of fate determinants.

3.4.2. Role of Prospero/Numb in the Induction of the GMC Fate

The asymmetric divisions in *Drosophila* neural precursors have begun to be elucidated by the discovery of the asymmetric segregation of two key proteins, Prospero (Pros) and Numb, which are synthesized in NBs and asymmetrically segregate to the GMC (Doe *et al.*, 1991; Rhyu *et al.*, 1994; Knoblich *et al.*, 1995).

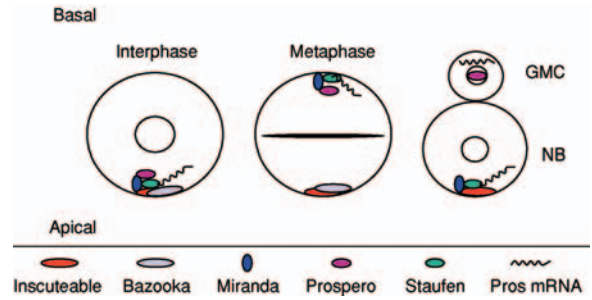


Figure 13 Distribution of cell fate determinants during the NB mitotic cycle. At late interphase, Miranda, Prospero (protein and mRNA), and Staufen are concentrated at the apical cortex and colocalize with Inscuteable and Bazooka. At metaphase, Miranda, Prospero (protein/mRNA), and Staufen move to the basal cortex whereas Inscuteable and Bazooka remain at the apical cortex. During telophase, most members of the basal complex are incorporated into the newly forming GMC. Then, Prospero translocates into the nucleus to activate GMC specific genes. Prospero and all the members of the basal complex (except Bazooka) are synthesized *de novo* in the precursor as the new mitotic cycle begins.

The *prospero* (*pros*) gene encodes a transcription factor that is expressed in all embryonic and larval NBs (Vaessin *et al.*, 1991). It is localized at the NB cortex in a highly cell cycle-dependent fashion (Hirata *et al.*, 1995; Knoblich *et al.*, 1995; Spana and Doe, 1995). At late interphase, Pros resides at the apical NB cortex in a diffuse crescent (Figure 13). As the NB enters mitosis, Pros is transported to the opposite side where it forms a tight crescent centered over the basal centrosome (Figure 13). As the GMC buds from the NB during anaphase, Pros is tightly associated with the basal cell cortex and ultimately segregates into the GMC, where it translocates into the nucleus (Figure 13). Nuclear Pros activates GMC-specific gene expression and represses NB-specific gene expression (Doe *et al.*, 1991; Hirata *et al.*, 1995; Knoblich *et al.*, 1995) (Figure 13). The complex regulation of subcellular Pros localization in the NB has two consequences: on one hand, it makes it possible to create progeny with distinct identity from a multipotent precursor and, on the other hand, it keeps the fate determinant inactive in this precursor.

It has been shown that *pros* mRNA is also asymmetrically localized in the NB in a cell cycle-dependent manner, and segregates to the GMC following mitosis (Li *et al.*, 1997; Broadus *et al.*, 1998) (Figure 13). In embryos lacking the RNA binding protein Staufen (Stau), the majority of NBs fail to localize *pros* mRNA (but not protein) apically at interphase or basally at mitosis, indicating that Stau controls *pros* mRNA localization independently of the Pros protein (Li *et al.*, 1997; Broadus *et al.*,

1998). In *staufen (stau)* mutants, the GMC fate is not affected, indicating that segregation of *pros* RNA is not sufficient to trigger GMC identity (Broadus *et al.*, 1998). However, loss of localization of *pros* mRNA or *Stau* alters GMC development in embryos that contain reduced levels of Pros protein, suggesting that *pros* mRNA and protein localization act redundantly to specify the GMC identity (Broadus *et al.*, 1998). This double control at the *pros* mRNA and protein levels reflects the requirement for fine-tuning in the building up the nervous system.

The *numb* gene encodes a membrane-associated protein (Uemura *et al.*, 1989) that shows the same cell cycle-dependent basal localization as seen for Pros (Rhyu *et al.*, 1994). Numb localizes normally in *pros* mutants, and Pros segregates normally in *numb* mutant embryos, indicating that the two proteins are independently localized (Knoblich *et al.*, 1995; Spana and Doe, 1995). Asymmetric localization of Numb initially requires the Partner of Numb (Pon) protein, which directly binds and colocalizes with Numb in dividing neural precursor cells (Lu *et al.*, 1998). However, Numb appears to be still attached to the cell cortex in the absence of Pon, indicating that other components likely play a role in Numb localization (Lu *et al.*, 1998). It has been recently shown that *jumeaux (jumu)*, a winged-helix transcription factor encoding gene, is necessary to correctly localize and segregate the Pon–Numb complex (Cheah *et al.*, 2000), suggesting that unknown target genes activated by *Jumeaux* play a crucial role in this process.

It is unclear why Numb needs to be asymmetrically distributed during NB division, since there is no reported function of *numb* in the GMC. Nevertheless, Numb does play an important role in the specification of sibling cell identity (reviews: Buescher *et al.*, 1998; Cayouette and Raff, 2002). In most cases, the two daughter cells, A and B, produced after each GMC division are distinct. It has been shown that Numb antagonizes Notch signaling, probably by direct interaction with the Notch intracellular domain (NICD) (Spana and Doe, 1995; Guo *et al.*, 1996). *Notch* loss-of-function mutations lead to the production of two similar daughter cells (A/A) whereas *numb* mutations lead to a reciprocal phenotype (B/B) (Buescher *et al.*, 1998; Lear *et al.*, 1999). Thus, asymmetric segregation of Numb biases Notch signaling between daughter cells to create distinct cell identities. This underlines the role of both autonomous and nonautonomous pathways in the establishment of cell diversity and the importance of crosstalk between these molecular pathways in asymmetric divisions.

3.4.3. Genes Involved in Asymmetric Cell Division

The establishment of different identities via asymmetric division requires the integration of two processes: segregation of cell fate determinants and correct orientation of the mitotic spindle, i.e., orientation parallel to the polar distribution of these determinants.

Inscuteable protein (Insc) is necessary for the coordination of orientation of the mitotic spindle with protein localization (Kraut and Campos-Ortega, 1996; Kraut *et al.*, 1996; Tio *et al.*, 1999). Insc is localized at the apical cortex of the NB and is first detected in the apical endfoot that remains associated with the epithelial surface in the delaminating NB (Figure 13). The apical crescent of Insc persists in the NB through metaphase. After metaphase, it appears to be delocalized or degraded (Kraut *et al.*, 1996) (Figure 13). In *inscuteable (insc)* mutant embryos, the orientation of the mitotic spindle is random with respect to the apicobasal axis (Kraut and Campos-Ortega, 1996). Moreover, Pros and Numb are either homogeneously localized at the cortex or localized in crescents whose positions are randomized with respect to the surrounding tissue during mitosis (Kraut *et al.*, 1996). Insc appears to independently regulate both spindle orientation and basal localization of Pros and Numb during mitosis. Once Insc localization is established in the NB, all known molecular and cellular asymmetries assayed to date are positioned with respect to Insc.

3.4.3.1. How is Insc localization regulated? Previous work has identified Bazooka (Baz), a PSD95/DLG/ZO-1 (PDZ) domain containing protein that binds Insc (Kraut and Campos-Ortega, 1996; Ikeshima-Kataoka *et al.*, 1997; Shen *et al.*, 1997; Kuchinke *et al.*, 1998) (Figure 13). Removal of both maternal and zygotic Bazooka products results in the uniform distribution of Insc in the NB cytoplasm and causes defects in the distribution of cell fate determinants and in spindle orientation similar to those seen in *Insc* mutant embryos (Schober *et al.*, 1999; Wodarz *et al.*, 1999). Indeed, when NBs delaminate from the epithelium, Bazooka colocalizes with Inscuteable and recruits it to the apical cortex in NBs.

The *bazooka (baz)* gene has been shown to be required for the polarity of epithelial cells, in which its protein is localized at the apical cortex (Muller and Wieschaus, 1996). This supports the idea that NBs inherit the apicobasal polarity from the epithelium. Two proteins, Par-6 and atypical protein kinase C (aPKC), colocalize with Bazooka

and form a complex at the apical cortex of NBs. The *par-6* gene encodes a PDZ containing protein (Petronczki and Knoblich, 2001) and *aPKC* encodes a serine/threonine kinase (Wodarz *et al.*, 2000). In *par-6* or *aPKC* mutant embryos, defects are seen in the apical localization of Bazooka and Insc in NBs, as well as in the orientation of the spindle (Wodarz *et al.*, 2000; Petronczki and Knoblich, 2001). These results indicate that a functional complex between Bazooka, Par-6, and aPKC plays a role in polarizing NBs to localize Inscuteable properly following delamination.

Strikingly, *insc* mutations show a relatively low penetrant phenotype (Kraut and Campos-Ortega, 1996; Kraut *et al.*, 1996). Since no Insc homologs exist in flies, it is likely that additional proteins are necessary to coordinate mitotic spindle organization and fate determinant localization. Using cell specific markers, it will be interesting to determine whether Insc affects specific NBs or whether it affects all NBs but is only required in some divisions within the neuroblast lineage (early versus late, for example).

Recently, it has been shown that asymmetrical localization of Insc requires the presence of Partner of Inscuteable (Pins), a protein with multiple repeats of the tetratricopeptide motif as well as motifs implicated in binding of G α proteins (Schaefer *et al.*, 2000; Yu *et al.*, 2000). Pins colocalizes with Insc at the apical cell cortex in interphase and mitotic neuroblasts, and loss of Pins results in the same phenotype as seen in *insc* mutant embryos (Bellaiche *et al.*, 2001). Moreover, the G protein α subunit G α i binds and localizes Pins at the cortex, and the effects of loss of G α i or Pins are very similar, supporting the idea that Pins/G α i act together to mediate neuroblast asymmetric division (Schaefer *et al.*, 2000). In general, the three subunits that compose the G protein (α , β , and γ) localize together at the proximity of seven transmembrane domain receptors also known as G protein-coupled receptors (GPCRs). Signal transduction signaling involves GPCR activation and dissociation of the three subunits, which triggers an intracellular molecular cascade (Offermanns, 2003) (see **Chapter 14**). Interestingly, binding of Pins to G α i can cause the release of the G β subunit from G α i, suggesting that G α i may function during asymmetric cell division via a receptor-independent mechanism (review: Knust, 2001). This suggests that an intracellular process may regulate G protein activation.

These data demonstrate that Pins and Insc are dependent on each other for asymmetric localization, and suggest that a receptor-independent G protein signaling is required to mediate and

coordinate basal protein localization with mitotic spindle orientation (reviews: Schaefer *et al.*, 2000; Knust, 2001; Schaefer *et al.*, 2001).

3.4.3.2. How is Pros localization regulated? Starting from the identification of Pros partners, it has been possible to dissect the molecular cascade triggering cell diversity in the nervous system. Miranda (Mira), a coiled-coil protein, was identified in a two-hybrid screen using the asymmetric localization domain of Pros (Ikeshima-Kataoka *et al.*, 1997; Shen *et al.*, 1997). *Miranda* mutations result in a failure to correctly localize Pros during or after NB division resulting in its nuclear localization in both NB and GMC (Ikeshima-Kataoka *et al.*, 1997). The search for mutations that alter Mira localization in metaphase NBs lead to the identification of *lethal giant larvae (lgl)*, *disc large (dlg)*, and *scribble (sbb)*, three tumor-suppressor encoding genes (Albertson and Doe, 2003). In embryos mutant for any of these genes, Mira is mislocalized (Albertson and Doe, 2003). Surprisingly, Insc localization and spindle orientation are correct, indicating that these proteins act specifically to polarize Mira by a pathway that does not involve Insc (Albertson and Doe, 2003). Therefore, although Mira is required for Pros localization to the cortex, it has no role in the establishment of an asymmetric division (**Figure 13**).

In conclusion, the asymmetric division of NBs requires multiple pathways that regulate independently the different aspects of subcellular localization of cell fate determinants.

3.5. Glial Differentiation in the Fly CNS

Embryonic *Drosophila* glial cells can be classified into three major categories depending on position, morphology and gene expression: surface-associated, cortex-associated, and neuropil-associated glia (Ito *et al.*, 1995; Hartenstein *et al.*, 1998). Nevertheless, considering the origin and molecular details of the developmental program, CNS glial cells can be subdivided into two populations: lateral glia (LG) and midline glia (MG) (reviews: Giangrande, 1996; Granderath and Klambt, 1999; Jacobs, 2000; Van De Bor and Giangrande, 2002). LG originate from the neuroectoderm and are located throughout the CNS, whereas MG originate from the mesectoderm and are located at the midline. Some 60 LG and six MG are present in each neuromere. All LG differentiation is under the control of *glial cell deficient/glial cell missing (glidel/gcm)* locus (review: Van De Bor and Giangrande, 2002), whereas MG differentiation requires *single-minded (sim)*

(Crews *et al.*, 1992). In the absence of LG, MG development is normal and vice versa, showing that these two developmental programs are completely independent.

3.5.1. Midline Glia Differentiation

As seen before, MG determination is under the control of the Sim/Tgo heterodimer, which likely activates the expression of DER (Jacobs, 2000). DER is specifically expressed and required in MG cells, as shown by the finding that DER hypomorph embryos lack all MG at the end of embryogenesis (Zak *et al.*, 1990; Scholz *et al.*, 1997; Stemerink and Jacobs, 1997). The main activating ligand for DER at the midline is Spitz (Spi) (Schweitzer *et al.*, 1995b), a fly ortholog of vertebrate transforming growth factor- α (TGF- α) (Rutledge *et al.*, 1992). Moreover, Rhomboid (Rho; a seven transmembrane protein) and Star (a transmembrane protein) are primary regulators of Spitz signaling (Sturtevant *et al.*, 1993; Guichard *et al.*, 1999). Indeed loss-of-function of *spitz*, *rho*, or *star* results in a decrease in MG number (Sonnenfeld and Jacobs, 1994; Lanoue and Jacobs, 1999; Pickup and Banerjee, 1999). Rho and Star are both expressed throughout the mesectoderm before being restricted to MG. Star is required for the relocalization of Spi from the endoplasmic reticulum to the Golgi apparatus (Figure 14). There, Rho cleaves Spi in its intramembrane domain, producing a soluble form of Spi that is secreted and activates DER (Lee *et al.*, 2001; Urban *et al.*, 2001). Ligand binding to DER activates the Ras pathway leading to phosphorylation and thereby activation of the transcription factor Pointed P2 (PntP2) (Jacobs, 2000). In the absence of *pntP2*, embryos display a fused commissures phenotype, which is characteristic of MG differentiation defects (see below) (Klambt, 1993). This indicates that PntP2 is required for MG development.

During embryonic development, some of the MG undergo programmed cell death (Sonnenfeld and Jacobs, 1995; Chapter 5). This process is controlled by *argos*, a direct target of PntP2 that is expressed in a subpopulation of MG (Scholz *et al.*, 1997). The *argos* gene encodes a secreted protein acting as an antagonist of DER signaling by interfering with Spi-mediated dimerization of DER (Schweitzer *et al.*, 1995a; Jin *et al.*, 2000). Ubiquitous expression of *argos* results in the shutdown of DER expression (Schweitzer *et al.*, 1995a), suggesting that *argos*-expressing cells repress expression of DER in other MG, which eventually die. However, the way in which cells that secrete Argos are protected from an autocrine repression of DER signaling is poorly

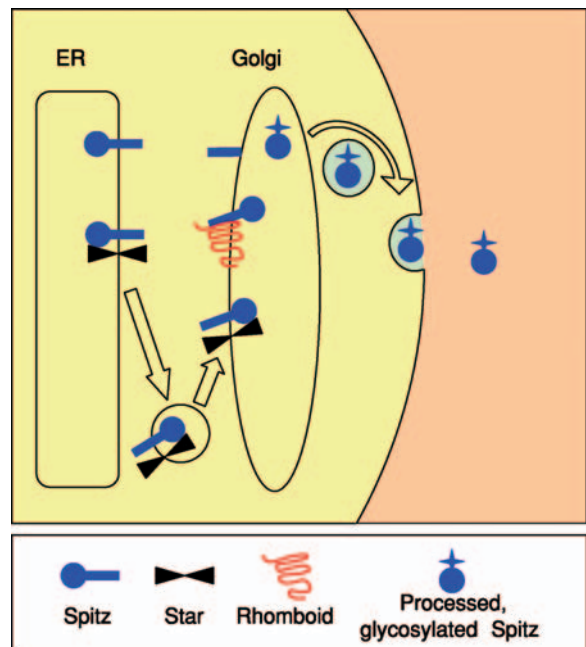


Figure 14 Mechanism of Spitz processing. Spitz (blue) is retained in the endoplasmic reticulum (ER) until Star (black) promotes its relocalization into the Golgi apparatus. There, Rhomboid (red) cleaves the Spitz protein, which is subsequently glycosylated (blue circle with spine) and secreted. (Modified from Lee, J.R., Urban, S., Garvey, C.F., Freeman, M. 2001. Regulated intracellular ligand transport and proteolysis control egf signal activation in *Drosophila*. *Cell* 107, 161–171.)

understood. The existence of a programmed cell death pathway in the midline population clearly indicates that the acquisition of the proper number of MG involves and requires the balance between two developmental pathways, cell differentiation and death.

3.5.2. Lateral Glia Differentiation

3.5.2.1. The *glide/gcm* glial promoting factor
As described above, LG arise from pure (glioblasts) and mixed precursors (neuroglioblasts), all expressing and depending for function on a single locus, irrespective of precursor type (review: Van De Bor and Giangrande, 2002). This locus contains the *glide/gcm* and *glide2* genes, which encode related transcription factors of a novel type (Hosoya *et al.*, 1995; Jones *et al.*, 1995; Vincent *et al.*, 1996; Kammerer and Giangrande, 2001; Alfonso and Jones, 2002) (Figure 15a). The *gcm* DNA-binding motif is conserved throughout evolution and provides a signature for the *gcm* gene family (Akiyama *et al.*, 1996; Schreiber *et al.*, 1997; Miller *et al.*, 1998; Kammerer and Giangrande, 2001). In embryos lacking *glide/gcm*, most LG are absent (Hosoya *et al.*, 1995; Jones *et al.*, 1995; Vincent *et al.*, 1996). Moreover, ectopic expression of *glide/gcm* is sufficient to

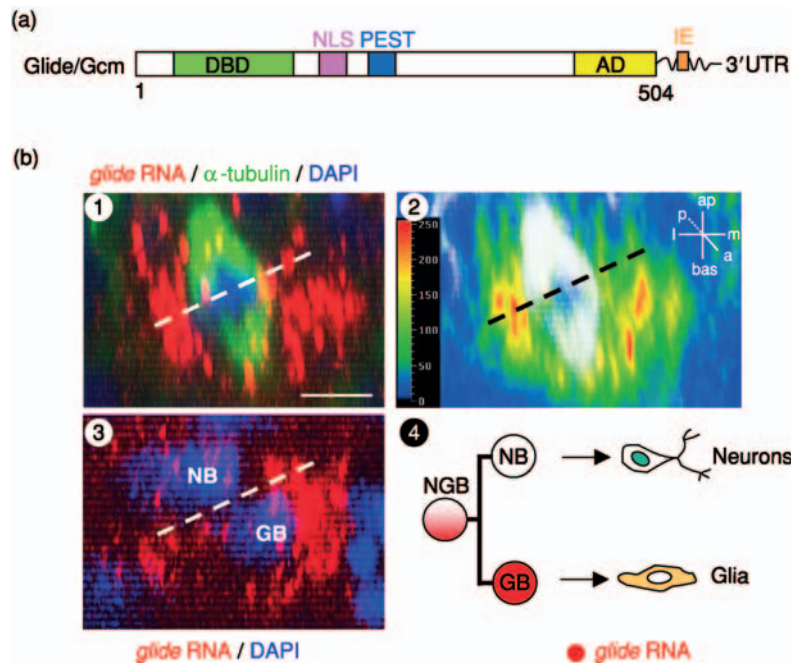


Figure 15 The role of *glide/gcm-glide2* in the determination of the glial fate. (a) Predicted *Glide/Gcm* mRNA structure with the encoded protein domains shown in blocks. DBD, DNA-binding domain; NLS, nuclear localization signal; PEST, rapid turnover signature; AD, activation domain; IE, instability element; UTR, untranslated region. (b) The NGB 6-4T lineage: α -tubulin labels the mitotic spindle 4'-6-diamidino-2-phenylindole (DAPI) the chromatin. (1, 2) Early NGB 6-4T anaphase: *glide* RNA is asymmetrically localized at the basoanterior pole of the cell. Dotted line is located in the middle of chromatin labeling. Scale bar = 4 μ m. In (2), α -tubulin labeling (white) is superimposed to the quantification data relative to *glide* RNA. Color coding at left represents different levels of RNA accumulation: the lowest (0) is represented in blue, the highest (250) in red. ap, apical; bas, basal; a, anterior; p, posterior; l, lateral; m, medial. (3) The NGB 6-4T daughter cells, NB and GB, soon after division. Note that *glide* RNA is preferentially inherited by the GB. NB, neuroblast; GB, glioblast. (4) Schematic of *glide* mode of action during NGB 6-4T division. NGB, neuroglioblast. (Modified from Van De Bor, V., Giangrande, A. 2002. *glide/gcm*: at the crossroads between neurons and glia. *Curr. Opin. Genet. Devel.* 12, 465–472.)

promote the glial fate within and outside the nervous system (Jones *et al.*, 1995; Vincent *et al.*, 1996; Akiyama-Oda *et al.*, 1998; Bernardoni *et al.*, 1998). Expression of *glide2* is also sufficient to promote the glial fate, but its absence has no effect on glia determination, suggesting that it plays a minor role during gliogenesis (Kammerer and Giangrande, 2001; Alfonso and Jones, 2002). In the absence of *Glide/Gcm-Glide2*, glial cells are absent because they are all transformed into neurons. This suggests that the *glide/gcm* locus activates the glial program and represses the neuronal one (Hosoya *et al.*, 1995; Jones *et al.*, 1995; Vincent *et al.*, 1996; Giesen *et al.*, 1997; Bernardoni *et al.*, 1998, 1999; Miller *et al.*, 1999; Van De Bor *et al.*, 2000; Kammerer and Giangrande, 2001). Thus, these genes trigger the glial cell fate by controlling the fate choice between glia and neurons in multipotent precursors (reviews: Wegner and Riethmacher, 2001; Van De Bor and Giangrande, 2002) (Figure 15b).

The role of *glide/gcm* has been extensively investigated in two lineages, the 6-4T and the 6-4A,

examples of type 1 NGB and pure GB, respectively (Akiyama-Oda *et al.*, 1999; Bernardoni *et al.*, 1999; Ragone *et al.*, 2001). During division of the 6-4A, *glide/gcm* mRNA is equally distributed in the two daughter cells leading them towards a glial fate. In multipotent precursors, the fate choice relies on a *glide/gcm* posttranscriptional control. The *glide/gcm* RNA forms a cytoplasmic gradient in the NGB, which leads to one daughter cell inheriting more *glide/gcm* transcripts than the other one (Figures 7 and 15). This cell (the GB) produces glia, whereas the other (the NB) produces neurons. Overexpression of *glide/gcm* in both 6-4T progeny cells results in expression of glial markers in both of them. The observation that daughters inheriting different amounts of the same cell fate determinant end up adopting distinct fates demonstrates the role of quantitative regulation in the establishment of cell diversity upon asymmetric division. This is further confirmed by the fact that *glide/gcm* is able to positively autoregulate its own expression (Miller *et al.*, 1998). Although the NGB provides another example of asymmetric

division, the behavior of *glide/gcm* transcripts suggests that novel molecular mechanisms are necessary for the induction of the glial fate.

3.5.2.2. The *glide/gcm* targets execute the glial differentiation program Once *glide/gcm* has been activated, the glial differentiation program takes place. Because *glide/gcm* expression is transient, terminal glial differentiation is likely under the control of genes acting downstream of it. Some targets, such as *reverse polarity (repo)*, *pointed P1 (pntP1)*, and *locomotion defects (loco-c1)*, have been shown to execute the glial differentiation program, whereas others, e.g., *tramtrack69 (ttk69)*, inhibit the neuronal pathway. Other identified target genes are likely involved in the late differentiation steps of specific glial subtypes (Egger *et al.*, 2002; Freeman *et al.*, 2003).

The first identified target of *glide/gcm* is *reverse polarity (repo)*, which encodes a paired-like homeodomain transcription factor expressed in all LG but not in MG (Campbell *et al.*, 1994; Xiong *et al.*, 1994; Halter *et al.*, 1995). Its promoter contains 11 *Glide/Gcm* consensus binding sequences, and *Glide/Gcm* is necessary and sufficient to induce *repo* expression *in vivo*, suggesting that *repo* represents a direct target of *Glide/Gcm* (Hosoya *et al.*, 1995; Jones *et al.*, 1995; Akiyama *et al.*, 1996; Vincent *et al.*, 1996; Bernardoni *et al.*, 1998). In *repo* mutant embryos, early gliogenesis occurs normally but late glial markers are not expressed, suggesting that *Repo* is required for terminal glia differentiation (Campbell *et al.*, 1994; Xiong *et al.*, 1994; Halter *et al.*, 1995; Yuasa *et al.*, 2003).

The *pointed* gene encodes two transcription factor isoforms containing an ETS DNA binding domain (Klambt, 1993; Klaes *et al.*, 1994). The ETS domain is a DNA binding domain found in a family of proteins to the vertebrate *ets* oncogene. While *Pointed P2 (PntP2)* is only present in MG (see above), *Pointed P1 (PntP1)* is specifically expressed in longitudinal glia (Klambt, 1993). In the absence of *PntP1*, longitudinal glia fail to develop normally leading to defects in connective formation (Klaes *et al.*, 1994). Thus, *PntP1* is involved in terminal differentiation of longitudinal glia (Klaes *et al.*, 1994; Granderath *et al.*, 2000).

Interestingly, ectopic expression of either *Repo* or *PntP1* leads to the activation of some glial-specific markers in few cells, whereas combined ectopic expression has a synergistic effect (Yuasa *et al.*, 2003). *Repo* and *PntP1* do not affect each other's expression, and this suggests that their genes are independent targets of *Glide/Gcm* that participate in glial specification, i.e., the differentiation of a specific type of glial cell (Yuasa *et al.*, 2003).

As mentioned above, gliogenesis needs both activation of glial-specific genes and repression of neuronal-genes. The *tramtrack (ttk)* gene encodes a transcription factor of the zinc finger family (Harrison and Travers, 1990). One of the two *Ttk* isoforms, *Ttk69*, constitutes the only protein expressed in all glial cells (LG and MG) and participates in neural repression (Giesen *et al.*, 1997; Badenhorst, 2001). Ectopic expression experiments and analysis of its expression profile suggest that *Ttk69* acts by repressing the neuronal fate rather than the early steps of neurogenesis (Badenhorst *et al.*, 1996). This is also confirmed by the observation that *Ttk69* does not repress the expression of proneural genes (Badenhorst, 2001). In this process, *Ttk69* seems to cooperate with *Repo*, although the molecular nature of this interaction is not yet understood (Yuasa *et al.*, 2003). *Ttk69* also plays a role in glial proliferation, as *ttk* loss-of-function mutants display an increase in LG number, while gain-of-function mutants show the opposite phenotype (Badenhorst, 2001).

Taken together, these results suggest that lateral gliogenesis is dependent on a single locus (*glide/gcm-glide2*), which triggers the different pathways executing glial cell differentiation. Glial specification depends on cell-specific factors that allocate distinct identities to the different glial lineages. Such factors act at early stages, by controlling the spatially and temporally regulated transcription of the *glide/gcm* locus, but are also activated at later stages by interacting with the *repo* and *pntP1* genes (Klaes *et al.*, 1994; Ragone *et al.*, 2003; Yuasa *et al.*, 2003). These factors likely include those that dictate positional cues in the neural precursors, since they are expressed throughout the neural lineage up to postmitotic cells.

Thus, *Drosophila* represents an ideal model for achieving an in-depth understanding of the molecular pathways controlling glia differentiation. Moreover, elucidating the *glide/gcm* mode of action during neuron–glia fate decision process will afford a better understanding of the mechanisms that control multipotent stem cell decisions during development.

3.6. Neuronal–Glial Cell Interactions Control the Development of the Nervous System

Neurons and glial cells constitute the major components of the nervous system. Although they play distinct roles, their continuous interactions are a gauge of appropriate development and function of such a complex tissue. Neuron–glia interactions

play important instructive roles during cell differentiation, migration, and axonal navigation, as well as in the regulation of cell survival (review: Laming *et al.*, 2000).

Most glia arise from precursors that also produce neurons, the control between the two fates depending on the cell-autonomous cue *Glide/Gcm* (see Section 3.5.2.2). Upstream of this choice, however, a nonautonomous pathway must be activated, at least in some precursors. In the CNS as well as in the PNS, the Notch signaling cascade controls the expression of *glide/gcm* and thereby the activation of the glial fate (Udolph *et al.*, 2001; Van De Bor and Giangrande, 2001; Umesono *et al.*, 2002). The requirement of this pathway, which is conserved throughout evolution (review: Artavanis-Tsakonas *et al.*, 1999), clearly shows that both intrinsic and extrinsic cues act on the same fate decision and participate the establishment of cell diversity.

After neuronal differentiation, polarized extensions grow toward specific targets through the use of distinct cellular and molecular cues. The fly midline represents a classical model for the study of neuron–glia interactions controlling axonal guidance (reviews: Tear, 1999; Kaprielian *et al.*, 2001). Bilaterally symmetric animals are able to transmit information between the left and right sides of their body in order to integrate sensory input and coordinate motor output. Thus, many neurons in the CNS project so-called commissural axons across the midline. Interestingly, these axons are never observed to recross the midline. However, some neurons project axons that remain on the ipsilateral side of the CNS, without ever crossing the midline (reviews: Tear, 1999; Kaprielian *et al.*, 2001). Many, if not most, of the fly navigational cues are provided by midline glia and their precursors (review: Lemke, 2001). Thus, Netrin (Net) chemoattractants, which are expressed in midline glia, attract axons, which express the Netrin receptor Frazzled (Fra) and allow them to cross the midline (review: Livesey, 1999). Disruption of the *netrin* (*net*) or *frazzled* (*fra*) gene leads to a reduction in the number of commissural axons tracts (Harris *et al.*, 1996; Kolodziej *et al.*, 1996; Mitchell *et al.*, 1996). However, neither mutation leads to a complete loss of all commissural axon connections (Kolodziej *et al.*, 1996; Hummel *et al.*, 1999a, 1999b), suggesting that other genes are also involved in this process. It was shown that two other mutations, *schizo* (*siz*) and *weniger* (*weg*), induce a reduced commissure phenotype (Hummel *et al.*, 1999a, 1999b). *Schizo* and *weg* mutant embryos show a more severe phenotype than that observed in *fra* embryos; however, commissural axons are still not affected. Genetic

analyses indicate that the *net/fra* and *siz/weg* genes act in parallel pathways (Hummel *et al.*, 1999a, 1999b). Therefore, *siz* and *weg* encode new, yet undefined, components guiding commissural growth cones towards the midline (Hummel *et al.*, 1999a, 1999b). The molecular characterization of these genes will likely identify new molecules, which also play a role in axon guidance during vertebrate CNS development.

While Netrins are essential for at least some commissural axons to grow across the midline, the choice of a contralateral versus ipsilateral pathway is dictated by the extracellular matrix protein Slit (Sli), which is secreted by midline glial cells and found associated with the surface of axons (Rothberg *et al.*, 1988, 1990). Sli repels axons that express the Roundabout (Robo) proteins (Kidd *et al.*, 1999). Repulsion prevents ipsilaterally projecting axons from entering the midline and contralaterally projecting axons from re-entering the midline once they have crossed it (Kidd *et al.*, 1998c). Three *robo* genes, (*robo1*, 2, 3) have been identified in *Drosophila*, all encoding proteins belonging to the IG-CAM family (Seeger *et al.*, 1993; Kidd *et al.*, 1998c; Rajagopalan *et al.*, 2000; Simpson *et al.*, 2000). Loss of the three proteins mimics the commissure-collapsed phenotype observed in *slit* mutant embryos (Rajagopalan *et al.*, 2000). Axons extending toward or across the midline express very low levels of Robo proteins; in contrast, crossed segments of commissural axons as well as ipsilaterally projecting axons express high levels of Robo proteins (Figure 16). This suggests that Robo proteins normally prevent midline crossing and function as receptors for the Slit inhibitory ligand, which is localized at the midline (Brose *et al.*, 1999; Kidd *et al.*, 1999).

The transmembrane protein Commissureless (Comm) is normally required for axons to cross the midline, and behaves as a powerful negative regulator of Robo, as was evidenced by its mutant phenotype (Seeger *et al.*, 1993; Tear *et al.*, 1996; Kidd *et al.*, 1998c) (Figure 16). Tissue culture experiments have shown that coexpression of Comm and Robo alters Robo's subcellular localization (Kidd *et al.*, 1998b). In the absence of Comm, Robo protein accumulates at the cell surface; however, when both proteins are present, Robo colocalizes with Comm to intracellular compartments, which are probably late endosomes (Figure 16). Moreover, Comm needs to interact with Robo to affect Robo's localization (Keleman *et al.*, 2002). These findings suggest that Comm prevents Robo from reaching the cell surface by binding and targeting it directly to endosomes. One model proposes that this depends on Comm ubiquitination by the DNedd4 ubiquitin

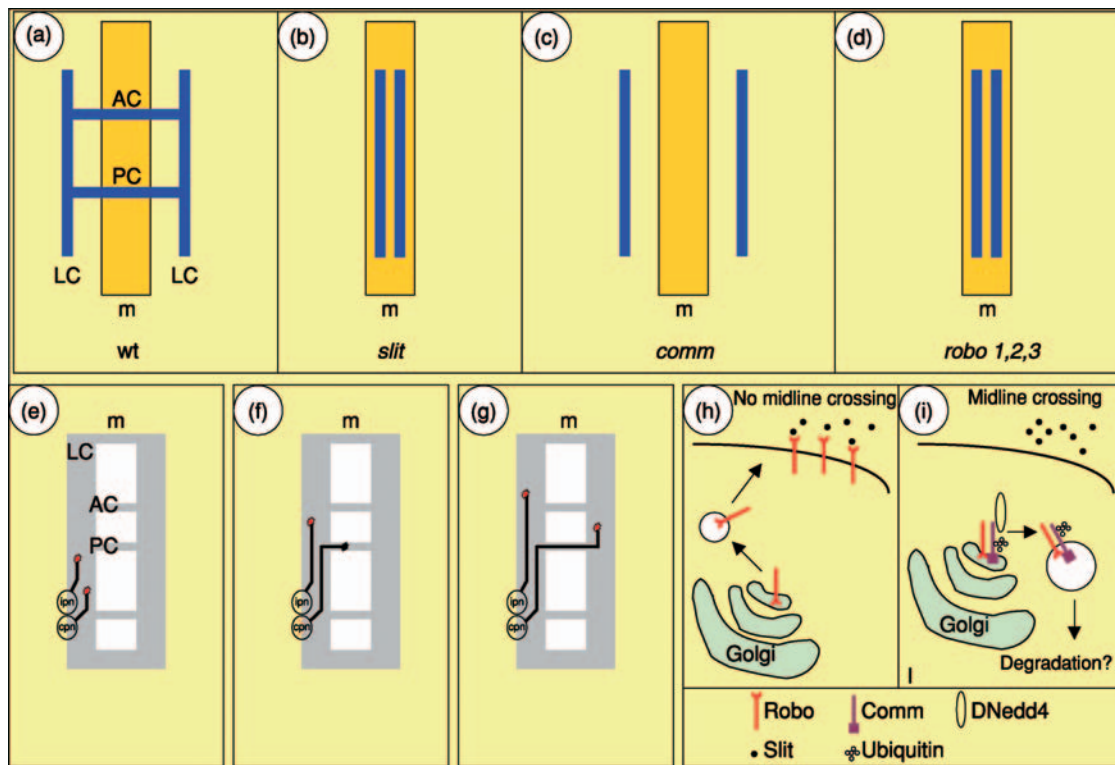


Figure 16 The role of the *slit-robo-comm* pathway in axon guidance at the fly midline. (a) In wild-type embryos, the ventral cord displays two longitudinal connectives (LC) running parallel on each side of the midline (m), an asymmetric structure that is crossed by the anterior and the posterior commissures (AC and PC, respectively). (b) In *slit* mutant embryos, all axons enter the midline but never leave it, resulting in a collapsed commissure phenotype. (c) An opposite phenotype is seen in *comm* mutant embryos in which axons never cross the midline. (d) Gene *robo1, 2, 3* triple mutant embryos show the same phenotype as that observed in *slit* embryos, i.e., collapsed commissures. (e) Initially, both growth cones of ipsilateral (ipn) and contralateral (cpn) axons express Robo (in red). (f) Comm is specifically expressed in the cpn while they are crossing the midline. This results in a loss of Robo at the cpn growth cone membrane. (g) Once cpn axons have crossed the midline, Comm expression is downregulated and Robo is present again at the cpn growth cone membrane. This allows repulsion by Slit, which prevents a new cross. The ipn axon never expresses Comm and never crosses the midline. (h) Neurons only expressing Robo (constitutively in ipn; before and after but not during midline crossing in cpn), accumulate it at the membrane. Axons can then respond to the Slit repellent signal and do not cross the midline. (i) Neurons expressing Comm and Robo do not accumulate Robo at the membrane and, as a consequence, are unable to respond to Slit and cross the midline. In these axons, Slit/Robo signaling is blocked by a mechanism involving ubiquitination of Comm by DNedd4. This ubiquitination step facilitates the sorting of Comm and Robo in late endosomes. Once the cpn has crossed the midline, Comm expression is downregulated and Robo is present again at the membrane.

ligase, which sequesters Robo away from the cell membrane, its site of action (Myat *et al.*, 2002) (Figure 16). These studies clearly identify neuron–glia interactions as privileged cell–cell communication for the establishment of correct axonal trajectories. The fact that these cellular and molecular pathways are conserved throughout evolution (Keleman *et al.*, 2002) emphasizes the importance of such interactions.

In the mature embryo, glial–neuronal interactions are pivotal for the homeostasis of the nervous system, and this is achieved through their influence on the control of glial and neuronal survival (Hidalgo *et al.*, 2001; Bergmann *et al.*, 2002). The importance of such interactions is highlighted by the finding that neurons send signals that control glial cell numbers,

and glial cells send signals that control neuronal cell numbers. Two different members of the EGF family, Vein (Vn) and Spi, were shown to function as gliatrophins in *Drosophila*. Thus, it was demonstrated that the neuregulin homolog Vein maintains survival of a subpopulation of longitudinal glia (Hidalgo *et al.*, 2001), while the TGF- α homolog Spi maintains survival of midline glia (Bergmann *et al.*, 2002). In both cases, the trophic ligands are secreted by adjacent axons at concentrations that are sufficient for the survival of only a subset of the target glial population.

Conversely, mutations in genes specifically expressed in glia affect neuronal survival. The *repo* gene is expressed and required in all fly glial cells (Halter *et al.*, 1995). In flies homozygous for a viable

allele of *repo*, neurodegeneration is observed in the eye, showing that alteration of glial cell function leads to neuronal death (Xiong and Montell, 1995). Similar results were described in other glia-affecting mutants like *drop-dead* (*drd*) or *swiss-cheese* (*sws*) (Buchanan and Benzer, 1993; Kretschmar *et al.*, 1997). These two genes are expressed by glial cells and in mutant flies homozygous for each of these genes, glial cells differentiate abnormally leading to neuronal death. Finally, in *glide/gcm* embryos, where almost all glia are missing, neuronal apoptosis occurs during development (Hosoya *et al.*, 1995; Jones *et al.*, 1995; Vincent *et al.*, 1996).

Once neurons and glia have differentiated, they move to reach their final destination within the CNS and the PNS (review: Hatten, 1999). Indeed, cell migration is a hallmark of nervous system development and is responsible for the generation of the layered structure of the vertebrate CNS (reviews: Tsai and Miller, 2002; Honda *et al.*, 2003). The migratory process entails several steps: cells have to initiate migration, find the direction of movement, and stop migrating as they reach the final destination. The complex and timed process of migration has been mostly analyzed in glial cells of the PNS, which displays a simpler organization than the CNS (Sepp *et al.*, 2000; Tsai and Miller, 2002; Sepp and Auld, 2003). These studies have demonstrated that, in some tissues, glial cells use axons as a migratory substratum (wing-associated glia) whereas in others they do not (eye-associated glia) (Giangrande *et al.*, 1993; Choi and Benzer, 1994; Giangrande, 1994; Rangarajan *et al.*, 1999; Van De Bor *et al.*, 2000). The different glial migratory behaviors observed so far underscore the diversity of developmental strategies used in time and space to make cells reach their final destination.

The study of a dynamic process such as migration has often been limited by two factors. On one hand, most commonly used techniques for labeling cell types rely on the fixation of samples (Ono *et al.*, 1997; Affolter and Shilo, 2000; Sugimoto *et al.*, 2001); on the other hand, real-time analyses have been realized in cell culture systems and/or sliced tissues, due to the nervous system complexity. The recent development of green fluorescent protein (GFP)-based tools has opened new perspectives for the analysis of dynamic processes in whole animals and in real time (review: Yu *et al.*, 2003). This non-invasive approach will certainly provide new and important information on the role of neuron–glia interactions in the control of glial cell migration.

Although neurons and glia have very distinct roles, these data clearly demonstrate that the continuous interaction between these two cell types plays a

fundamental role in neural development, and that neurons or glial cells must be considered as a whole, in order to understand their differentiation and role.

3.7. Evolutionary Aspects of Insect CNS Development

Much of what was initially learned on embryonic nervous system development comes from studies on large insects. Indeed, neural precursors were first identified in grasshopper embryos (Wheeler, 1891), in which it is possible to predict the identity of individual neural precursor with 100% certainty solely on the basis of its stereotyped size, morphology, and position in the neurogenic region (Raper *et al.*, 1984; Taghert and Goodman, 1984; Doe and Goodman, 1985a). By comparison, the early and most of the late-forming neural precursors can be unequivocally identified in *Drosophila* by their morphology, while the use of molecular markers is necessary for the identification of the others (Doe, 1992).

Morphological analysis of neural precursor lineages shows that subtle differences exist between *Drosophila* and the grasshopper. In *Drosophila*, the ectodermal cells adjacent to the neural precursors extend processes around the precursor, which are withdrawn soon after the latter begin to divide. In the grasshopper, these cells differentiate into “sheath cells,” which maintain processes that wrap around the GMCs and the neurons/glial cells produced from a single neural precursor (Doe and Goodman, 1985a) (Figure 17). As a consequence, the progeny of individual *Drosophila* neural precursor are not “packaged” together in a line, instead GMCs and neurons from adjacent neural precursors intermingle in the CNS (Doe, 1992). In addition, the grasshopper has one cell type that is not present in *Drosophila*, the cap cell, which is attached to the ventral surface of every neural precursor and is required for its asymmetric divisions (Kawamura and Carlson, 1962) (Figure 17).

3.7.1. Neural Precursor Identity and Positional Cues

With respect to position and timing of formation, pattern of gene expression and cell lineage, the development of neural precursors in *Drosophila* is similar to that observed in the larger grasshopper embryo (Bate, 1976; Doe and Goodman, 1985a; Doe, 1992). Because of this, neural precursor names in *Drosophila* have been chosen in order to indicate functional homology with those of the grasshopper (Goodman *et al.*, 1984; Doe, 1992; Jia *et al.*, 2002). However, this does not imply that each *Drosophila* neural precursor has a grasshopper

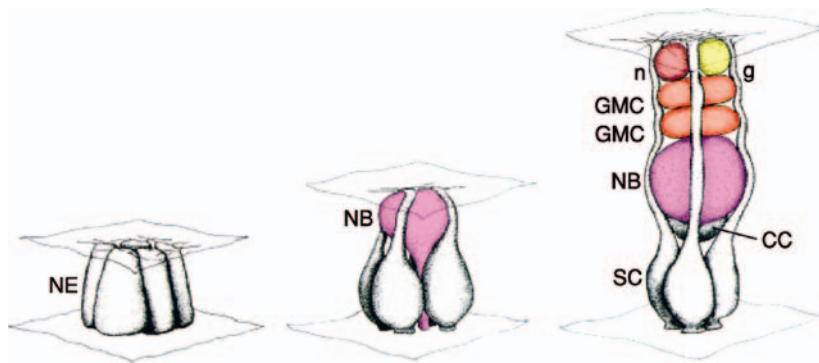


Figure 17 *Camera lucida* illustration of the delamination and the differentiation process of a neural precursor in the grasshopper. One neural precursor or neuroblast (NB) (pink) forms from a cluster of neuroectodermal cells (NE); the neuroblast enlarges and delaminates toward the interior of the embryo. Successive asymmetric divisions of the neural precursor give rise to ganglion mother cells (GMCs, orange), which divide once to produce sibling neurons or a neuron (n) and a glial cell (g) (red and yellow, respectively). In the grasshopper, sheath cells (SC) enwrap the progeny of each neural precursor. The grasshopper has one cell type not present in *Drosophila*, the cap cell (CC), which is attached to the ventral surface of every neural precursor. Ventral is down. (Modified from Doe, C.Q., Fuerstenberg, S., Peng, C.Y., 1998. Neural stem cells: from fly to vertebrates. *J. Neurobiol.* 36, 111–127.)

equivalent. Both organisms have about 30 neural precursors per hemisegment, including a glial precursor that generates longitudinal glia and a MP2 precursor that produces the dMP2 and vMP2 neurons. Both in *Drosophila* and the grasshopper, neural precursors form in waves, with each new population of precursors arranged roughly in columns and rows (Doe and Goodman, 1985a; Doe, 1992).

Cell heterotopic transplantation experiments have shown that neural precursors are formed in the neuroectoderm before delamination (Prokop and Technau, 1994; Udolph *et al.*, 1995), thus suggesting that positional information plays an essential role in neural precursor formation. Heterotopic transplantation between different *Drosophilidae* (*Drosophila melanogaster*, *D. virilis*, *D. hydei*, *D. pseudoobscura*, *D. latifasciaformis*, *D. buscii*) leads to results comparable to those obtained from intraspecific transplantations. Therefore, positional cues are conserved amongst these different species (Becker and Technau, 1990).

3.7.2. Neural Precursor Formation and Cell–Cell Interactions

The observation that the decision to adopt an epidermal or a neural fate is mediated by cell–cell interactions was first described in the grasshopper. Indeed, laser cell ablation experiments in this insect have shown that, while more cells than one in a specific region have the potential to form a neural precursor, the developing neural precursor inhibits its adjacent cells from developing as neural precursors (Taghert *et al.*, 1984; Doe and Goodman, 1985a, 1985b). Thus, while under normal circumstances these adjacent cells develop as epidermoblasts, they adopt the

neural fate when the neural precursor is killed. This demonstrates that the presumptive epidermoblasts are prevented by the neural precursor from adopting the neural fate. This process has been called lateral inhibition, and genetic studies in *Drosophila* have identified the Notch pathway as a major signaling process involved in cell fate choice (see Section 3.2.2.2).

3.7.2.1. Invariant neural precursor cell lineage The analysis of several identified grasshopper neural precursors has shown that each neural lineage is invariant and generates a specific set of neurons and/or glia (Bate and Grunewald, 1981; Goodman and Spitzer, 1979; Taghert *et al.*, 1984), and this has allowed the establishment of a neural precursor map (Bate, 1976; Doe and Goodman, 1985a, 1985b). Subsequently, a similar map was also established in *Drosophila* (see Section 3.2.3). Some of the lineages in these two insects show similarities; thus, for example, both in the grasshopper and in *Drosophila*, the first GMC from NB1-1 always divides to produce the aCC and pCC motoneurons (Goodman and Spitzer, 1979; Udolph *et al.*, 1993).

While embryonic thoracic and abdominal segments of hemimetabolous insects have more neural precursors present in the thoracic segments than in the abdominal ones (Bate, 1976; Doe and Goodman, 1985a; Booker and Truman, 1987), in the holometabolous *Drosophila* embryo, the neural precursor patterns in the thoracic and abdominal segments look very much the same (Doe, 1992). This implies that hemi- and holometabolous insects employ distinct mechanisms in order to produce segmental cell diversity: in the first case, cell diversity is triggered

by the production of segment-specific neural precursors, whereas in the second one, the difference in the number and/or the type of neuron and glial cell between thoracic and abdominal segments is a consequence of segment-specific neural precursor lineages. In both cases, these segmental differences are due to the action of homeotic genes that control differentiation of the neurogenic region in hemimetabolous insects and segment-specific lineage in holometabolous insects (see Section 3.3.1.1).

3.7.3. Conservation of Proneural Activity during Evolution

In general, the mechanisms involved in insect neurogenesis appear to be well conserved during evolution. How could one explain such conservation?

In *Drosophila*, the proneural genes, including the genes of the *Achaete-Scute Complex* (*ac*, *sc*, *l'sc* and *ase*), confer neural potential to cells and/or specify neuronal subtypes (Brunet and Ghysen, 1999; Chan and Jan, 1999). Because AS-C members are involved in the initiation of nervous system development in invertebrates and vertebrates (Section 3.2.2.1), the study of these genes has allowed the understanding of the evolution of development of the arthropod nervous system (Skaer *et al.*, 2002).

Phylogenetic trees constructed by amino acid sequence comparison of the products encoded by the *achaete-scute* orthologs strongly suggest that the four *Drosophila* genes have arisen from three independent duplication events during insect evolution (Skaer *et al.*, 2002). In contrast, *Tribolium castaneum* (Coleoptera) and *Anopheles gambiae* (an, "early" dipteran) contain a single proneural *ac-sc* like gene (Wulbeck and Simpson, 2002; Wheeler *et al.*, 2003), whereas *Ceratitis*, a more recently evolved dipteran, contains two proneural *ac-sc* like genes (Wulbeck and Simpson, 2000). Thus, a duplication of an ancestral proneural *ac-sc* like gene has occurred within the dipteran lineage prior to the appearance of *Ceratitis*.

The *ase* gene is the only member that is expressed only in the neural precursors (Brand *et al.*, 1993; Jarman *et al.*, 1993). Within the complex, the *ase* orthologs form a group that is clearly separated from the other genes, suggesting that the duplication of an ancestral gene has given rise to *ase* and to a gene from which *l'sc*, *sc*, and *ac* evolved subsequently. Actually, it appears that the last common ancestor of arthropods contained a single prototypical *ac-sc* like gene that carried out both proneural and *ase* functions (Skaer *et al.*, 2002). The duplication giving rise to *ac* and *sc* is the most recent

event. Additional evidence for this is the observation that the *ac* and *sc* genes of *Drosophila* share *cis*-regulatory elements, are almost always coexpressed, and their products are functionally redundant (Rodriguez *et al.*, 1990; Martinez and Modolell, 1991; Skeath and Carroll, 1991; Cubas and Modolell, 1992; Gomez-Skarmeta *et al.*, 1995). After duplication, the new genes have acquired individual expression patterns but, in *Drosophila*, their products can compensate for one another, showing that a functional conservation has been maintained during evolution.

While *ac*, *sc*, and *l'sc* have the potential to induce neural precursor formation, only *ac* or *sc* can promote a correct gene expression profile and cell division pattern of the MP2 precursor (Parras *et al.*, 1996; Skeath and Doe, 1996), suggesting that *ac* and *sc* control both the formation and individual fate specification of neural precursors. Recently, it was shown that the single *ac-sc* like ortholog identified in the red flour beetle *T. castaneum* has conserved its proneural function, but is unable to allocate specific identity to neural precursors (Wheeler *et al.*, 2003). This suggests that the ability of *ac* and *sc* to control specification of neural precursors represents a recent evolutionary specialization within Diptera.

3.8. Conclusion and Perspectives

The nervous system represents the most complex tissue in multicellular organisms. The sophisticated network of neurons and glia generated by neural stem cells allows organisms to respond and adapt to the numerous internal and external stimuli they encounter constantly during life. Characterizing the molecular and the cellular cues underlying the differentiation of the nervous system has a dual goal. First, it should allow us to understand how complex tissues are built during development and preserved during ontogenesis. Second, it should also have implications in the development of targeted therapeutic strategies for brain cancer and neurodegenerative diseases. The evolutionary conservation of the developmental strategies makes it likely that the use of a simple genetic model like *Drosophila* will help us to unravel the molecular and cellular basis of vertebrate neural development.

One of the most challenging issues for the next generation of drosophilists will be to follow the different steps that lead to nervous system differentiation *in vivo*, and to combine real-time observations with genetic dissection approaches. The ultimate goal will be to integrate the inter- and intracellular signaling pathways that coordinate

the establishment of the highly sophisticated neural network.

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References

- Affolter, M., Shilo, B.Z., 2000. Genetic control of branching morphogenesis during *Drosophila* tracheal development. *Curr. Opin. Cell. Biol.* 12, 731–735.
- Akiyama, Y., Hosoya, T., Poole, A.M., Hotta, Y., 1996. The gcm-motif: a novel DNA-binding motif conserved in *Drosophila* and mammals. *Proc. Natl Acad. Sci. USA* 93, 14912–14916.
- Akiyama-Oda, Y., Hosoya, T., Hotta, Y., 1998. Alteration of cell fate by ectopic expression of *Drosophila* glial cells missing in non-neural cells. *Devel. Genes Evol.* 208, 578–585.
- Akiyama-Oda, Y., Hosoya, T., Hotta, Y., 1999. Asymmetric cell division of thoracic neuroblast 6–4 to bifurcate glial and neuronal lineage in *Drosophila*. *Development* 126, 1967–1974.
- Albertson, R., Doe, C.Q., 2003. Dlg, Scrib and Lgl regulate neuroblast cell size and mitotic spindle asymmetry. *Nat. Cell Biol.* 5, 166–170.
- Alfonso, T.B., Jones, B.W., 2002. gcm2 promotes glial cell differentiation and is required with glial cells missing for macrophage development in *Drosophila*. *Devel. Biol.* 248, 369–383.
- Alonso, M.C., Cabrera, C.V., 1988. The achaete–scute gene complex of *Drosophila melanogaster* comprises four homologous genes. *EMBO J.* 7, 2585–2591.
- Anderson, H., Edwards, J.S., Palka, J., 1980. Developmental neurobiology of invertebrates. *Annu. Rev. Neurosci.* 3, 97–139.
- Arendt, D., Nubler-Jung, K., 1999. Comparison of early nerve cord development in insects and vertebrates. *Development* 126, 2309–2325.
- Artavanis-Tsakonas, S., Rand, M.D., Lake, R.J., 1999. Notch signaling: cell fate control and signal integration in development. *Science* 284, 770–776.
- Badenhorst, P., 2001. Tramtrack controls glial number and identity in the *Drosophila* embryonic CNS. *Development* 128, 4093–4101.
- Badenhorst, P., Harrison, S., Travers, A., 1996. End of the line? Tramtrack and cell fate determination in *Drosophila*. *Genes Cells* 1, 707–716.
- Bailey, A.M., Posakony, J.W., 1995. Suppressor of hairless directly activates transcription of enhancer of split complex genes in response to Notch receptor activity. *Genes Devel.* 9, 2609–2622.
- Baker, N.E., 1988. Localization of transcripts from the wingless gene in whole *Drosophila* embryos. *Development* 103, 289–298.
- Baron, M., Aslam, H., Flaszka, M., Fostier, M., Higgs, J.E., et al. Multiple levels of Notch signal regulation (review). *Mol. Membr. Biol.* 19, 27–38.
- Bate, C.M., 1976. Embryogenesis of an insect nervous system. 1. A map of the thoracic and abdominal neuroblasts in *Locusta migratoria*. *J. Embryol. Exp. Morphol.* 35, 107–123.
- Bate, C.M., Grunewald, E.B., 1981. Embryogenesis of an insect nervous system. 2. A second class of neuron precursor cells and the origin of the intersegmental connectives. *J. Embryol. Exp. Morphol.* 61, 317–330.
- Baumgartner, S., Bopp, D., Burri, M., Noll, M., 1987. Structure of two genes at the *gooseberry* locus related to the paired gene and their spatial expression during *Drosophila* embryogenesis. *Genes Devel.* 1, 1247–1267.
- Becker, T., Technau, G.M., 1990. Single cell transplantation reveals interspecific cell communication in *Drosophila* chimeras. *Development* 109, 821–832.
- Bellaïche, Y., Radovic, A., Woods, D.F., Hough, C.D., Parmentier, M.L., et al., 2001. The Partner of Inscutable/Discs-large complex is required to establish planar polarity during asymmetric cell division in *Drosophila*. *Cell* 106, 355–366.
- Bello, B.C., Hirth, F., Gould, A.P., 2003. A pulse of the *Drosophila* Hox protein Abdominal-A schedules the end of neural proliferation via neuroblast apoptosis. *Neuron* 37, 209–219.
- Bergmann, A., Tugentman, M., Shilo, B.Z., Steller, H., 2002. Regulation of cell number by MAPK-dependent control of apoptosis: a mechanism for trophic survival signaling. *Devel. Cell* 2, 159–170.
- Berleth, T., Burri, M., Thoma, G., Bopp, D., Richstein, S., et al., 1988. The role of localization of bicoid RNA in organizing the anterior pattern of the *Drosophila* embryo. *EMBO J.* 7, 1749–1756.
- Bernardoni, R., Kammerer, M., Vonesch, J.L., Giangrande, A., 1999. Gliogenesis depends on glide/gcm through asymmetric division of neuroglioblasts. *Devel. Biol.* 216, 265–275.
- Bernardoni, R., Miller, A.A., Giangrande, A., 1998. Glial differentiation does not require a neural ground state. *Development* 125, 3189–3200.
- Bertrand, N., Castro, D.S., Guillemot, F., 2002. Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci.* 3, 517–530.
- Bhat, K.M., 1999. Segment polarity genes in neuroblast formation and identity specification during *Drosophila* neurogenesis. *BioEssays* 21, 472–485.
- Biehls, B., Francois, V., Bier, E., 1996. The *Drosophila* short gastrulation gene prevents Dpp from

- autoactivating and suppressing neurogenesis in the neuroectoderm. *Genes Devel.* 10, 2922–2934.
- Bier, E., 1997. Anti-neural-inhibition: a conserved mechanism for neural induction. *Cell* 89, 681–684.
- Bier, E., Jan, L.Y., Jan, Y.N., 1990. *rhomboid*, a gene required for dorsoventral axis establishment and peripheral nervous system development in *Drosophila melanogaster*. *Genes Devel.* 4, 190–203.
- Boncinelli, E., Simeone, A., Acampora, D., Gulisano, M., 1993. Homeobox genes in the developing central nervous system. *Ann. Genet.* 36, 30–37.
- Booker, R., Truman, J.W., 1987. Postembryonic neurogenesis in the CNS of the tobacco hornworm, *Manduca sexta*. 1. Neuroblast arrays and the fate of their progeny during metamorphosis. *J. Comp. Neurol.* 255, 548–559.
- Bossing, T., Technau, G.M., 1994. The fate of the CNS midline progenitors in *Drosophila* as revealed by a new method for single cell labelling. *Development* 120, 1895–1906.
- Bossing, T., Udolph, G., Doe, C.Q., Technau, G.M., 1996. The embryonic central nervous system lineages of *Drosophila melanogaster*. 1. Neuroblast lineages derived from the ventral half of the neuroectoderm. *Devel. Biol.* 179, 41–64.
- Boulianne, G.L., de la Concha, A., Campos-Ortega, J.A., Jan, L.Y., Jan, Y.N., 1991. The *Drosophila* neurogenic gene neuralized encodes a novel protein and is expressed in precursors of larval and adult neurons. *EMBO J.* 10, 2975–2983.
- Boyan, G.S., Ball, E.E., 1993. The grasshopper, *Drosophila* and neuronal homology (advantages of the insect nervous system for the neuroscientist). *Prog. Neurobiol.* 41, 657–682.
- Brand, M., Jarman, A.P., Jan, L.Y., Jan, Y.N., 1993. *asense* is a *Drosophila* neural precursor gene and is capable of initiating sense organ formation. *Development* 119, 1–17.
- Bray, S.J., 1997. Expression and function of Enhancer of split bHLH proteins during *Drosophila* neurogenesis. *Perspect. Devel. Neurobiol.* 4, 313–323.
- Bray, S., Furriols, M., 2001. Notch pathway: making sense of suppressor of hairless. *Curr. Biol.* 11, R217–R221.
- Broadus, J., Doe, C.Q., 1997. Extrinsic cues, intrinsic cues and microfilaments regulate asymmetric protein localization in *Drosophila* neuroblasts. *Curr. Biol.* 7, 827–835.
- Broadus, J., Fuerstenberg, S., Doe, C.Q., 1998. Staufendependent localization of prospero mRNA contributes to neuroblast daughter-cell fate. *Nature* 391, 792–795.
- Brody, T., Odenwald, W.F., 2000. Programmed transformations in neuroblast gene expression during *Drosophila* CNS lineage development. *Devel. Biol.* 226, 34–44.
- Brody, T., Odenwald, W.F., 2002. Cellular diversity in the developing nervous system: a temporal view from *Drosophila*. *Development* 129, 3763–3770.
- Brose, K., Bland, K.S., Wang, K.H., Arnott, D., Henzel, W., et al., 1999. Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. *Cell* 96, 795–806.
- Brunet, J.F., Ghysen, A., 1999. Deconstructing cell determination: proneural genes and neuronal identity. *BioEssays* 21, 313–318.
- Buchanan, R.L., Benzer, S., 1993. Defective glia in the *Drosophila* brain degeneration mutant *drop-dead*. *Neuron* 10, 839–850.
- Buescher, M., Yeo, S.L., Udolph, G., Zavortink, M., Yang, X., et al., 1998. Binary sibling neuronal cell fate decisions in the *Drosophila* embryonic central nervous system are nonstochastic and require inscuteable-mediated asymmetry of ganglion mother cells. *Genes Devel.* 12, 1858–1870.
- Cabrera, C.V., 1990. Lateral inhibition and cell fate during neurogenesis in *Drosophila*: the interactions between scute, Notch and Delta. *Development* 110, 733–742.
- Cabrera, C.V., Martinez-Arias, A., Bate, M., 1987. The expression of three members of the *achaete-scute* gene complex correlates with neuroblast segregation in *Drosophila*. *Cell* 50, 425–433.
- Calleja, M., Herranz, H., Estella, C., Casal, J., Lawrence, P., et al., 2000. Generation of medial and lateral dorsal body domains by the pannier gene of *Drosophila*. *Development* 127, 3971–3980.
- Campbell, G., Goring, H., Lin, T., Spana, E., Andersson, S., et al., 1994. RK2, a glial-specific homeodomain protein required for embryonic nerve cord condensation and viability in *Drosophila*. *Development* 120, 2957–2966.
- Campos-Ortega, J.A., 1993. Early neurogenesis in *Drosophila melanogaster*. In: Bate, C.M., Martinez-Arias, A. (Eds.), *The Development of Drosophila*, vol. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1091–1129.
- Campos-Ortega, J.A., 1998. The genetics of the *Drosophila achaete-scute* gene complex: a historical appraisal. *Int. J. Devel. Biol.* 42, 291–297.
- Campos-Ortega, J.A., Hartenstein, V., 1985. *The Embryonic Development of Drosophila melanogaster*. Springer, New York.
- Campuzano, S., Carramolino, L., Cabrera, C.V., Ruiz-Gomez, M., Villares, R., et al., 1985. Molecular genetics of the *achaete-scute* gene complex of *D. melanogaster*. *Cell* 40, 327–338.
- Cavodeassi, F., Modolell, J., Campuzano, S., 2000. The Iroquois homeobox genes function as dorsal selectors in the *Drosophila* head. *Development* 127, 1921–1929.
- Cayouette, M., Raff, M., 2002. Asymmetric segregation of Numb: a mechanism for neural specification from *Drosophila* to mammals. *Nat. Neurosci.* 5, 1265–1269.
- Chan, Y.M., Jan, Y.N., 1998. Roles for proteolysis and trafficking in Notch maturation and signal transduction. *Cell* 94, 423–426.
- Chan, Y.M., Jan, Y.N., 1999. Conservation of neurogenic genes and mechanisms. *Curr. Opin. Neurobiol.* 9, 582–588.

- Cheah, P.Y., Chia, W., Yang, X., 2000. Jumeaux, a novel *Drosophila* winged-helix family protein, is required for generating asymmetric sibling neuronal cell fates. *Development* 127, 3325–3335.
- Choi, K.W., Benzer, S., 1994. Migration of glia along photoreceptor axons in the developing *Drosophila* eye. *Neuron* 12, 423–431.
- Chu, H., Parras, C., White, K., Jimenez, F., 1998. Formation and specification of ventral neuroblasts is controlled by vnd in *Drosophila* neurogenesis. *Genes Devel.* 12, 3613–3624.
- Chu-LaGriff, Q., Doe, C.Q., 1993. Neuroblast specification and formation regulated by wingless in the *Drosophila* CNS. *Science* 261, 1594–1597.
- Condrón, B.G., Patel, N.H., Zinn, K., 1994. Engrailed controls glial/neuronal cell fate decisions at the midline of the central nervous system. *Neuron* 13, 541–554.
- Condrón, B.G., Zinn, K., 1994. The grasshopper median neuroblast is a multipotent progenitor cell that generates glia and neurons in distinct temporal phases. *J. Neurosci.* 14, 5766–5777.
- Cornell, R.A., Ohlen, T.V., 2000. Vnd/nkx, ind/gsh, and msh/msx: conserved regulators of dorsoventral neural patterning? *Curr. Opin. Neurobiol.* 10, 63–71.
- Crews, S., Franks, R., Hu, S., Matthews, B., Nambu, J., 1992. *Drosophila* single-minded gene and the molecular genetics of CNS midline development. *J. Exp. Zool.* 261, 234–244.
- Crews, S.T., Thomas, J.B., Goodman, C.S., 1988. The *Drosophila* single-minded gene encodes a nuclear protein with sequence similarity to the *per* gene product. *Cell* 52, 143–151.
- Cubas, P., de Celis, J.F., Campuzano, S., Modolell, J., 1991. Proneural clusters of achaete–scute expression and the generation of sensory organs in the *Drosophila* imaginal wing disc. *Genes Devel.* 5, 996–1008.
- Cubas, P., Modolell, J., 1992. The *extramacrochaetae* gene provides information for sensory organ patterning. *EMBO J.* 11, 3385–3393.
- D’Alessio, M., Frasch, M., 1996. msh may play a conserved role in dorsoventral patterning of the neuroectoderm and mesoderm. *Mech. Devel.* 58, 217–231.
- Dambly-Chaudière, C., Ghysen, A., 1987. Independent subpatterns of sense organs require independent genes of the *achaete–scute* complex in *Drosophila* larvae. *Genes Devel.* 1, 297–306.
- Davenne, M., Maconochie, M.K., Neun, R., Pattyn, A., Chambon, P., et al., 1999. Hoxa2 and Hoxb2 control dorsoventral patterns of neuronal development in the rostral hindbrain. *Neuron* 22, 677–691.
- Doe, C.Q., 1992. Molecular markers for identified neuroblasts and ganglion mother cells in the *Drosophila* central nervous system. *Development* 116, 855–863.
- Doe, C.Q., Bowerman, B., 2001. Asymmetric cell division: fly neuroblast meets worm zygote. *Curr. Opin. Cell Biol.* 13, 68–75.
- Doe, C.Q., Chu-LaGriff, Q., Wright, D.M., Scott, M.P., 1991. The *prospero* gene specifies cell fates in the *Drosophila* central nervous system. *Cell* 65, 451–464.
- Doe, C.Q., Fuerstenberg, S., Peng, C.Y., 1998. Neural stem cells: from fly to vertebrates. *J. Neurobiol.* 36, 111–127.
- Doe, C.Q., Goodman, C.S., 1985a. Early events in insect neurogenesis. 1. Development and segmental differences in the pattern of neuronal precursor cells. *Devel. Biol.* 111, 193–205.
- Doe, C.Q., Goodman, C.S., 1985b. Early events in insect neurogenesis. 2. The role of cell interactions and cell lineage in the determination of neuronal precursor cells. *Devel. Biol.* 111, 206–219.
- Doe, C.Q., Smouse, D.T., 1990. The origins of cell diversity in the insect central nervous system. *Semin. Cell Biol.* 1, 211–218.
- Dominguez, M., Campuzano, S., 1993. *asense*, a member of the *Drosophila* *achaete–scute* complex, is a proneural and neural differentiation gene. *EMBO J.* 12, 2049–2060.
- Dong, R., Jacobs, J.R., 1997. Origin and differentiation of supernumerary midline glia in *Drosophila* embryos deficient for apoptosis. *Devel. Biol.* 190, 165–177.
- Egger, B., Leemans, R., Loop, T., Kammermeier, L., Fan, Y., et al., 2002. Gliogenesis in *Drosophila*: genome-wide analysis of downstream genes of glial cells missing in the embryonic nervous system. *Development* 129, 3295–3309.
- Ferguson, E.L., 1996. Conservation of dorsal–ventral patterning in arthropods and chordates. *Curr. Opin. Genet. Devel.* 6, 424–431.
- François, V., Bier, E., 1995. *Xenopus* chordin and *Drosophila* short gastrulation genes encode homologous proteins functioning in dorsal–ventral axis formation. *Cell* 80, 19–20.
- François, V., Solloway, M., O’Neill, J.W., Emery, J., Bier, E., 1994. Dorsal–ventral patterning of the *Drosophila* embryo depends on a putative negative growth factor encoded by the short gastrulation gene. *Genes Devel.* 8, 2602–2616.
- Fraser, S.E., 1991. Pattern formation in the vertebrate nervous system. *Curr. Opin. Genet. Devel.* 1, 217–220.
- Freeman, M.R., Delrow, J., Kim, J., Johnson, E., Doe, C.Q., 2003. Unwrapping glial biology: gcm target genes regulating glial development, diversification, and function. *Neuron* 38, 567–580.
- Fuerstenberg, S., Broadus, J., Doe, C.Q., 1998. Asymmetry and cell fate in the *Drosophila* embryonic CNS. *Int. J. Devel. Biol.* 42, 379–383.
- Furst, A., Mahowald, A.P., 1985. Differentiation of primary embryonic neuroblasts in purified neural cell cultures from *Drosophila*. *Devel. Biol.* 109, 184–192.
- García-García, M.J., Romain, P., Simpson, P., Modolell, J., 1999. Different contributions of pannier and wingless to the patterning of the dorsal mesothorax of *Drosophila*. *Development* 126, 3523–3532.
- Gautier, P., Ledent, V., Massaer, M., Dambly-Chaudière, C., Ghysen, A., 1997. tap, a *Drosophila* bHLH gene expressed in chemosensory organs. *Gene* 191, 15–21.
- Gay, N.J., Keith, F.J., 1990. Formation of a gradient of the *Drosophila* dorsal morphogen by differential nuclear localisation. *BioEssays* 12, 181–182.

- Gehring, W.J., 1993. Exploring the homeobox. *Gene* 135, 215–221.
- Ghysen, A., Richelle, J., 1979. Determination of sensory bristles and pattern formation in *Drosophila*. 2. The *achaete-scute* locus. *Devel. Biol.* 70, 438–452.
- Giangrande, A., 1994. Glia in the fly wing are clonally related to epithelial cells and use the nerve as a pathway for migration. *Development* 120, 523–534.
- Giangrande, A., 1996. Development and organization of glial cells in *Drosophila melanogaster*. *Int. J. Devel. Biol.* 40, 917–927.
- Giangrande, A., Murray, M.A., Palka, J., 1993. Development and organization of glial cells in the peripheral nervous system of *Drosophila melanogaster*. *Development* 117, 895–904.
- Giesen, K., Hummel, T., Stollewerk, A., Harrison, S., Travers, A., et al., 1997. Glial development in the *Drosophila* CNS requires concomitant activation of glial and repression of neuronal differentiation genes. *Development* 124, 2307–2316.
- Gomez-Skarmeta, J.L., Rodriguez, I., Martinez, C., Culi, J., Ferrer-Marco, D., et al., 1995. *Cis*-regulation of *achaete* and *scute*: shared *enhancer*-like elements drive their co-expression in proneural clusters of the imaginal discs. *Genes Devel.* 9, 1869–1882.
- Goodman, C.S., Spitzer, N.C., 1979. Embryonic development of identified neurones: differentiation from neuroblast to neurone. *Nature* 280, 208–214.
- Goodman, C.S., Bastiani, M.J., Doe, C.Q., du Lac, S., Helfand, S.L., et al., 1984. Cell recognition during neuronal development. *Science* 225, 1271–1279.
- Goodman, C.S., Doe, C.Q., 1993. Embryonic development of the *Drosophila* central nervous system. In: Bate, C.M., Martinez-Arias, A. (Eds.), *The Development of Drosophila*, vol. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1131–1206.
- Goriely, A., Dumont, N., Dambly-Chaudiere, C., Ghysen, A., 1991. The determination of sense organs in *Drosophila*: effect of the neurogenic mutations in the embryo. *Development* 113, 1395–1404.
- Govind, S., Steward, R., 1991. Dorsoventral pattern formation in *Drosophila*: signal transduction and nuclear targeting. *Trends Genet.* 7, 119–125.
- Granderath, S., Bunse, I., Klambt, C., 2000. *gcm* and *pointed* synergistically control glial transcription of the *Drosophila* gene *loco*. *Mech. Devel.* 91, 197–208.
- Granderath, S., Klambt, C., 1999. Glia development in the embryonic CNS of *Drosophila*. *Curr. Opin. Neurobiol.* 9, 531–536.
- Guichard, A., Biehs, B., Sturtevant, M.A., Wickline, L., Chacko, J., et al., 1999. Rhomboid and Star interact synergistically to promote EGFR/MAPK signaling during *Drosophila* wing vein development. *Development* 126, 2663–2676.
- Guo, M., Jan, L.Y., Jan, Y.N., 1996. Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. *Neuron* 17, 27–41.
- Gustavson, E., Goldsborough, A.S., Ali, Z., Kornberg, T.B., 1996. The *Drosophila engrailed* and *invected* genes: partners in regulation, expression and function. *Genetics* 142, 893–906.
- Halter, D.A., Urban, J., Rickert, C., Ner, S.S., Ito, K., et al., 1995. The homeobox gene *repo* is required for the differentiation and maintenance of glia function in the embryonic nervous system of *Drosophila melanogaster*. *Development* 121, 317–332.
- Hama, J., Weinstein, D.C., 2001. Is Chordin a morphogen? *BioEssays* 23, 121–124.
- Harris, R., Sabatelli, L.M., Seeger, M.A., 1996. Guidance cues at the *Drosophila* CNS midline: identification and characterization of two *Drosophila* Netrin/UNC-6 homologs. *Neuron* 17, 217–228.
- Harrison, S.D., Travers, A.A., 1990. The *tramtrack* gene encodes a *Drosophila* finger protein that interacts with the *ftz* transcriptional regulatory region and shows a novel embryonic expression pattern. *EMBO J.* 9, 207–216.
- Hartenstein, V., Nassif, C., Lekven, A., 1998. Embryonic development of the *Drosophila* brain. 2. Pattern of glial cells. *J. Comp. Neurol.* 402, 32–47.
- Hassan, B., Vaessin, H., 1996. Regulatory interactions during early neurogenesis in *Drosophila*. *Devel. Genet.* 18, 18–27.
- Hatten, M.E., 1999. Central nervous system neuronal migration. *Annu. Rev. Neurosci.* 22, 511–539.
- Hemmati-Brivanlou, A., Melton, D., 1997. Vertebrate embryonic cells will become nerve cells unless told otherwise. *Cell* 88, 13–17.
- Hidalgo, A., Kinrade, E.F., Georgiou, M., 2001. The *Drosophila* neuregulin *Vein* maintains glial survival during axon guidance in the CNS. *Devel. Cell* 1, 679–690.
- Hirata, J., Nakagoshi, H., Nabeshima, Y., Matsuzaki, F., 1995. Asymmetric segregation of the homeodomain protein Prospero during *Drosophila* development. *Nature* 377, 627–630.
- Holley, S.A., Jackson, P.D., Sasai, Y., Lu, B., De Robertis, E.M., et al., 1995. A conserved system for dorsal-ventral patterning in insects and vertebrates involving *sog* and *chordin*. *Nature* 376, 249–253.
- Honda, T., Tabata, H., Nakajima, K., 2003. Cellular and molecular mechanisms of neuronal migration in neocortical development. *Semin. Cell Devel. Biol.* 14, 169–174.
- Hosoya, T., Takizawa, K., Nitta, K., Hotta, Y., 1995. *glial cells missing*: a binary switch between neuronal and glial determination in *Drosophila*. *Cell* 82, 1025–1036.
- Huang, F., Dambly-Chaudière, C., Ghysen, A., 1994. Position-reading and the emergence of sense organ precursors in *Drosophila*. *Prog. Neurobiol.* 42, 293–297.
- Huang, J.D., Schwyster, D.H., Shirokawa, J.M., Courey, A.J., 1993. The interplay between multiple enhancer and silencer elements defines the pattern of *decapentaplegic* expression. *Genes Devel.* 7, 694–704.

- Huff, R., Furst, A., Mahowald, A.P., 1989. *Drosophila* embryonic neuroblasts in culture: autonomous differentiation of specific neurotransmitters. *Devel. Biol.* 134, 146–157.
- Hummel, T., Schimmelpfeng, K., Klambt, C., 1999a. Commissure formation in the embryonic CNS of *Drosophila*: II. Functions of the different midline cells. *Development* 126, 771–779.
- Hummel, T., Schimmelpfeng, K., Klambt, C., 1999b. Commissure formation in the embryonic CNS of *Drosophila*: I. Identification of the required gene functions. *Devel. Biol.* 209, 381–398.
- Ikeshima-Kataoka, H., Skeath, J.B., Nabeshima, Y., Doe, C.Q., Matsuzaki, F., 1997. Miranda directs Prospero to a daughter cell during *Drosophila* asymmetric divisions. *Nature* 390, 625–629.
- Ingham, P.W., Taylor, A.M., Nakano, Y., 1991. Role of the *Drosophila patched* gene in positional signaling. *Nature* 353, 184–187.
- Ip, Y.T., Kraut, R., Levine, M., Rushlow, C.A., 1991. The dorsal morphogen is a sequence-specific DNA-binding protein that interacts with a long-range repression element in *Drosophila*. *Cell* 64, 439–446.
- Ip, Y.T., Levine, M., Small, S.J., 1992. The bicoid and dorsal morphogens use a similar strategy to make stripes in the *Drosophila* embryo. *J. Cell Sci.* 16 (Suppl.), 33–38.
- Irish, V.F., Gelbart, W.M., 1987. The *decapentaplegic* gene is required for dorsal–ventral patterning of the *Drosophila* embryo. *Genes Devel.* 1, 868–879.
- Isshiki, T., Pearson, B., Holbrook, S., Doe, C.Q., 2001. *Drosophila* neuroblasts sequentially express transcription factors which specify the temporal identity of their neuronal progeny. *Cell* 106, 511–521.
- Isshiki, T., Takeichi, M., Nose, A., 1997. The role of the *msh* homeobox gene during *Drosophila* neurogenesis: implication for the dorsoventral specification of the neuroectoderm. *Development* 124, 3099–3109.
- Ito, K., Urban, J., Techanu, G.M., 1995. Distribution, classification, and development of *Drosophila* glial cells in the late embryonic and early larval ventral nerve cord. *Roux's Arch. Devel. Biol.* 204, 284–307.
- Jacobs, J.R., 2000. The midline glia of *Drosophila*: a molecular genetic model for the developmental functions of glia. *Prog. Neurobiol.* 62, 475–508.
- Jarman, A.P., Brand, M., Jan, L.Y., Jan, Y.N., 1993. The regulation and function of the helix–loop–helix gene, *asense*, in *Drosophila* neural precursors. *Development* 119, 19–29.
- Jarriault, S., Brou, C., Logeat, F., Schroeter, E.H., Kopan, R., et al., 1995. Signaling downstream of activated mammalian Notch. *Nature* 377, 355–358.
- Jia, X.X., Siegler, M.V., 2002. Midline lineages in grasshopper produce neuronal siblings with asymmetric expression of Engrailed. *Development* 129, 5181–5193.
- Jiang, J., Kosman, D., Ip, Y.T., Levine, M., 1991. The dorsal morphogen gradient regulates the mesoderm determinant *twist* in early *Drosophila* embryos. *Genes Devel.* 5, 1881–1891.
- Jimenez, F., Campos-Ortega, J.A., 1990. Defective neuroblast commitment in mutants of the *achaete–scute* complex and adjacent genes of *D. melanogaster*. *Neuron* 5, 81–89.
- Jin, M.H., Sawamoto, K., Ito, M., Okano, H., 2000. The interaction between the *Drosophila* secreted protein Argos and the epidermal growth factor receptor inhibits dimerization of the receptor and binding of secreted Spitz to the receptor. *Mol. Cell Biol.* 20, 2098–2107.
- Jones, B.W., Fetter, R.D., Tear, G., Goodman, C.S., 1995. *glial cells missing*: a genetic switch that controls glial versus neuronal fate. *Cell* 82, 1013–1023.
- Kageyama, R., Ishibashi, M., Takebayashi, K., Tomita, K., 1997. bHLH transcription factors and mammalian neuronal differentiation. *Int. J. Biochem. Cell Biol.* 29, 1389–1399.
- Kammerer, M., Giangrande, A., 2001. Glide2, a second glial promoting factor in *Drosophila melanogaster*. *EMBO J.* 20, 4664–4673.
- Kaprielian, Z., Runko, E., Imondi, R., 2001. Axon guidance at the midline choice point. *Devel. Dyn.* 221, 154–181.
- Kasai, Y., Nambu, J.R., Lieberman, P.M., Crews, S.T., 1992. Dorsal–ventral patterning in *Drosophila*: DNA binding of snail protein to the single-minded gene. *Proc. Natl Acad. Sci. USA* 89, 3414–3418.
- Kawamura, K., Carlson, J.G., 1962. Studies on cytokinesis in neuroblasts of the grasshopper, *Chortophaga viridifasciata* (De Geer). 3. Factors determining the location of the cleavage furrow. *Exp. Cell Res.* 26, 411–423.
- Keleman, K., Rajagopalan, S., Cleppien, D., Teis, D., Paiha, K., et al., 2002. Comm sorts Robo to control axon guidance at the *Drosophila* midline. *Cell* 110, 415–427.
- Kidd, S., Lieber, T., Young, M.W., 1998a. Ligand-induced cleavage and regulation of nuclear entry of Notch in *Drosophila melanogaster* embryos. *Genes Devel.* 12, 3728–3740.
- Kidd, T., Bland, K.S., Goodman, C.S., 1999. Slit is the midline repellent for the Robo receptor in *Drosophila*. *Cell* 96, 785–794.
- Kidd, T., Brose, K., Mitchell, K.J., Fetter, R.D., Tessier-Lavigne, M., et al., 1998c. Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. *Cell* 92, 205–215.
- Kidd, T., Russell, C., Goodman, C.S., Tear, G., 1998b. Dosage-sensitive and complementary functions of roundabout and commissureless control axon crossing of the CNS midline. *Neuron* 20, 25–33.
- Kim, P., Helms, A.W., Johnson, J.E., Zimmerman, K., 1997. XATH-1, a vertebrate homolog of *Drosophila* atonal, induces a neuronal differentiation within ectodermal progenitors. *Devel. Biol.* 187, 1–12.
- Klaes, A., Menne, T., Stollewerk, A., Scholz, H., Klambt, C., 1994. The *Ets* transcription factors encoded by the *Drosophila* gene pointed direct glial cell differentiation in the embryonic CNS. *Cell* 78, 149–160.

- Klambt, C., 1993. The *Drosophila* gene *pointed* encodes two ETS-like proteins which are involved in the development of the midline glial cells. *Development* 117, 163–176.
- Klambt, C., Goodman, C.S., 1991. The diversity and pattern of glia during axon pathway formation in the *Drosophila* embryo. *Glia* 4, 205–213.
- Knoblich, J.A., 2001. Asymmetric cell division during animal development. *Nat. Rev. Mol. Cell Biol.* 2, 11–20.
- Knoblich, J.A., Jan, L.Y., Jan, Y.N., 1995. Asymmetric segregation of Numb and Prospero during cell division. *Nature* 377, 624–627.
- Knust, E., 2001. G protein signaling and asymmetric cell division. *Cell* 107, 125–128.
- Knust, E., Bremer, K.A., Vassin, H., Ziemer, A., Tepass, U., et al., 1987a. The enhancer of *split* locus and neurogenesis in *Drosophila melanogaster*. *Devel. Biol.* 122, 262–273.
- Knust, E., Dietrich, U., Tepass, U., Bremer, K.A., Weigel, D., et al., 1987b. EGF homologous sequences encoded in the genome of *Drosophila melanogaster*, and their relation to neurogenic genes. *EMBO J.* 6, 761–766.
- Kolodziej, P.A., Timpe, L.C., Mitchell, K.J., Fried, S.R., Goodman, C.S., et al., 1996. *frazzled* encodes a *Drosophila* member of the DCC immunoglobulin subfamily and is required for CNS and motor axon guidance. *Cell* 87, 197–204.
- Kosman, D., Ip, Y.T., Levine, M., Arora, K., 1991. Establishment of the mesoderm–neuroectoderm boundary in the *Drosophila* embryo. *Science* 254, 118–122.
- Kraut, R., Campos-Ortega, J.A., 1996. *inscuteable*, a neural precursor gene of *Drosophila*, encodes a candidate for a cytoskeleton adaptor protein. *Devel. Biol.* 174, 65–81.
- Kraut, R., Chia, W., Jan, L.Y., Jan, Y.N., Knoblich, J.A., 1996. Role of *inscuteable* in orienting asymmetric cell divisions in *Drosophila*. *Nature* 383, 50–55.
- Kretschmar, D., Hasan, G., Sharma, S., Heisenberg, M., Benzer, S., 1997. The *swiss cheese* mutant causes glial hyperwrapping and brain degeneration in *Drosophila*. *J. Neurosci.* 17, 7425–7432.
- Krumlauf, R., Marshall, H., Studer, M., Nonchev, S., Sham, M.H., et al., 1993. Hox homeobox genes and regionalization of the nervous system. *J. Neurobiol.* 24, 1328–1340.
- Kuchinke, U., Grawe, F., Knust, E., 1998. Control of spindle orientation in *Drosophila* by the Par-3-related PDZ-domain protein Bazooka. *Curr. Biol.* 8, 1357–1365.
- Kunisch, M., Haenlin, M., Campos-Ortega, J.A., 1994. Lateral inhibition mediated by the *Drosophila* neurogenic gene *delta* is enhanced by proneural proteins. *Proc. Natl Acad. Sci. USA* 91, 10139–10143.
- Laming, P.R., Kimelberg, H., Robinson, S., Salm, A., Hawrylak, N., et al., 2000. Neuronal–glial interactions and behaviour. *Neurosci. Biobehav. Rev.* 24, 295–340.
- Lanoue, B.R., Jacobs, J.R., 1999. Rhomboid function in the midline of the *Drosophila* CNS. *Devel. Genet.* 25, 321–330.
- Lear, B.C., Skeath, J.B., Patel, N.H., 1999. Neural cell fate in *rca1* and *cycA* mutants: the roles of intrinsic and extrinsic factors in asymmetric division in the *Drosophila* central nervous system. *Mech. Devel.* 88, 207–219.
- Lecourtois, M., Schweisguth, F., 1998. Indirect evidence for Delta-dependent intracellular processing of notch in *Drosophila* embryos. *Curr. Biol.* 8, 771–774.
- Lee, J.E., 1997. Basic helix–loop–helix genes in neural development. *Curr. Opin. Neurobiol.* 7, 13–20.
- Lee, J.E., Hollenberg, S.M., Snider, L., Turner, D.L., Lipnick, N., et al., 1995. Conversion of *Xenopus* ectoderm into neurons by NeuroD, a basic helix–loop–helix protein. *Science* 268, 836–844.
- Lee, J.R., Urban, S., Garvey, C.F., Freeman, M., 2001. Regulated intracellular ligand transport and proteolysis control egf signal activation in *Drosophila*. *Cell* 107, 161–171.
- Lemke, G., 2001. Glial control of neuronal development. *Annu. Rev. Neurosci.* 24, 87–105.
- Leptin, M., 1991. *twist* and *snail* as positive and negative regulators during *Drosophila* mesoderm development. *Genes Devel.* 5, 1568–1576.
- Leptin, M., Roth, S., 1994. Autonomy and non-autonomy in *Drosophila* mesoderm determination and morphogenesis. *Development* 120, 853–859.
- Leyns, L., Gomez-Skarmeta, J.L., Dambly-Chaudière, C., 1996. *iroquois*: a prepattern gene that controls the formation of bristles on the thorax of *Drosophila*. *Mech. Devel.* 59, 63–72.
- Li, P., Yang, X., Wasser, M., Cai, Y., Chia, W., 1997. *inscuteable* and *Staufen* mediate asymmetric localization and segregation of prospero RNA during *Drosophila* neuroblast cell divisions. *Cell* 90, 437–447.
- Livesey, F.J., 1999. Netrins and netrin receptors. *Cell. Mol. Life Sci.* 56, 62–68.
- Livneh, E., Glazer, L., Segal, D., Schlessinger, J., Shilo, B.Z., 1985. The *Drosophila* EGF receptor gene homolog: conservation of both hormone binding and kinase domains. *Cell* 40, 599–607.
- Lu, B., Rothenberg, M., Jan, L.Y., Jan, Y.N., 1998. Partner of Numb colocalizes with Numb during mitosis and directs Numb asymmetric localization in *Drosophila* neural and muscle progenitors. *Cell* 95, 225–235.
- Martin-Bermudo, M.D., Carmena, A., Jimenez, F., 1995. Neurogenic genes control gene expression at the transcriptional level in early neurogenesis and in mesectoderm specification. *Development* 121, 219–224.
- Martin-Bermudo, M.D., Martinez, C., Rodriguez, A., Jimenez, F., 1991. Distribution and function of the lethal of *scute* gene product during early neurogenesis in *Drosophila*. *Development* 113, 445–454.
- Martinez, C., Modolell, J., 1991. Cross-regulatory interactions between the proneural *achaete* and *scute* genes of *Drosophila*. *Science* 251, 1485–1487.
- Matsuzaki, F., 2000. Asymmetric division of *Drosophila* neural stem cells: a basis for neural diversity. *Curr. Opin. Neurobiol.* 10, 38–44.

- McConnell, S.K., 1995. Strategies for the generation of neuronal diversity in the developing central nervous system. *J. Neurosci.* 15, 6987–6998.
- McDonald, J.A., Holbrook, S., Isshiki, T., Weiss, J., Doe, C.Q., et al., 1998. Dorsal-ventral patterning in the *Drosophila* central nervous system: the *vnd* homeobox gene specifies ventral column identity. *Genes Devel.* 12, 3603–3612.
- McGinnis, W., Krumlauf, R., 1992. Homeobox genes and axial patterning. *Cell* 68, 283–302.
- Mehler, M.F., Mabie, P.C., Zhang, D., Kessler, J.A., 1997. Bone morphogenetic proteins in the nervous system. *Trends Neurosci.* 20, 309–317.
- Mellerick, D.M., Nirenberg, M., 1995. Dorsal-ventral patterning genes restrict NK-2 homeobox gene expression to the ventral half of the central nervous system of *Drosophila* embryos. *Devel. Biol.* 171, 306–316.
- Menne, T.V., Klambt, C., 1994. The formation of commissures in the *Drosophila* CNS depends on the midline cells and on the Notch gene. *Development* 120, 123–133.
- Miller, A.A., Bernardoni, R., Giangrande, A., 1998. Positive autoregulation of the glial promoting factor *glide/gcm*. *EMBO J.* 17, 6316–6326.
- Miller, A.A., Bernardoni, R., Hindelang, C., Kammerer, M., Sorrentino, S., et al., 1999. Role and mechanism of action of glial cell deficient/glial cell missing (*glide/gcm*), the fly glial promoting factor. *Adv. Exp. Med. Biol.* 468, 33–46.
- Mitchell, K.J., Doyle, J.L., Serafini, T., Kennedy, T.E., Tessier-Lavigne, M., et al., 1996. Genetic analysis of Netrin genes in *Drosophila*: Netrins guide CNS commissural axons and peripheral motor axons. *Neuron* 17, 203–215.
- Modolell, J., Campuzano, S., 1998. The *achaete-scute* complex as an integrating device. *Int. J. Dev. Biol.* 42, 275–282.
- Mohler, J., Vani, K., 1992. Molecular organization and embryonic expression of the hedgehog gene involved in cell-cell communication in segmental patterning of *Drosophila*. *Development* 115, 957–971.
- Moore, A.W., Barbel, S., Jan, L.Y., Jan, Y.N., 2000. A genomewide survey of basic helix-loop-helix factors in *Drosophila*. *Proc. Natl Acad. Sci. USA* 97, 10436–10441.
- Morel, V., Schweisguth, F., 2000. Repression by suppressor of hairless and activation by Notch are required to define a single row of single-minded expressing cells in the *Drosophila* embryo. *Genes Devel.* 14, 377–388.
- Muller, H.A., Wieschaus, E., 1996. *armadillo*, *bazooka*, and *stardust* are critical for early stages in formation of the zonula adherens and maintenance of the polarized blastoderm epithelium in *Drosophila*. *J. Cell Biol.* 134, 149–163.
- Myat, A., Henry, P., McCabe, V., Flintoft, L., Rotin, D., et al., 2002. *Drosophila* Nedd4, a ubiquitin ligase, is recruited by Commissureless to control cell surface levels of the roundabout receptor. *Neuron* 35, 447–459.
- Nambu, J.R., Franks, R.G., Hu, S., Crews, S.T., 1990. The *single-minded* gene of *Drosophila* is required for the expression of genes important for the development of CNS midline cells. *Cell* 63, 63–75.
- Nambu, J.R., Lewis, J.O., Wharton, K.A., Jr., Crews, S.T., 1991. The *Drosophila single-minded* gene encodes a helix-loop-helix protein that acts as a master regulator of CNS midline development. *Cell* 67, 1157–1167.
- Nusslein-Volhard, C., Wieschaus, E., 1980. Mutations affecting segment number and polarity in *Drosophila*. *Nature* 287, 795–801.
- Offermanns, S., 2003. G-proteins as transducers in transmembrane signaling. *Prog. Biophys. Mol. Biol.* 83, 101–130.
- Ono, K., Yasui, Y., Rutishauser, U., Miller, R.H., 1997. Focal ventricular origin and migration of oligodendrocyte precursors into the chick optic nerve. *Neuron* 19, 283–292.
- Park, Y., Fujioka, M., Kobayashi, M., Jaynes, J.B., Datta, S., 2001. *even skipped* is required to produce a trans-acting signal for larval neuroblast proliferation that can be mimicked by ecdysone. *Development* 128, 1899–1909.
- Parras, C., Garcia-Alonso, L.A., Rodriguez, I., Jimenez, F., 1996. Control of neural precursor specification by proneural proteins in the CNS of *Drosophila*. *EMBO J.* 15, 6394–6399.
- Peterson, C., Carney, G.E., Taylor, B.J., White, K., 2002. *reaper* is required for neuroblast apoptosis during *Drosophila* development. *Development* 129, 1467–1476.
- Petronczki, M., Knoblich, J.A., 2001. DmPAR-6 directs epithelial polarity and asymmetric cell division of neuroblasts in *Drosophila*. *Nat. Cell Biol.* 3, 43–49.
- Peyrefitte, S., Kahn, D., Haenlin, M., 2001. New members of the *Drosophila* Myc transcription factor subfamily revealed by a genome-wide examination for basic helix-loop-helix genes. *Mech. Devel.* 104, 99–104.
- Pickup, A.T., Banerjee, U., 1999. The role of *star* in the production of an activated ligand for the EGF receptor signaling pathway. *Devel. Biol.* 205, 254–259.
- Prokop, A., Technau, G.M., 1994. Early tagma-specific commitment of *Drosophila* CNS progenitor NB1–1. *Development* 120, 2567–2578.
- Ragone, G., Bernardoni, R., Giangrande, A., 2001. A novel mode of asymmetric division identifies the fly neuroglioblast 6–4t. *Devel. Biol.* 235, 74–85.
- Ragone, G., Van De Bor, V., Sorrentino, S., Kammerer, M., Galy, A., et al., 2003. Transcriptional regulation of glial cell specification. *Devel. Biol.* 255, 138–150.
- Rajagopalan, S., Vivancos, V., Nicolas, E., Dickson, B.J., 2000. Selecting a longitudinal pathway: Robo receptors specify the lateral position of axons in the *Drosophila* CNS. *Cell* 103, 1033–1045.
- Ramain, P., Heitzler, P., Haenlin, M., Simpson, P., 1993. *pannier*, a negative regulator of *achaete* and *scute* in *Drosophila*, encodes a zinc finger protein with

- homology to the vertebrate transcription factor GATA-1. *Development* 119, 1277–1291.
- Rangarajan, R., Gong, Q., Gaul, U., 1999. Migration and function of glia in the developing *Drosophila* eye. *Development* 126, 3285–3292.
- Rao, Y., Jan, L.Y., Jan, Y.N., 1990. Similarity of the product of the *Drosophila* neurogenic gene *big brain* to transmembrane channel proteins. *Nature* 345, 163–167.
- Rao, Y., Vaessin, H., Jan, L.Y., Jan, Y.N., 1991. Neuroectoderm in *Drosophila* embryos is dependent on the mesoderm for positioning but not for formation. *Genes Devel.* 5, 1577–1588.
- Raper, J.A., Bastiani, M.J., Goodman, C.S., 1984. Pathfinding by neuronal growth cones in grasshopper embryos. 4. The effects of ablating the A and P axons upon the behavior of the G growth cone. *J. Neurosci.* 4, 2329–2345.
- Ray, R.P., Arora, K., Nusslein-Volhard, C., Gelbart, W.M., 1991. The control of cell fate along the dorsal-ventral axis of the *Drosophila* embryo. *Development* 113, 35–54.
- Rhyu, M.S., Jan, L.Y., Jan, Y.N., 1994. Asymmetric distribution of Numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. *Cell* 76, 477–491.
- Rodriguez, I., Hernandez, R., Modolell, J., Ruiz-Gomez, M., 1990. Competence to develop sensory organs is temporally and spatially regulated in *Drosophila* epidermal primordia. *EMBO J.* 9, 3583–3592.
- Romani, S., Campuzano, S., Macagno, E.R., Modolell, J., 1989. Expression of *achaete* and *scute* genes in *Drosophila* imaginal discs and their function in sensory organ development. *Genes Devel.* 3, 997–1007.
- Rooke, J.E., Xu, T., 1998. Positive and negative signals between interacting cells for establishing neural fate. *BioEssays* 20, 209–214.
- Roth, S., Stein, D., Nusslein-Volhard, C., 1989. A gradient of nuclear localization of the Dorsal protein determines dorsoventral pattern in the *Drosophila* embryo. *Cell* 59, 1189–1202.
- Rothberg, J.M., Hartley, D.A., Walther, Z., Artavanis-Tsakonas, S., 1988. *slit*: an EGF-homologous locus of *D. melanogaster* involved in the development of the embryonic central nervous system. *Cell* 55, 1047–1059.
- Rothberg, J.M., Jacobs, J.R., Goodman, C.S., Artavanis-Tsakonas, S., 1990. *Slit*: an extracellular protein necessary for development of midline glia and commissural axon pathways contains both EGF and LRR domains. *Genes Devel.* 4, 2169–2187.
- Rusch, J., Levine, M., 1996. Threshold responses to the dorsal regulatory gradient and the subdivision of primary tissue territories in the *Drosophila* embryo. *Curr. Opin. Genet. Devel.* 6, 416–423.
- Rutledge, B.J., Zhang, K., Bier, E., Jan, Y.N., Perrimon, N., 1992. The *Drosophila* *spitz* gene encodes a putative EGF-like growth factor involved in dorsal-ventral axis formation and neurogenesis. *Genes Devel.* 6, 1503–1517.
- Salzberg, A., Bellen, H.J., 1996. Invertebrate versus vertebrate neurogenesis: variations on the same theme? *Devel. Genet.* 18, 1–10.
- Sanchez, D., Ganformina, M.D., Bastiani, M.J., 1995. Contributions of an orthopteran to the understanding of neuronal pathfinding. *Immunol. Cell Biol.* 73, 565–574.
- Sasai, Y., Lu, B., Steinbeisser, H., De Robertis, E.M., 1995. Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in *Xenopus*. *Nature* 376, 333–336.
- Sasamura, T., Sasaki, N., Miyashita, F., Nakao, S., Ishikawa, H.O., et al., 2003. *neurotic*, a novel maternal neurogenic gene, encodes an O-fucosyltransferase that is essential for Notch-Delta interactions. *Development* 130, 4785–4795.
- Schaefer, M., Petronczki, M., Dorner, D., Forte, M., Knoblich, J.A., 2001. Heterotrimeric G proteins direct two modes of asymmetric cell division in the *Drosophila* nervous system. *Cell* 107, 183–194.
- Schaefer, M., Shevchenko, A., Knoblich, J.A., 2000. A protein complex containing Inscuteable and the G α -binding protein Pins orients asymmetric cell divisions in *Drosophila*. *Curr. Biol.* 10, 353–362.
- Schmidt, A., Chiba, A., Doe, C.Q., 1999. Clonal analysis of *Drosophila* embryonic neuroblasts: neural cell types, axon projections and muscle targets. *Development* 126, 4653–4689.
- Schmidt, H., Rickert, C., Bossing, T., Vef, O., Urban, J., et al., 1997. The embryonic central nervous system lineages of *Drosophila melanogaster*. 2. Neuroblast lineages derived from the dorsal part of the neuroectoderm. *Devel. Biol.* 189, 186–204.
- Schober, M., Schaefer, M., Knoblich, J.A., 1999. Bazooka recruits Inscuteable to orient asymmetric cell divisions in *Drosophila* neuroblasts. *Nature* 402, 548–551.
- Scholz, H., Sadlowski, E., Klaes, A., Klambt, C., 1997. Control of midline glia development in the embryonic *Drosophila* CNS. *Mech. Devel.* 62, 79–91 (Corrected and republished in *Mech. Devel.* (1997) 64, 137–51).
- Schreiber, J., Sock, E., Wegner, M., 1997. The regulator of early gliogenesis glial cells missing is a transcription factor with a novel type of DNA-binding domain. *Proc. Natl Acad. Sci. USA* 94, 4739–4744.
- Schroeter, E.H., Kisslinger, J.A., Kopan, R., 1998. Notch-1 signaling requires ligand-induced proteolytic release of intracellular domain. *Nature* 393, 382–386.
- Schweitzer, R., Howes, R., Smith, R., Shilo, B.Z., Freeman, M., 1995a. Inhibition of *Drosophila* EGF receptor activation by the secreted protein Argos. *Nature* 376, 699–702.
- Schweitzer, R., Shaharabany, M., Seger, R., Shilo, B.Z., 1995b. Secreted Spitz triggers the DER signaling pathway and is a limiting component in embryonic ventral ectoderm determination. *Genes Devel.* 9, 1518–1529.
- Seeger, M., Tear, G., Ferres-Marco, D., Goodman, C.S., 1993. Mutations affecting growth cone guidance in

- Drosophila*: genes necessary for guidance toward or away from the midline. *Neuron* 10, 409–426.
- Segal, M., Bloom, K., 2001. Control of spindle polarity and orientation in *Saccharomyces cerevisiae*. *Trends Cell Biol.* 11, 160–166.
- Sepp, K.J., Auld, V.J., 2003. RhoA and Rac1 GTPases mediate the dynamic rearrangement of actin in peripheral glia. *Development* 130, 1825–1835.
- Sepp, K.J., Schulte, J., Auld, V.J., 2000. Developmental dynamics of peripheral glia in *Drosophila melanogaster*. *Glia* 30, 122–133.
- Shannon, M.P., 1972. Characterization of the female-sterile mutant *almondex* of *Drosophila melanogaster*. *Genetica* 43, 244–256.
- Shen, C.P., Jan, L.Y., Jan, Y.N., 1997. Miranda is required for the asymmetric localization of Prospero during mitosis in *Drosophila*. *Cell* 90, 449–458.
- Shimell, M.J., Ferguson, E.L., Childs, S.R., O'Connor, M.B., 1991. The *Drosophila* dorsal–ventral patterning gene *tolloid* is related to human bone morphogenetic protein 1. *Cell* 67, 469–481.
- Simpson, J.H., Kidd, T., Bland, K.S., Goodman, C.S., 2000. Short-range and long-range guidance by *slit* and its Robo receptors Robo and Robo2 play distinct roles in midline guidance. *Neuron* 28, 753–766.
- Skaer, N., Pistillo, D., Gibert, J.M., Lio, P., Wulbeck, C., *et al.*, 2002. Gene duplication at the *achaete-scute* complex and morphological complexity of the peripheral nervous system in Diptera. *Trends Genet.* 18, 399–405.
- Skeath, J.B., 1998. The *Drosophila* EGF receptor controls the formation and specification of neuroblasts along the dorsal–ventral axis of the *Drosophila* embryo. *Development* 125, 3301–3312.
- Skeath, J.B., 1999. At the nexus between pattern formation and cell-type specification: the generation of individual neuroblast fates in the *Drosophila* embryonic central nervous system. *BioEssays* 21, 922–931.
- Skeath, J.B., Carroll, S.B., 1991. Regulation of *achaete-scute* gene expression and sensory organ pattern formation in the *Drosophila* wing. *Genes Devel.* 5, 984–995.
- Skeath, J.B., Carroll, S.B., 1992. Regulation of proneural gene expression and cell fate during neuroblast segregation in the *Drosophila* embryo. *Development* 114, 939–946.
- Skeath, J.B., Carroll, S.B., 1994. The *achaete-scute* complex: generation of cellular pattern and fate within the *Drosophila* nervous system. *FASEB J.* 8, 714–721.
- Skeath, J.B., Doe, C.Q., 1996. The *achaete-scute* complex proneural genes contribute to neural precursor specification in the *Drosophila* CNS. *Curr. Biol.* 6, 1146–1152.
- Skeath, J.B., Panganiban, G.F., Carroll, S.B., 1994. The ventral nervous system defective gene controls proneural gene expression at two distinct steps during neuroblast formation in *Drosophila*. *Development* 120, 1517–1524.
- Skeath, J.B., Panganiban, G., Selegue, J., Carroll, S.B., 1992. Gene regulation in two dimensions: the proneural *achaete* and *scute* genes are controlled by combinations of axis-patterning genes through a common intergenic control region. *Genes Devel.* 6, 2606–2619.
- Skeath, J.B., Thor, S., 2003. Genetic control of *Drosophila* nerve cord development. *Curr. Opin. Neurobiol.* 13, 8–15.
- Sonnenfeld, M.J., Jacobs, J.R., 1994. Mesectodermal cell fate analysis in *Drosophila* midline mutants. *Mech. Devel.* 46, 3–13.
- Sonnenfeld, M.J., Jacobs, J.R., 1995. Apoptosis of the midline glia during *Drosophila* embryogenesis: a correlation with axon contact. *Development* 121, 569–578.
- Sonnenfeld, M., Ward, M., Nystrom, G., Mosher, J., Stahl, S., *et al.*, 1997. The *Drosophila tango* gene encodes a bHLH-PAS protein that is orthologous to mammalian *Arnt* and controls CNS midline and tracheal development. *Development* 124, 4571–4582.
- Spana, E.P., Doe, C.Q., 1995. The Prospero transcription factor is asymmetrically localized to the cell cortex during neuroblast mitosis in *Drosophila*. *Development* 121, 3187–3195.
- St Johnston, R.D., Gelbart, W.M., 1987. Decapentaplegic transcripts are localized along the dorsal–ventral axis of the *Drosophila* embryo. *EMBO J.* 6, 2785–2791.
- Stathopoulos, A., Levine, M., 2002. Dorsal gradient networks in the *Drosophila* embryo. *Devel. Biol.* 246, 57–67.
- Stemerdink, C., Jacobs, J.R., 1997. Argos and Spitz group genes function to regulate midline glial cell number in *Drosophila* embryos. *Development* 124, 3787–3796.
- Stent, G.S., Weisblat, D.A., 1985. Cell lineage in the development of invertebrate nervous systems. *Annu. Rev. Neurosci.* 8, 45–70.
- Steward, R., 1987. *dorsal*, an embryonic polarity gene in *Drosophila*, is homologous to the vertebrate proto-oncogene, *c-rel*. *Science* 238, 692–694.
- Steward, R., 1989. Relocalization of the dorsal protein from the cytoplasm to the nucleus correlates with its function. *Cell* 59, 1179–1188.
- Struhl, G., Adachi, A., 1998. Nuclear access and action of notch *in vivo*. *Cell* 93, 649–660.
- Sturtevant, M.A., Roark, M., Bier, E., 1993. The *Drosophila rhomboid* gene mediates the localized formation of wing veins and interacts genetically with components of the EGF-R signaling pathway. *Genes Devel.* 7, 961–973.
- Sugimoto, Y., Taniguchi, M., Yagi, T., Akagi, Y., Nojyo, Y., *et al.*, 2001. Guidance of glial precursor cell migration by secreted cues in the developing optic nerve. *Development* 128, 3321–3330.
- Taghert, P.H., Goodman, C.S., 1984. Cell determination and differentiation of identified serotonin-immunoreactive neurons in the grasshopper embryo. *J. Neurosci.* 4, 989–1000.
- Tata, F., Hartley, D.A., 1995. Inhibition of cell fate in *Drosophila* by *Enhancer of split* genes. *Mech. Devel.* 51, 305–315.
- Tear, G., 1999. Axon guidance at the central nervous system midline. *Cell. Mol. Life Sci.* 55, 1365–1376.

- Tear, G., Harris, R., Sutaria, S., Kilomanski, K., Goodman, C.S., *et al.*, 1996. *commisureless* controls growth cone guidance across the CNS midline in *Drosophila* and encodes a novel membrane protein. *Neuron* 16, 501–514.
- Thomas, J.B., Crews, S.T., Goodman, C.S., 1988. Molecular genetics of the *single-minded* locus: a gene involved in the development of the *Drosophila* nervous system. *Cell* 52, 133–141.
- Tio, M., Zavortink, M., Yang, X., Chia, W., 1999. A functional analysis of *inscuteable* and its roles during *Drosophila* asymmetric cell divisions. *J. Cell Sci.* 112, 1541–1551.
- Truman, J.W., Bate, M., 1988. Spatial and temporal patterns of neurogenesis in the central nervous system of *Drosophila melanogaster*. *Devel. Biol.* 125, 145–157.
- Truman, J.W., Thorn, R.S., Robinow, S., 1992. Programmed neuronal death in insect development. *J. Neurobiol.* 23, 1295–1311.
- Truman, J.W., Taylor, B., Awad, A.T., 1993. Formation of the adult nervous system. In: Bate, C.M., Martinez-Arias, A. (Eds.), *The Development of Drosophila melanogaster*, vol. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1245–1277.
- Tsai, H.H., Miller, R.H., 2002. Glial cell migration directed by axon guidance cues. *Trends Neurosci.* 25, 173–176.
- Udolph, G., Luer, K., Bossing, T., Technau, G.M., 1995. Commitment of CNS progenitors along the dorsoventral axis of *Drosophila* neuroectoderm. *Science* 269, 1278–1281.
- Udolph, G., Prokop, A., Bossing, T., Technau, G.M., 1993. A common precursor for glia and neurons in the embryonic CNS of *Drosophila* gives rise to segment-specific lineage variants. *Development* 118, 765–775.
- Udolph, G., Rath, P., Chia, W., 2001. A requirement for Notch in the genesis of a subset of glial cells in the *Drosophila* embryonic central nervous system which arise through asymmetric divisions. *Development* 128, 1457–1466.
- Uemura, T., Shepherd, S., Ackerman, L., Jan, L.Y., Jan, Y.N., 1989. *numb*, a gene required in determination of cell fate during sensory organ formation in *Drosophila* embryos. *Cell* 58, 349–360.
- Umesono, Y., Hiromi, Y., Hotta, Y., 2002. Context-dependent utilization of Notch activity in *Drosophila* glial determination. *Development* 129, 2391–2399.
- Urban, S., Lee, J.R., Freeman, M., 2001. *Drosophila* rhomboid-1 defines a family of putative intramembrane serine proteases. *Cell* 107, 173–182.
- Vaessin, H., Grell, E., Wolff, E., Bier, E., Jan, L.Y., *et al.*, 1991. *prospero* is expressed in neuronal precursors and encodes a nuclear protein that is involved in the control of axonal outgrowth in *Drosophila*. *Cell* 67, 941–953.
- Van De Bor, V., Giangrande, A., 2001. Notch signaling represses the glial fate in fly PNS. *Development* 128, 1381–1390.
- Van De Bor, V., Giangrande, A., 2002. *glide/gcm*: at the crossroads between neurons and glia. *Curr. Opin. Genet. Devel.* 12, 465–472.
- Van De Bor, V., Walther, R., Giangrande, A., 2000. Some fly sensory organs are gliogenic and require *glide/gcm* in a precursor that divides symmetrically and produces glial cells. *Development* 127, 3735–3743.
- Vincent, S., Vonesch, J.L., Giangrande, A., 1996. *Glide* directs glial fate commitment and cell fate switch between neurones and glia. *Development* 122, 131–139.
- Voigt, A., Pflanz, R., Schafer, U., Jackle, H., 2002. Perlecan participates in proliferation activation of quiescent *Drosophila* neuroblasts. *Devel. Dynam.* 224, 403–412.
- Ward, M.P., Mosher, J.T., Crews, S.T., 1998. Regulation of bHLH-PAS protein subcellular localization during *Drosophila* embryogenesis. *Development* 125, 1599–1608.
- Weigmann, K., Lehner, C.F., 1995. Cell fate specification by even-skipped expression in the *Drosophila* nervous system is coupled to cell cycle progression. *Development* 121, 3713–3721.
- Wegner, M., Riethmacher, D., 2001. Chronicles of a switch hunt: *gcm* genes in development. *Trends Genet.* 17, 286–290.
- Weiss, J.B., Von Ohlen, T., Mellerick, D.M., Dressler, G., Doe, C.Q., *et al.*, 1998. Dorsoventral patterning in the *Drosophila* central nervous system: the intermediate neuroblasts defective homeobox gene specifies intermediate column identity. *Genes Devel.* 12, 3591–3602.
- Wheeler, S.R., Carrico, M.L., Wilson, B.A., Brown, S.J., Skeath, J.B., 2003. The expression and function of the *achaete-scute* genes in *Tribolium castaneum* reveals conservation and variation in neural pattern formation and cell fate specification. *Development* 130, 4373–4381.
- Wheeler, W.M., 1891. Neuroblasts in the arthropod embryo. *J. Morphol.* 4, 337–343.
- Wodarz, A., Ramrath, A., Kuchinke, U., Knust, E., 1999. Bazooka provides an apical cue for *Inscuteable* localization in *Drosophila* neuroblasts. *Nature* 402, 544–547.
- Wodarz, A., Ramrath, A., Grimm, A., Knust, E., 2000. *Drosophila* atypical protein kinase C associates with Bazooka and controls polarity of epithelia and neuroblasts. *J. Cell Biol.* 150, 1361–1374.
- Wulbeck, C., Simpson, P., 2000. Expression of *achaete-scute* homologues in discrete proneural clusters on the developing notum of the medfly *Ceratitis capitata*, suggests a common origin for the stereotyped bristle patterns of higher Diptera. *Development* 127, 1411–1420.
- Wulbeck, C., Simpson, P., 2002. The expression of *pannier* and *achaete-scute* homologues in a mosquito suggests an ancient role of *pannier* as a selector gene in the regulation of the dorsal body pattern. *Development* 129, 3861–3871.
- Xiong, W.C., Montell, C., 1995. Defective glia induce neuronal apoptosis in the *repo* visual system of *Drosophila*. *Neuron* 14, 581–590.
- Xiong, W.C., Okano, H., Patel, N.H., Blendy, J.A., Montell, C., 1994. *repo* encodes a glial-specific homeo domain protein required in the *Drosophila* nervous system. *Genes Devel.* 8, 981–994.
- Yagi, Y., Suzuki, T., Hayashi, S., 1998. Interaction between *Drosophila* EGF receptor and *vnd* determines

- three dorsoventral domains of the neuroectoderm. *Development* 125, 3625–3633.
- Yedvobnick, B., Smoller, D., Young, P., Mills, D., 1988. Molecular analysis of the neurogenic locus mastermind of *Drosophila melanogaster*. *Genetics* 118, 483–497.
- Yu, F., Morin, X., Cai, Y., Yang, X., Chia, W., 2000. Analysis of partner of *inscuteable*, a novel player of *Drosophila* asymmetric divisions, reveals two distinct steps in *inscuteable* apical localization. *Cell* 100, 399–409.
- Yu, Y.A., Oberg, K., Wang, G., Szalay, A.A., 2003. Visualization of molecular and cellular events with green fluorescent proteins in developing embryos: a review. *Luminescence* 18, 1–18.
- Yuasa, Y., Okabe, M., Yoshikawa, S., Tabuchi, K., Xiong, W.C., *et al.*, 2003. *Drosophila* homeodomain protein Repo controls glial differentiation by cooperating with ETS and BTB transcription factors. *Development* 130, 2419–2428.
- Zak, N.B., Wides, R.J., Schejter, E.D., Raz, E., Shilo, B.Z., 1990. Localization of the DER/flb protein in embryos: implications on the faint little ball lethal phenotype. *Development* 109, 865–874.
- Zinn, K., Condron, B.G., 1994. Cell fate decisions in the grasshopper central nervous system. *Curr. Opin. Cell Biol.* 6, 783–787.
- Zhong, W., 2003. Diversifying neural cells through order of birth and asymmetry of division. *Neuron* 37, 11–14.
- Zhou, L., Schnitzler, A., Agapite, J., Schwartz, L.M., Steller, H., *et al.*, 1997a. Cooperative functions of the reaper and head involution defective genes in the programmed cell death of *Drosophila* central nervous system midline cells. *Proc. Natl Acad. Sci. USA* 94, 5131–5136.
- Zhou, L., Xiao, H., Nambu, J.R., 1997b. CNS midline to mesoderm signaling in *Drosophila*. *Mech. Devel.* 67, 59–68.

4 Hormonal Control of the Form and Function of the Nervous System

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4.1. Introduction

Nervous systems are notable for the diversity of cell types they contain and for the specificity of connections made between these cells. This complexity arises from a diversity of local signaling systems beginning with those that select cells to become the stem cells that generate neurons and glia, progressing through a blend of short-range and long-range attraction and repulsion factors involved in axon guidance (e.g., Tessier-Lavigne and Goodman, 1996; Chapter 3), and ending with systems that mediate target recognition and synapse stability (see Chapter 3). Besides this plethora of local signaling systems, the nervous system also makes use of “global” cues provided by the endocrine system. At first sight, the widespread presence of a hormone in the blood would seem to be unsuitable for the individualized responses that are the hallmark of central nervous system (CNS) development. Hormones, though, have essential roles in coordinating development across the organism

and in linking environmental information with developmental programs.

The most obvious role of hormones is associated with their wide distribution within the organism, which enables them to maintain developmental coordination between distant regions of the CNS and between the CNS and the periphery. In establishing this coordination, they often serve as “gatekeepers” via hormone-sensitive mechanisms that control the ability of neurons to transition from one phase of development to the next. In this regard, hormones provide us with important experimental tools to identify key transition points in neuronal development, and lead us to the switch genes that oversee these transitions. Global signaling, though, can come into conflict with the need for individualized timing differences in the nervous systems so that neural circuits can be assembled in their appropriate sequence. The diversification of hormone receptors and temporal regulation of expression of receptor isoforms provides a mechanism by

which individual cellular responses can be adjusted in time despite the global landscape of hormone titers.

Attempt is made to take a comparative and evolutionary perspective in writing this review. Most of the complex hormone–neuron interactions that are studied in insects are in the context of metamorphosis in the holometabolous orders. In ancestral forms, though, most of these processes occur during embryogenesis and may, or may not, have been under hormonal control. In this regard, the actions of hormones in embryos of both basal and derived insects need to be better understood before we can understand how ancestral embryonic systems were modified to produce the larval stage, whether hormones had a role in these modifications, and how the endocrine system “captured” these developmental pathways and put them under hormonal control. Consequently, the author has started with the embryo in the discussion of these systems, and has tried to highlight where we have serious gaps in knowledge.

Because of limitations in length, the scope of this review has been narrowed to systems for which some knowledge of the cellular or molecular changes is available, and the review is confined to hormonal effects that fall within the developmental realm. Other issues such as the regulation of the complex behavioral tasks at ecdysis and the complex polyphenisms shown by social insects have been covered in detail in this volume. Also, this treatment has an extreme “neurocentric” focus. Glial cells have a crucial role in development of the CNS, but they have largely been omitted from this review. An excellent review of the role of glial cells in neuronal development has been written recently by Oland and Tolbert (2003; see also Chapter 3).

4.2. Patterns of Development of the Insect Nervous System

Although insects vary in their early embryonic development in terms of how body segments arise (i.e., short versus long germ-band insects), they are remarkably similar by the time they start to build their CNS at the extended germ band stage (e.g., Thomas *et al.*, 1984; see Chapter 3). Studies on grasshopper embryos provided initial insights into how the nervous system is constructed. The cellular composition of the central ganglia is determined by an interplay between neurogenesis and programmed cell death (see Chapter 5). The neurons arise from large neuronal stem cells, the neuroblasts (NBs), which enlarge and move inward from the ventral

neuroectoderm. As seen in Figure 1, each NB undergoes repeated asymmetric divisions, producing a series of ganglion mother cells (GMCs), each of which then divides once to produce two daughter neurons. Although the existence of NBs and their pattern of division had been known for over 100 years, a milestone in the field was the publication by Bate (1976a) showing that NBs are arranged in stereotyped arrays that were invariant from individual to individual, and that each NB could be identified uniquely based on its position in this array (see Chapter 3). The NB arrays are almost identical from segment to segment but those in the thorax typically generate more neurons than their abdominal counterparts (Shepherd and Bate, 1990). The NBs were first identified in terms of position alone but later studies in *Drosophila* showed that each NB expresses a unique combination of molecular markers (Doe, 1992) and the same NB typically shows similar markers in diverse species (Broadus *et al.*, 1995).

Two aspects of neurogenesis are important for comparative studies of the CNS. First, the types of neurons arising from a particular NB are unique to that stem cell, and the destruction of the stem cell results in the failure of these neurons to appear (e.g., Taghert *et al.*, 1984; Chapter 3). Therefore, the neurons in a hemiganlion arise in 31 autonomous clones, each being a product of a single NB; Chapter 3. Second, the arrays of NBs have been extremely stable through evolution. The set found in embryos of very basal insects, such as silverfish (Truman and Ball, 1998), is identical to that in grasshoppers and is very similar to that found

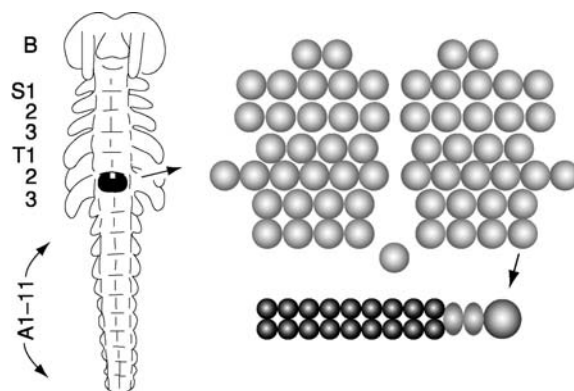


Figure 1 Drawing of a grasshopper embryo showing the segmental arrangement of the neuroblasts (NB) that produce the neurons of the CNS. Asymmetrical divisions by the NB produces a sequence of ganglion mother cells (GMC), each of which then divides to produce two daughter neurons.

in advanced insects such as *Drosophila* (Doe, 1992). Also, a given NB appears to produce similar types of neurons in diverse groups of insects (Thomas *et al.*, 1984; Witten and Truman, 1998). This conservation allows a level of comparative analysis that is unrivaled by any other cells or tissues in insects. Most work on neuronal lineages has dealt with the relatively simple segmental ganglia, but rapid strides are being made in establishing the lineage relationships in the brain and in lineage comparisons between species (e.g., Urbach and Technau, 2003).

Within a lineage, the identity of a neuron depends on the birth order of the GMC from which it arises (Kuwada and Goodman, 1985). Brody and Odenwald (2000, 2002) showed that as they divide, the NBs progress through a stereotyped program of gene expression, including *hunchback* (*hb*), *Krüppel* (*Kr*), *POU domain protein* (*pdm*), and *caster* (*cas*) – a set of genes that were used earlier to establish regions of the body (see **Chapter 1**). Each GMC retains the expression of the gene activated at the time of its birth and passes it on to its progeny. Studies by Isshiki *et al.* (2001), using mutation and gain-of-function approaches, showed that these genes do indeed provide the information by which a neuron is informed of the birth-order of its GMC, and, hence, determine its identity.

Cell death also plays a prominent role in sculpting segmental differences within the CNS. For example, the leg motoneurons are generated in both thoracic and abdominal lineages, but they degenerate in the abdominal segments (Whittington *et al.*, 1982). Also, Thompson and Siegler (1993) showed that the difference in the number of neurons in the thoracic versus the A1 version of the median NB lineage is due primarily to differential neuronal death (see also **Chapter 5**).

The first-born neurons serve a pioneer role as they establish the major pathways within the CNS and out into the periphery (Bate, 1976b; **Chapter 3**). The progressive events of axon pathfinding, target recognition, and synaptogenesis then bring about the orderly construction of neural circuits.

4.2.1. The Hemimetabolous Pattern of CNS Development

Our understanding of nervous system development in hemimetabolous insects is based primarily on studies of orthopteroid insects such as crickets, grasshoppers, and cockroaches, in which the body form of the nymph differs little from that of the adult except that it lacks functional wings and genitalia. Unfortunately, we have little metamorphic data for the CNS

from groups like dragonflies and mayflies, in which the nymphal and adult forms are more disparate. In orthopteroid orders, the NBs generate their entire lineage of neurons prior to hatching and; consequently, the nymph hatches with a full set of central neurons. The only regions known to add neurons after hatching are the mushroom bodies and the optic lobes. The mushroom bodies are associated with various types of learning, and certain species continue to add mushroom body neurons even in the adult (e.g., in the cricket *Acheta domesticus*; Cayre *et al.*, 1996). The neurogenesis in the optic lobes provides the new interneurons needed to deal with the rows of ommatidia that are added during nymphal molts.

At the time of hatching, neurons already have a dendritic shape characteristic of the adult cell (e.g., Shankland and Goodman, 1982; Boyan, 1983; Kutsch and Heckmann, 1995) although they are only a fraction of their adult size (see **Chapter 3**). This similarity is not surprising for neurons having similar roles in the nymph and the adult, but it was not expected for neurons involved in unique adult behaviors such as flight (**Figure 2**; Kutsch and Heckmann, 1995) and reproduction. Not only do these neurons have their adult morphology, but the adult behavioral circuits, have already been assembled by hatching, even though the insect may not show the behavior until weeks or months later, when it finally attains its adult morphology. For example, a newly hatched locust shows the typical flight posture when suspended without tarsal contact, and generates rhythmic alternating bursts by the motoneurons that will supply the elevators and depressor muscles, even though these muscles are undeveloped in the early nymph and there are no wings to move (Stevenson and Kutsch, 1988). Similarly, pharmacological treatment of the terminal ganglia of female grasshoppers can already elicit an “adult” oviposition motor pattern from the embryonic ganglion (Thompson, 1993).

Although the CNS is stable in terms of the number of interneurons, additional sensory neurons are added to the periphery at each molt. Consequently, as the nymph grows, a constant number of interneurons are faced with an increasing number of sensory neurons competing for synaptic space. A well studied example involves the medial giant interneuron (MGI) that receives direct synaptic contact from the filiform hairs on the cercus in the house cricket, *A. domesticus*. As the number of filiform hairs increases with each nymphal molt, the dendrites of MGI must provide additional synaptic space to accommodate the expanding sensory input. An interesting issue with this growth, though,

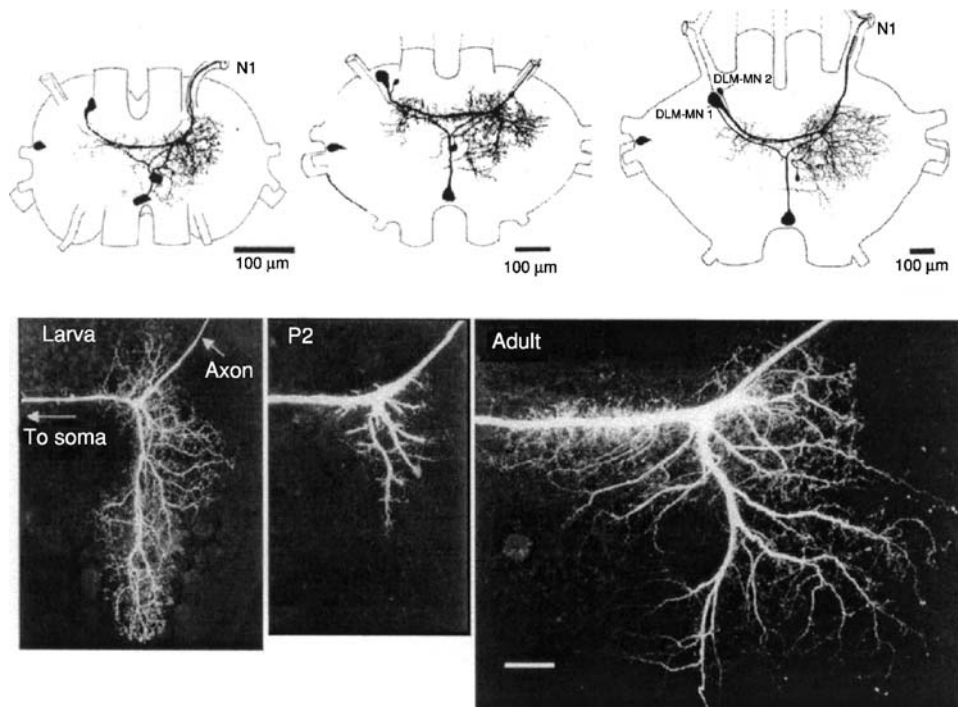


Figure 2 Postembryonic development of flight motoneurons in a hemimetabolous insect (top) *Schistocerca gregaria* and a Holometabolous insect (bottom), the moth *Manduca sexta*. The grasshopper neurons in the late embryo (left), 3rd instar nymph (middle), and adult (right) are revealed by backfilling with cobalt ions from the nerve leading to the site of the dorsal longitudinal flight muscles. The major cells filled include a large dorsal unpaired midline neuron that is neuromodulatory and one of the large motoneurons to the flight muscles (DLM-MN1). The overall structure of this neuron changes very little during postembryonic life. (Modified from Kutsch, W., Hechmann, R., 1995. Homologous structures, exemplified by motoneurons of Mandibulata. In: Breidbach, O., Kutsch, W. (Eds.), *The Nervous Systems of Invertebrates: An Evolutionary and Comparative Approach*. Birkhauser Verlag, Basel, pp. 221–248.) Photomicrographs show the dendritic arbor of the moth motoneuron that is the homolog of DLM-MN1. The neuron has a well-developed dendritic arbor in the larva. The arbor is pruned back in the pupa and an extensive adult-specific arbor then forms during adult differentiation. (From Duch, C., Bayline, R.J., Levine, R.B., 2000. Postembryonic development of the dorsal longitudinal flight muscle and its innervation in *Manduca sexta*. *J. Comp. Neurol.* 422, 1–17.)

is that a sensory hair's transduction properties change as it grows longer: short hairs are sensitive to wind acceleration but as they grow longer they become more sensitive to wind velocity. The MGI is sensitive to stimulus acceleration and newly born, short hairs make strong connections onto this interneuron. With successive molts, as the hair grows longer, the strength of synaptic connections that its neuron makes on the MGI wanes but the same neuron strengthens its synapses onto velocity-sensitive interneurons such as IN 10-3 (Chiba *et al.*, 1988). Hence, some sensory neurons show striking plasticity during growth and remodel their synapses to accommodate the changing properties of their sensory apparatus (see **Chapter 3**).

At the time of metamorphosis, there is a qualitative change in the sensory system, with the addition of new sensilla associated with adult-specific structures, like wings and genitalia. Also, to a lesser extent, there is a loss of sensilla that function only in the nymph.

4.2.2. The Holometabolous Pattern of CNS Development

The evolution of the holometabolous larva has been accompanied by a number of innovations in CNS development including proliferative and developmental arrests to accommodate the delayed production of the adult form (**Figure 3**). Also, embryonic neurons do not immediately assume an adult shape but acquire a novel morphology and connectivity to accommodate the larval body.

A fundamental change that occurred during embryogenesis is the truncation of neurogenesis. Rather than generating their entire lineage of neurons during embryogenesis, the NBs produce an initial set of neurons and then arrest. Their early progeny still pioneer the tracts and commissures to provide the basic architecture of the neuropils (Thomas *et al.*, 1984), and most then function in regulating the behavior of the larva. The majority of their progeny, though, are produced well after hatching.

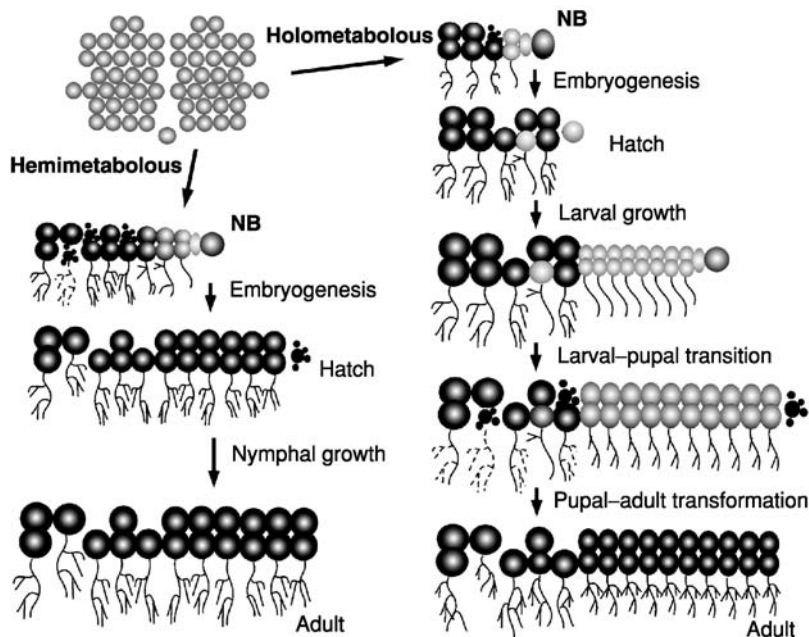


Figure 3 Summary of the changes in the development of neuronal lineages that accommodated the shift from a hemimetabolous to a holometabolous pattern of development. In the ancestral, hemimetabolous condition each neuroblast (NB) generated its entire lineage of neurons during embryogenesis, and, during the process, some of the progeny were removed by programmed cell death. With the evolution of the holometabolous larva, the neuroblasts generate only the early portion of their lineage. Some of these progeny arrest as immature neurons (light colored progeny) and cell death is prevented in some of the neurons that normally died in ancestral forms. Neurogenesis resumes during larval life but the newly-born neurons are stockpiled at an early stage in their development. The onset of metamorphosis then stimulates the development of the arrested neurons as well as cell death and dendritic and axonal pruning in the larval neurons. Adult-specific arbors are then elaborated during the pupal–adult transformation.

The extent of cell death may also have been modified to accommodate metamorphosis. In grasshoppers, a thoracic ganglion has about fivefold more neurons than its abdominal counterpart. This disparity arises during embryogenesis, in part from abdominal NBs undergoing fewer divisions than thoracic ones (Shepherd and Bate, 1990) but also from enhanced cell death in the abdominal lineages (Thompson and Siegler, 1993). By contrast, larvae of holometabolous insects have become “abdominalized” as the abdomen has taken over most or all of the larva’s locomotor function, and, accordingly, their abdominal ganglia may have twice as many neurons as in the corresponding ganglia in a grasshopper. In lepidopteran caterpillars, for example, each abdominal ganglion has over 1000 neurons, only a few hundred less than seen in a thoracic ganglion in these larvae. The embryonic neurogenic periods are similar for the thorax and abdomen in caterpillars, which suggests that the extent of embryonic neuronal death does not differ dramatically between the two regions. This numerology presents the interesting possibility that the larval CNS may have acquired the extra neurons that

it needed simply by maintaining neurons that normally had died in their hemimetabolous ancestors. These evolutionarily “un-dead” neurons then serve as functional neurons, but then finally undergo their ancestral fate of degeneration as metamorphosis begins and the adult form is established.

A neuron’s form and pattern of connectivity in the larva often differs markedly from that seen in the adult (Figure 2; Levine and Truman, 1985; Duch and Levine, 2000). Most larval neurons are not just partially differentiated adult cells. They are fully differentiated neurons that have specializations adapted to the larval body plan. Their specializations can include their electrical properties (Duch and Levine, 2000) and transmitters (Tublitz and Slywester, 1990), as well as their morphology and synaptic connections. The difference between a neuron’s larval and adult morphology varies widely from cell to cell and likely relates to the divergence in the cell’s function between the two stages.

Importantly, not all neurons born during embryogenesis acquire a mature phenotype. The leg motoneurons in *Drosophila*, for example, are born during embryogenesis but their muscle targets

do not appear until metamorphosis. In the larva these neurons are found with a sparse dendritic arbor and an axon that extends into the growing leg imaginal discs. Similarly, the larval form of the indirect flight muscle motoneuron MN5 possess an axon but has no dendrites or peripheral targets (Consoulas *et al.*, 2002). It is not known, however, if these “immature” neurons contribute to the functioning of the larval CNS despite their immature appearance.

During larval growth, the arrested embryonic NBs reactivate and generate the remainder of their lineage. The neurons born during this second, post-embryonic bout of neurogenesis extend an axon to an initial target but then arrest. They are then stockpiled in this immature condition until the start of metamorphosis (Figure 4).

Hence, the larva enters metamorphosis with a CNS composed of larval neurons and arrested, immature neurons of both embryonic and postembryonic origins. Some of the dismantling of the larval nervous system comes about through neuronal death, especially in the abdomen, as the ancestral ratios of thoracic to abdominal neurons are finally achieved. Many larval neurons, though, are remodeled. Their larval dendritic and axonal arbors are pruned back and new, adult-specific arbors grow to achieve the shape and connectivity appropriate for the adult stage (Figure 2). While larval neurons are starting their remodeling, the immature neurons initiate their adult outgrowth. These diverse processes are orchestrated by the circulating ecdysteroid titers.

4.3. Developmental Hormones and Their Receptors

The developmental hormones in insects are the sesquiterpene juvenile hormones (JHs; see Chapter 8), and the ecdysteroids, the steroid molting hormones (Gilbert *et al.*, 2002; see Chapter 7). The major circulating ecdysteroids are ecdysone (E), which is released from the prothoracic glands, and 20 hydroxyecdysone (20E), an active metabolite of E made by peripheral tissues. The pattern of hormone secretion in hemimetabolous insects is relatively simple (Figure 5a). Periodic surges of ecdysteroids drive the molting from one nymphal stage to the next. The presence of JH maintains the nymphal form, but JH titers decline after the formation of the last nymphal stage, allowing the differentiation of adult characters, when the insect is next challenged with ecdysteroid. JH typically reappears in the adult to regulate aspects of reproduction.

The holometabolous life history is associated with a more complex pattern of hormone secretion (Figure 5b). Ecdysteroids still cause molting during larval stages and JH is required to maintain the larval form, its so-called “status quo” function (Riddiford, 1994). The decline in JH at the start of the last larval stage then allows the preparation for metamorphosis. During the last larval stage, small surges of steroid occur, which are too small to induce molting but nevertheless direct premetamorphic programs in tissues (Wolfgang and Riddiford, 1986). The largest of these small peaks, the commitment peak, terminates larval feeding, causes

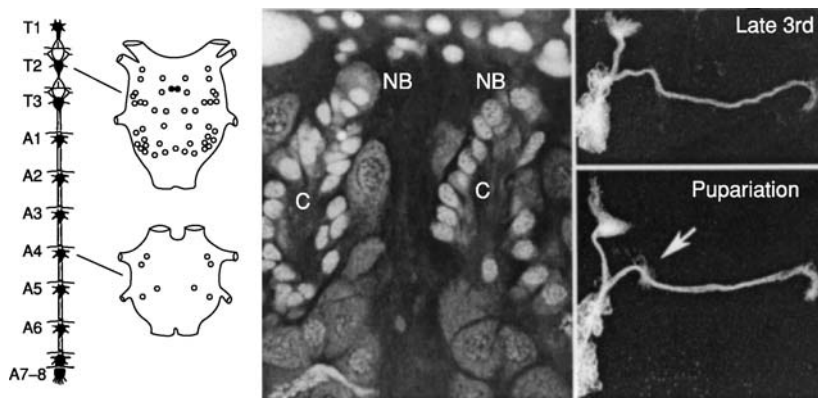


Figure 4 Postembryonic neurogenesis in larvae of holometabolous insects. (Left) Drawing of the ventral nervous system of a *Manduca* larva showing the location of the reactivated embryonic neuroblasts. A thoracic ganglion has about 20 more neuroblast pairs than does an abdominal ganglion. The locations of the paired “F” neuroblasts are indicated by the black circles. (Middle) A confocal section showing a dorsal view of a thoracic ganglion stained with propidium iodide showing DNA and RNA. The section shows the 2 “F” neuroblasts (NB) and their respective clusters (C) of arrested, adult-specific neurons. (Right) Dorsal views of *Drosophila* thoracic neuromeres showing the postembryonic progeny of a single neuroblast that expresses green fluorescent protein (GFP). The immature neurons extend axons either to the next anterior neuromere or across the midline to a contralateral site. The cells only begin to sprout dendrites (arrow) when metamorphosis begins at pupariation.

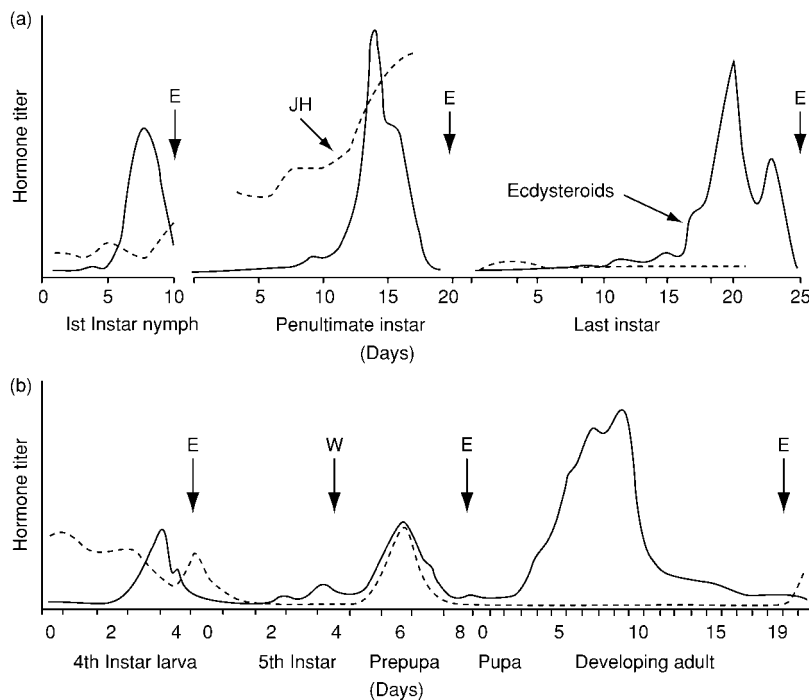


Figure 5 Juvenile hormone (dotted line) and ecdysteroid (solid line) titers in (a) a hemimetabolous insect, the cockroach *Nauphoeta cinerea* and (b) a holometabolous insect, the moth *Manduca sexta*. E, ecdysis; H, hatching; W, wandering. (Titers based on Lanzrein, B., Gentinetta, V., Abegglen, H., Baker, F.C., Miller, C.A., *et al.*, 1985. Titers of ecdysone, 20-hydroxyecdysone and juvenile hormone III throughout the life cycle of a hemimetabolous insect, the ovoviviparous cockroach *Nauphoeta cinerea*. *Experientia* 41, 913–917; and Riddiford, L.M., 1994. Cellular and molecular actions of juvenile hormone. I. General considerations and permetamorphic actions. *Adv. Insect Physiol.* 24, 213–274 (review).)

wandering behavior, and commits larval tissues to pupal differentiation (Riddiford, 1994). The latter is significant because it renders larval tissues insensitive to subsequent exposure to JH. A major ecdysteroid peak then causes the formation of the pupal stage. This peak is accompanied by the reappearance of JH which acts on selected imaginal tissues to prevent their premature adult differentiation (Williams, 1961; Kiguchi and Riddiford, 1978; Champlin *et al.*, 1999). In higher flies, like *Drosophila*, this large ecdysteroid peak also causes the larval cuticle to transform into a protective case, the puparium, inside of which the true pupal stage forms. JH disappears prior to pupal ecdysis and a prolonged surge of ecdysteroids then drives adult differentiation. In *Manduca*, the major circulating steroid at the beginning of adult differentiation is E but 20E then becomes prominent as metamorphosis progresses (Warren and Gilbert, 1986). As with hemimetabolous insects, JH then returns in the adult, typically in association with some aspect of reproductive control.

Although the overall patterns of hormone secretion are similar in moths and flies, there are major differences in how their tissues respond to this

hormonal landscape. The most striking differences are seen in the response of tissues to JH. Application of exogenous JH during the last larval stage of *Manduca* causes the larva to molt to a supernumerary larval stage rather than to pupa. Also, the application of JH to the pupa, at the outset of adult differentiation, results in a second pupal stage, although some adult features may also be present (Riddiford and Ajami, 1973). In *Drosophila*, by contrast, treatment of the last larval stage with JH does not result in an extra larval instar, and an apparently normal puparium and pupa are formed. During subsequent adult differentiation, though, the abdominal epidermis makes a second pupal cuticle, while the imaginal discs in the head and thorax make normal adult structures (Postlethwait, 1974; Riddiford and Ashburner, 1991). JH also suppresses aspects of adult differentiation of internal tissues including the CNS (Restifo and Wilson, 1998; Williams and Truman, 2004). Hence, in higher flies, JH sensitivity is lost during the larval pupal transition and appears only in selected tissues during the differentiation to the adult.

The changing sensitivity of tissues to hormonal signals could be encoded at the level of their

receptors. In the case of JH, though, the issue is unclear because a JH receptor has yet to be unequivocally demonstrated (see **Chapter 8**). Indeed, multiple receptor systems might be involved in mediating the action of this versatile hormone (Wheeler and Nijhout, 2003). By contrast, the receptors that mediate ecdysteroid action have been known for over a decade (Koelle *et al.*, 1991; see **Chapter 7**), and receptor distributions direct the time and nature of a cell's response to this steroid.

The action of ecdysteroids is mediated through a heterodimer of two members of the nuclear receptor family, the Ecdysone Receptor (EcR) and Ultraspiracle (Usp) (see **Chapter 7**; Riddiford *et al.*, 2000 for reviews). Like other family members, EcR has a DNA binding domain consisting of two Zn fingers, and a ligand binding domain that carries an activation function (the AF2 domain) that mediates ligand-dependent activation. The *Ecr* gene encodes different protein isoforms that share a common DNA and ligand-binding domain, but differ in their N-termini. *Drosophila* has three isoforms, EcR-B1, -B2, and -A, whereas other Diptera and Lepidoptera have only two isoforms, EcR-B1 and -A. Cell transfection experiments have shown that the A/B regions of EcR-B1 and -B2 have potent AF1 activation domains, whereas this region of EcR-A appears to have a repressive function (Mouillet *et al.*, 2001; Hu *et al.*, 2003).

The EcR/Usp complex binds to specific DNA response elements. The unliganded receptor binds corepressors and mediates local transcriptional silencing. With binding of ligand, coactivators are assembled allowing transcriptional activation. Transcriptional activation can be mediated through two regions of the receptor – through the AF1 site in the A/B region (causing “ligand-independent” activation) and the AF2 site in the LBD. The coactivator and corepressor complexes include a number of components, many of which participate in multiple signaling pathways. They may interact with the core promoter and/or locally modify the chromatin (e.g., through acetylation or deacetylation of histones) to facilitate or suppress transcription (review: Kraus and Wong, 2002).

EcR mutant and receptor misexpression studies indicate that the receptor isoforms have both unique and overlapping functions (Bender *et al.*, 1998; Schubiger *et al.*, 1998; Li and Bender, 2000; Cherbas *et al.*, 2003). Since EcR-A and EcR-B1 show dramatic differences in their distribution in both time and space, their expression and action is a key to understanding how different neurons may show differing responses to the same hormonal signal.

4.4. Hormones and the Generation of the Larval CNS

The specializations that brought about the larval CNS include the truncation of neurogenesis, a suppression of neuronal death, and an alteration in the growth patterns of embryonic neurons. The roles that ecdysteroids and JH may have had in these events, though, are poorly understood. During embryogenesis, holometabolous insects show a precocious appearance of JH relative to that seen in embryos from hemimetabolous groups. This early appearance of JH is thought to be associated with the truncation of growth and premature differentiation that was necessary to generate the novel larval stage (Truman and Riddiford, 2000; Erezyilmaz *et al.*, 2004). At this point, though, the linkage between key developmental switches in the CNS and the hormone titers stands only as a correlation.

A striking feature of the neurogenesis in embryos of both *Drosophila* and Lepidoptera is its abrupt arrest that occurs at about 50% of embryogenesis (e.g., **Figure 6**). This arrest contrasts with the pattern seen in ametabolous and hemimetabolous insects, in which some neuroblasts continue proliferation until almost hatching (Shepherd and Bate, 1990; Truman and Ball, 1998), and is consistent with there being an extrinsic signal responsible for the arrest. The arrest occurs at the time that the rising embryonic ecdysteroid titer causes the production of the first larval cuticle. Therefore, this embryonic ecdysteroid surge may be responsible for the arrest, but experimental data are needed to support this speculation.

4.5. Nervous System Changes during Larval or Nymphal Growth

4.5.1. Somatic Sensory Systems

An initial set of sensory neurons differentiate during embryogenesis in both larvae and nymphs. These include proprioceptors, such as stretch receptors and chordotonal organs, that monitor body wall stretch and joint position, and external sensory organs with cuticular hairs and having mechanoreceptive functions. This initial set of receptors is highly stereotyped and homologous cells can be found between *Schistocerca gregaria*, *Drosophila* (Meier *et al.*, 1991) and *Manduca sexta* (Grueber and Truman, 1999). Typically, the number of proprioceptors usually remains unchanged during the growth of the larva or nymph, but the sensory hairs show dramatic increases in

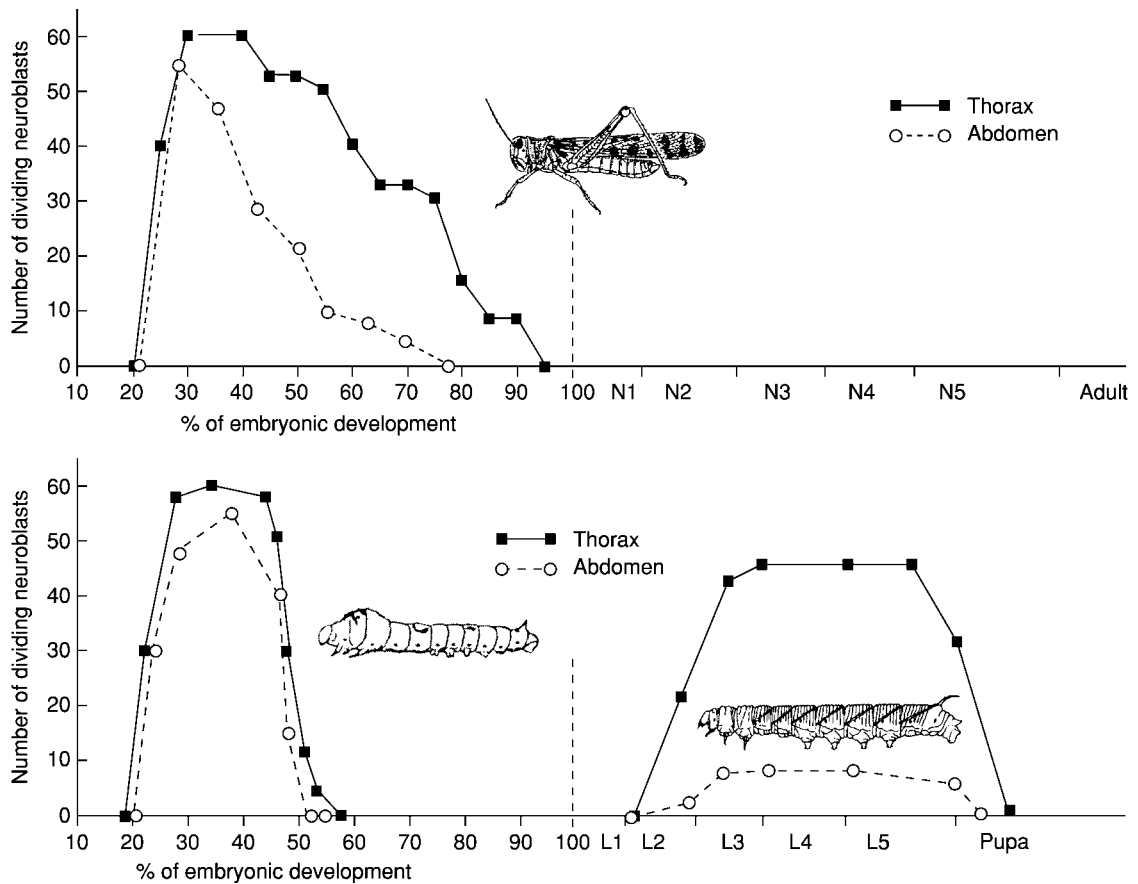


Figure 6 Comparison of the duration of neurogenesis in a hemimetabolous insect, the grasshopper *Schistocerca gregaria*, and in holometabolous lepidopteran larvae. (The grasshopper data are based on Shepherd, D., Bate, C.M., 1990. Spatial and temporal patterns of neurogenesis in the embryo of the locust (*Schistocerca gregaria*). *J. Comp. Neurol.* 319, 436–453, while the lepidopteran data are based on Booker, R., Truman, J.W., 1987a. Postembryonic neurogenesis in the CNS of the tobacco hornworm, *Manduca sexta*. I. Neuroblast arrays and the fate of their progeny during metamorphosis. *J. Comp. Neurol.* 255, 548–559 and J.W. Truman, unpublished data.)

numbers through successive instars (Figure 7). The larvae of higher Diptera, though, are exceptional in that they add no new sensory neurons after hatching.

The positions of the first hairs that arise during embryogenesis are very stereotyped, but during the postembryonic period interactions between the epidermal cells and existing sensory hairs determine the placement of new sensory hairs. The studies by Wigglesworth (1940) on the spacing of new sensory hairs in *Rhodnius* provided the first experimental demonstration of lateral inhibition. He showed that existing bristles suppressed the formation of a new sensory organ precursor (SOP) in their immediately vicinity, thereby insuring that new bristles were placed in spaces that lacked them.

The production of new bristles is coordinated with the molt cycle. In *Rhodnius*, the quartet of cells that comprise the sensory organ (SO) appears

6 days after the insect has taken a blood meal, and is associated with a general mitotic peak at the start of a molt (Wigglesworth, 1953). The sensory neuron extends an axon towards the CNS, and the trichogen and tormogen cells that form the cuticular hair and socket, enlarge soon thereafter. The new sensillum appears to be anatomically functional by the time the molt is completed.

Although *Rhodnius* undergoes the steps from the establishment of the SOP to the finished sensory organ within a single molt, the process extends over two molts in large rapidly growing larvae, such as *M. sexta* (Grueber and Truman, 1999). In larvae of *Manduca* each SOP produces five cells including two neurons. One neuron is a typical mechanoreceptor neuron associated with the new bristle but the other is a dendritic arborization (da) sensory neuron whose dendrites ramify under the soft cuticle of the larva. At this point, it is not known whether

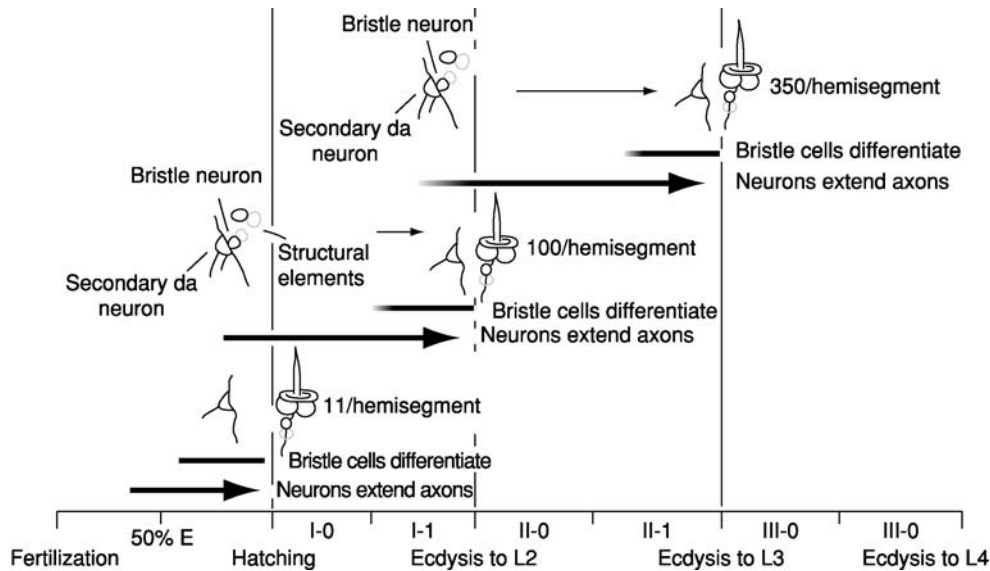


Figure 7 The timing of the birth and differentiation of sensory bristles on the second abdominal segment of *Manduca sexta* caterpillars. The larva hatches with 11 sensory bristles and 12 dendritic arborization (da) sensory neurons. Two waves of sensory neurons are then added, the first prior to hatching and the second during the molt to the second instar. The neurons immediately extend axons to the CNS but the sensory hair does not differentiate until the succeeding molt. No sensory hairs are added after the second wave. (Based on data from Grueber, W.B., Truman, J.W., 1999. Development and organization of a nitric-oxide-sensitive peripheral neural plexus in larvae of the moth, *Manduca sexta*. *J. Comp. Neurol.* 404, 127–141.)

the dense network of processes from the latter is involved in detecting cuticular stretch or in sensing noxious, damaging stimuli. During the first molt the SOP is established and undergoes the divisions to produce the two neurons and three support cells. The cuticular structures made by the trichogen and tormogen then appear at the next molt, in response to the next molting surge of ecdysone (Figure 7). This delay may be necessary in large insects to allow axons from the bristle to grow into the CNS and make connections with their central targets.

4.5.2. Growth of the Eyes

In many nymphs new ommatidia are added to the eye at each molt. In *S. gregaria*, for example, the number of ommatidia increases from about 2500 in the newly hatched nymph to over 9000 in the adult. The new ommatidia arise from a region along the anterior margin of the eye, which is divided into proliferation and differentiation zones (Anderson, 1978). The proliferation zone shows a basal level of cell division that probably produces photoreceptor neurons at a constant rate, since outgrowing axons from new photoreceptors are evident at all times during the intermolt and molting periods. At the start of the molt there is an increase in mitoses in the proliferation zone and also a spike of proliferation in the differentiation zone, where the ommatidia are being assembled. The divisions in the

differentiation zone, likely produce support cells, such as those that make the crystalline cone and the screening pigments. Except for responses to circulating factors, the growth of the eye is under autonomous control, as illustrated by normal patterns of proliferation being observed in eyes transplanted to the surface of the prothorax (Anderson, 1978).

Unlike nymphs, the eyes of larvae consist of only a small set of separated facets, the stemmata. These likely arose from the most posterior set of ommatidia in the eyes of the ancestral larva. The stemmata, though, add neither facets nor more photoreceptors as the larva progresses through its larval instars. The compound eye of the adult appears only at metamorphosis and forms immediately anterior to the stemmata.

4.5.3. New Central Neurons. Regulation of Neurogenesis

In hemimetabolous insects, postembryonic neurogenesis in the CNS is confined to the mushroom bodies and the optic lobes. There is no information as to whether neurogenesis in the mushroom bodies is influenced by ecdysone and molting. In the optic lobe of *Schistocerca*, the production of new neurons for the lamina and the medulla, the outer two optic neuropils, occurs at a constant rate throughout the instar, without any modulation during the time of the molts (Anderson, 1978). This pattern of constant

interneuron production matches the pattern of constant addition of photoreceptor axons in the periphery. While the eye does not need the optic lobes to make new ommatidia, the optic lobes do need the eyes to some extent. In *Schistocerca* the surgical removal of the anterior proliferation and differentiation zones result in no new photoreceptor axons growing into the optic lobe. Despite the lack of ingrowing axons, there appears to be no difference in the rate of division of NB or GMCs for either the lamina or medulla. There is, though, a dramatic effect of cell survival as one sees enhanced death of the lamina ganglion cells (Anderson, 1978). In *Manduca* and *Drosophila*, the relationship between photoreceptor axons and lamina neurons differs from that seen above, because ingrowing axons influence proliferation as well as cell survival (see Section 4.8.4).

Postembryonic neurogenesis in the ventral CNS has been studied primarily in *Manduca* (Booker and Truman, 1987a) and *Drosophila* (White and Kankel, 1978; Truman and Bate, 1988), but also occurs in larvae of Coleoptera, Hymenoptera, and Neuroptera (J.W. Truman, unpublished data). Hence, it is a ubiquitous feature of the holometabolous life style. In highly derived larvae, like those of *Manduca* and *Drosophila*, neurogenesis begins early in larval life and concludes shortly after pupal ecdysis (Figure 4). The factors responsible for the reactivation of the arrested embryonic neuroblasts are best understood in *Drosophila*. The need for nutritional cues was shown by experiments, in which newly hatched larvae were maintained on a glucose diet that lacked amino acids. On such a diet, the energetic requirements of the larva are met by the sugar but growth is not possible. Under these conditions neuroblasts that are already cycling, such as those of the mushroom bodies, continued to divide, but arrested neuroblasts could not reenter the cell cycle (Britton and Edgar, 1998).

The control over NB activity may not be cell autonomous and may involve the surrounding glia. Mutations in the gene *anachronism* (*ana*), that encodes a cell-surface protein, results in the precocious onset of postembryonic neurogenesis (Ebens *et al.*, 1993). Interestingly, *ana* is not expressed in the NB but, rather, it is expressed in the surrounding glia, suggesting that these cells may actively inhibit NB reactivation. The suppression imposed by *ana* appears to be antagonized by the expression of the *trol* gene (*terribly reduced optic lobes*) (Caldwell and Datta, 1998) which acts through cyclin E to allow the transition of the stem cells from G1 into the S phase. The activation of proliferation may also involve the action of ecdysteroids in the early larvae (Datta, 1995; Park *et al.*, 2001).

4.6. Role of Hormones in the Metamorphosis of Hemimetabolous Nervous Systems

The metamorphic changes seen in hemimetabolous insects are most evident in the peripheral nervous system, because there is an obvious acquisition of adult-specific sensory neurons and, to a lesser extent, the loss of nymph-specific sensilla. An example of the latter is seen in *Rhodnius prolixus*, in which most of the sensory hairs are lost on the dorsum of the adult abdomen since it is covered by the wings. Interestingly, although the cuticular hairs are lost, the sensory neurons that supplied them do not die and persist in the adult (Wigglesworth, 1953). Presumably, they cease to serve as functional sensory neurons since their transducing hairs are no longer present. The topical application of JH prevents both the loss of these nymphal sensory hairs and the appearance of adult-specific sensilla. Another example of JH preventing the appearance of adult-specific sensilla is seen in cockroaches in which JH treatment of the last nymphal stage suppresses the appearance of pheromone-sensitive hairs on the antennae (Schaffer and Sanchez, 1973).

The metamorphic changes in the CNS of hemimetabolous insects are subtle and do not involve massive neuronal death or neuronal remodeling. There may be, however, important functional changes that occur during the molt to the adult (Olberg, 1986). This is seen most strikingly in the visual system of dragonflies. In the adult, there are eight descending visual interneurons that carry wide-field information from the optic lobes down to the thoracic ganglia. These interneurons are also present in the nymph, but only one is functional and the others are electrically silent. They finally become electrically active during the molt to the adult. The one functional interneuron in the nymph receives information from the whole visual field of the nymph, but after metamorphosis those ommatidia now comprise the extreme posterior border of the adult eye, and the interneuron is now responsive only to stimuli and the periphery of the visual field.

4.7. Hormones and the Metamorphosis of Holometabolous Nervous Systems

4.7.1. Death of Larval Neurons

Most studies on the metamorphic fates of larval neurons have focused on the motor system (see Chapter 5). The fates of motoneurons, though, need to be considered in the context of their target muscles (Figure 8; reviews: Consoulas *et al.*, 2000a).

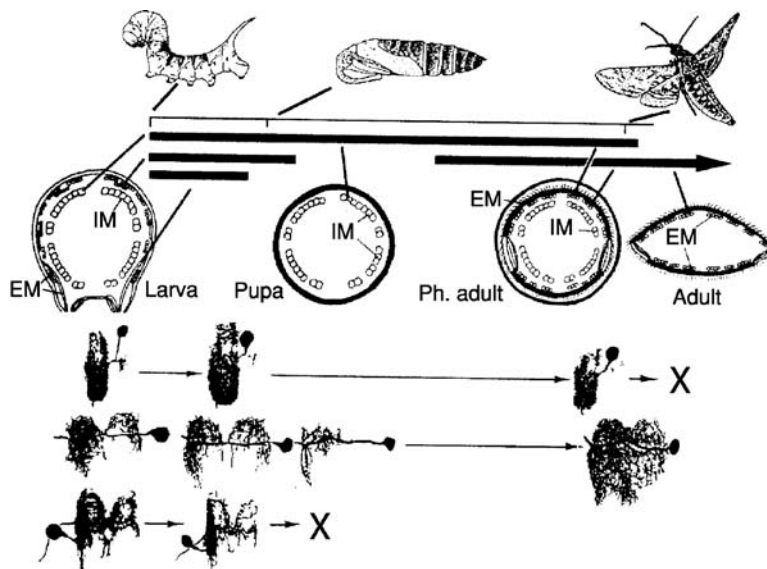


Figure 8 Summary of the fates of larval neurons and muscles during metamorphosis in *Manduca sexta*. (Top) Time-line showing the three major muscle fates during metamorphosis. The cross-section views show the positions of abdominal internal (IM) and external (EM) muscle groups in the larva, pupa, pharate adult (Ph. adult), and adult. The bars show the time when functional muscle is present. Many internal muscles persist through metamorphosis but then die after adult emergence. One group of external muscles die in the prepupa while others degenerate 1–2 days after pupal ecdysis. The remains of some of these muscles are used for templates for the growth of adult muscles. (Bottom) Examples of the changes in morphology and survival of motoneurons associated with muscles having the three kinds of fates. (Neuron data from Weeks, J.C., Truman, J.W., 1985. Independent steroid control of the fates of motoneurons and their muscles during insect metamorphosis. *J. Neurosci.* 5, 2290–2300; and Levine, R.B., Truman, J.W., 1985. Dendritic reorganization of abdominal motoneurons during metamorphosis of the moth, *Manduca sexta*. *J. Neurosci.* 5, 2424–2431.)

In both *Manduca* and *Drosophila* the larval musculature is dismantled in three steps. An initial bout of degeneration occurs prior to pupal ecdysis, and includes most of the musculature of the head and thorax, and selected muscles in the abdomen. The early loss of the head and thoracic musculature provides space for the growth and expansion of the imaginal discs that will produce parts of the adult head and thorax. A second period of muscle death occurs early in pupal life and involves muscles that were necessary for pupal ecdysis. In *Manduca*, the pupa is then left with a set of internal longitudinal muscles in segments A3 to A6, and these mediate the limited behavior of the pupa. *Drosophila* similarly has a set of “persistent larval muscles” but these lose their contractile machinery through the middle of metamorphosis. The adult muscles begin forming after pupal ecdysis. Some are formed from templates provided by the remains of the larval muscles, while others are formed *de novo* as groups of myoblasts aggregate to found adult muscles. In *Manduca*, the muscles of the head and thorax (except for the indirect flight muscles) are formed *de novo*, whereas most of the abdominal musculature is formed on larval

templates. In *Drosophila*, by contrast, the vast majority of the adult muscles arise *de novo*, with the indirect flight muscles being one of the few muscles organized on a larval template (Fernandes and Keshishian, 1998). In both species, at the end of adult development, the abdomen has both persisting larval muscles and the new adult-specific musculature. The larval muscles, along with a few specialized ecdysial muscles, then degenerate a few hours after emergence leaving the animal with its adult-specific musculature.

4.7.1.1. Death during the larval–pupal transition Although larval muscles die in three waves, the neurons that innervate them only die at two times (Figure 8) (see Chapter 5). In both *Drosophila* and *Manduca*, most larval neurons die during the initial period that occurs shortly after pupal ecdysis. The most extensive cell loss is seen in the abdominal CNS, reflecting a reduction in the behavioral importance of this region of the body. With the vermiform body of the larva, the abdominal CNS controlled a complex musculature and had the major responsibility for locomotion. At metamorphosis the latter functions are given over to the thorax, and the

abdomen becomes severely reduced both in its musculature and its behavioral capacity.

The most detailed studies of neuronal death during this first period are from *Manduca* and examine the fates of PPR and APR, abdominal motoneurons that control the retraction of the larval prolegs. Two endocrine conditions are needed for the death of PPR: the lack of JH during the commitment peak of ecdysteroids, and the subsequent high 20E titers of the prepupal peak (Weeks and Truman, 1985). Although the death of PPR follows that of its muscle, the neuron's death is not induced by the loss of its target. This conclusion is based on experiments showing that PPR continues to show its appropriate 20E-dependent fate even when its muscle is surgically removed.

An elegant set of *in vitro* experiments using the motoneuron APR confirmed that the degeneration response was not caused by the death of its target. This larval motoneuron undergoes programmed cell death but in a segment-specific context: those in segments A5 and A6 degenerate whereas those in segments A3 and A4 survive (Sandstrom and Weeks, 1998). Neurons taken from larvae and maintained without 20E *in vitro* survive regardless of their segmental origin. When exposed to 20E, however, some undergo programmed cell death, but only if they were removed from segments in which these cells would normally die (Streichert *et al.*, 1997). Thus, the hormone-induced death of these neurons is intrinsically programmed and does not depend on interactions either within the ganglion or with their muscle targets.

4.7.1.2. Death during the pupal–adult transition The last wave of neuron and muscle death occurs shortly after adult ecdysis, and primarily involves the ecdysial muscles that will not be needed again in the adult. Post-ecdysial muscle death appears to be a universal feature of adult ecdysis in higher insects, but the loss of their motoneurons is not. In the moth *Hyalophora cecropia*, for example, the intersegmental muscles degenerate but their motoneurons survive through the remainder of adult life (S. Fahrbach, personal communication; see Chapter 5). The hormonal signals that control the death of these muscles and their motoneurons are varied. In all cases, the decline in ecdysteroids at the end of adult differentiation is an essential component of this control, and late injections with 20E delay the degeneration response. In some species, such as the moth *Antheraea polyphemus*, a peptide hormone provides the final trigger for muscle degeneration (Schwartz and Truman, 1982).

In *Manduca*, by contrast, the withdrawal of ecdysteroids alone appears to be sufficient for muscle death.

As with the muscles, the death of neurons in both *Manduca* and *Drosophila* requires a decline in ecdysteroids, and injection of 20E just before adult ecdysis prevents the death (Truman and Schwartz, 1984). The control over the degeneration is complicated, though, because the doomed neurons are involved in both ecdysis and post-ecdysis behaviors, such as wing inflation, and these behaviors can be separated in time if the insect is not given an appropriate site to inflate its wings. Accordingly, in *Manduca*, behavioral manipulations, such as forcing newly emerged moths to dig through soil, result in some neurons dying on schedule but delay the death of others (Truman, 1983). Nerve cord transection experiments in *Manduca* showed that one of the last neurons to die, MN12, requires a descending signal via the nerve cord in order to degenerate (Fahrbach and Truman, 1987). A similar situation appears to hold for some of the doomed neurons in *Drosophila* since decapitation of the newly emerged fly prevents the death of a subset of the cells (Kimura and Truman, 1990; Robinow *et al.*, 1997). Hence, the death of some of the late dying neurons requires both the absence of 20E and a descending signal from the head.

The death of neurons in *Drosophila* is immediately preceded by the appearance of transcripts for the cell death genes, *reaper* and *grim* (Robinow *et al.*, 1997). Focusing on *reaper* expression, these authors showed that the injection of 20E, less than an hour before the transcripts normally appear, blocked both the appearance of *reaper* and the onset of neuronal death. This timing is consistent with the hypothesis that 20E directly suppresses the transcription of these cell death genes. Interestingly, the decapitation of newly emerged flies, which prevents the death of a subset of doomed cells, does not prevent *reaper* transcripts from appearing in these cells (Robinow *et al.*, 1997). Apparently the descending signal acts downstream of *reaper* transcription, perhaps at the level of translation of this protein.

4.7.2. Remodeling of Larval Neurons

Figure 9 summarizes the main developmental phases, through which a neuron progresses as it changes from its larval to its adult form. The first changes appear at the larval–pupal transition during the “pruning phase,” as neurons rapidly remove axonal and dendritic branches. For the axonal branches of mushroom body neurons and the dendrites of

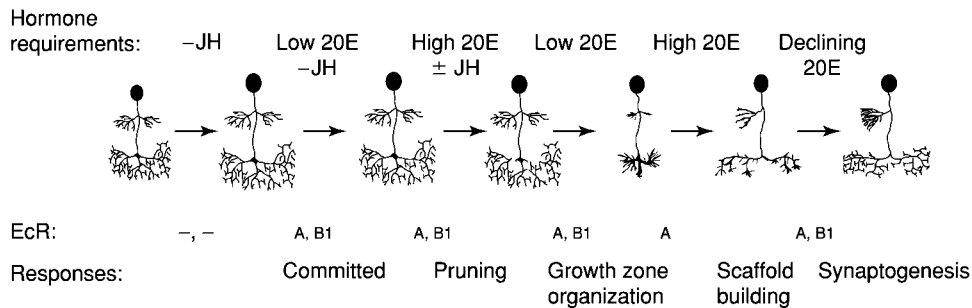


Figure 9 Summary of the developmental changes that occur during the life of a larval neuron during larval growth and metamorphosis. The EcR isoform data were obtained from *Drosophila* and the hormonal requirements are based on studies on *Manduca sexta*. The one process whose endocrine requirements are known to be different in moths and flies is during commitment: JH treatment cannot prevent *Drosophila* neurons from subsequent pruning.

dendritic arborization (da) sensory neurons, pruning occurs as branches are pinched off and undergo fragmentation (Watts *et al.*, 2003; D.W. Williams and J.W. Truman, unpublished data). This process is mediated through the ubiquitin-proteasome pathway (Watts *et al.*, 2003) similar to Wallerian degeneration seen in severed vertebrate axons. Fragmentation is not the only mechanism employed during pruning, though, since time-lapse movies show that the loss of the axonal arbors of the Tv neurosecretory neurons occurs by retraction of the axonal branches (H. Brown and J.W. Truman, unpublished data) more like the more gradual retraction of processes seen during synapse elimination at vertebrate neuromuscular junctions (e.g., Walsh and Lichtman, 2003).

Pruning is followed by a “growth zone organization phase,” as the cell prepares for its adult outgrowth. One part of the arbor may show retraction bulbs as pruned stumps are retracted, while another part is enlarging and organizing into a growth cone. This is also a time when soma size increases, a change that is especially obvious for the flight motoneurons (Duch *et al.*, 2000).

The growth cones begin to extend and branch during the “scaffold building phase.” *In vivo* imaging of the outgrowth of dendritic arbors from the da sensory neurons show that, as the arbor expands, branch retraction also occurs as the cell establishes the basic “foot-print” of the arbor (Williams and Truman, 2004). Studies on *Manduca* motoneurons show that the outgrowth phase is marked by changes in Ca^{2+} currents in the cell (Duch and Levine, 2000). During the early phase of elongation, Ca^{2+} currents are small and confined to distal ends of extending branches. In the later phases, the cell shows Ca^{2+} spikes that provide Ca^{2+} transients throughout the cell. Eventually, these Ca^{2+} spikes are masked by the appearance of strong K^+ conductances.

The “synaptogenesis” phase then involves the formation and maturation of synaptic contacts. This phase occupies the last half of adult differentiation.

4.7.2.1. The pruning phase and its hormonal control The pruning of larval dendritic and axonal branches in both *Manduca* and *Drosophila* is associated with the large peak of ecdysteroid that causes the transition from the larval to the pupal stage. Ligation and hormone replacement experiments in *Manduca* demonstrated that the pruning of the proleg motoneuron PPR requires two discrete endocrine cues. 20E, acting in the absence of JH at the wandering peak, is necessary to “commit” the neuron to prune, when it is next exposed to high levels of ecdysteroid. The following high 20E titers of the prepupal peak then cause the pruning (Weeks and Truman, 1985). In the case of PPR, dendritic pruning is followed a few days later by the death of the cell. These two responses, though, are triggered separately, since the length of steroid exposure needed to induce pruning is less than that needed to cause cell death (Weeks *et al.*, 1992). Although appropriately, timed treatment with JH mimics can prevent axon and dendritic pruning in *Manduca*, JH is not able to suppress the pruning response in *Drosophila* neurons (Williams and Truman, 2004).

The demonstration that dendritic pruning removes larval-specific synaptic contacts comes from work on the proleg withdrawal reflex in *Manduca* caterpillars. When the plantal hairs on the end of the proleg contact a foreign object, the firing of their sensory neurons (PH-SNs) causes a reflexive withdrawal of the proleg. The PH-SNs make monosynaptic contacts with PPR, the motoneuron that innervates the proleg retractor muscles. Early in metamorphosis, the proleg retraction reflex weakens. This occurs at the time that the proleg retractor muscles are degenerating, but there are also changes

to the synapse between the PH-SNs and PPR. Stimulation of the sensory nerve carrying the axons of the PH-SNs show a marked weakening of synaptic strength associated with the pruning of dendrites of the motoneuron (Jacobs and Weeks, 1990). Localized treatment of a proleg with JH mimics can produce a pupa that carries a larval proleg with its normal complement of plantal hairs. Even though these JH-treated sensory neurons maintain their larval morphology, PPR still prunes back its dendrites with the associated drop in synaptic efficacy. These results argue that the dendritic pruning is a cell autonomous response rather than one that is elicited by changes in presynaptic partners.

Similar attempts to use ecdysteroid-induced mosaics to examine axonal pruning in motoneurons were not as clear cut (Hegstrom and Truman, 1996). In *Manduca*, permanent larval abdomens were established by ligation, and then 20E was applied locally to the epidermis over a muscle to cause local degeneration of the muscle. The muscles showed a mixed early metamorphic response with some fibers regressing and others remaining contractile. The motoneuron arbor withdrew endplates from the regressing fibers but maintained endplates on fibers that were intact. Thus, the regression of the muscle is sufficient to cause the withdrawal of endplates. The unresolved issue, though, is whether target regression is *necessary* for synapse removal to occur. In other words, would suitably hormone primed motoneuron still withdraw contact from a

healthy target muscle? Manipulation of steroid receptors, as described in Section 4.9.1, suggests that the target response is not necessary for the neuron to prune back dendrites and axons. The loss of endplates described above may be a homeostatic response of the neuron to target degeneration, and may not be relevant to the changes seen during metamorphosis.

4.7.2.2. Hormonal control of arbor outgrowth The outgrowth of pruned axonal arbors involves organization of the outgrowth zone followed by scaffold production and synaptogenesis (Figures 9 and 10). The ecdysteroid requirements for some of these events have been addressed primarily in *Manduca*. The growth zone organization phase occurs during the first few days of adult differentiation, when the ecdysteroid titer is dominated by ecdysone with relatively low levels of 20E. The concentration of 20E begins its rapid rise about day P + 7 to 8, and correlates with the shift to the rapid branch extension that underlies scaffold building (Figure 10). Synaptogenesis begins on about day P + 12 as the 20E titer begins to fall (Truman and Reiss, 1995).

The organization of the growth zone requires the initiation of the adult peak of ecdysteroids, as shown by diapausing pupae, which lack 20E and maintain their neurons in a post-pruning state for months. A growth zone only begins to form, when these pupae are provided with 20E (Truman and

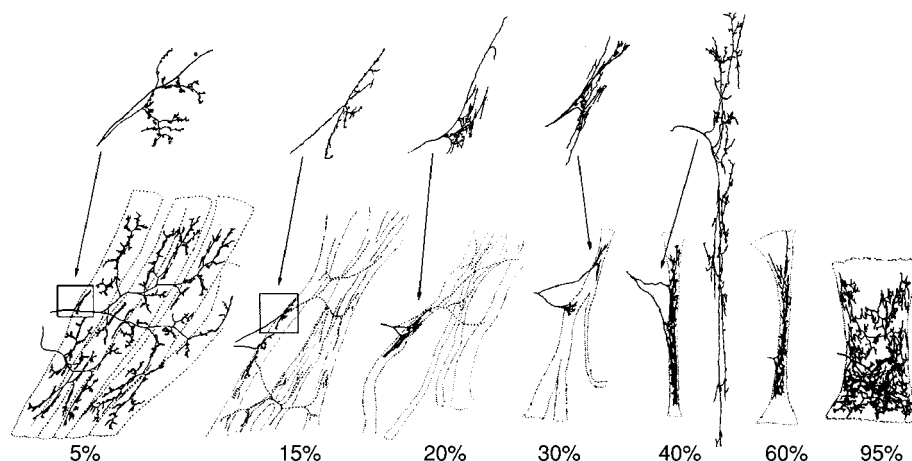


Figure 10 Camera lucida drawings showing the remodeling of the axonal arbor of motoneuron MN-12 through the pupal–adult transition (indicated as percentage of development) of *Manduca sexta*. The target muscle and its remains are shown in dotted outline. The arbor in the boxed area is shown at higher magnification above. Between 5 and 15% of development, the axonal arbor undergoes a massive collapse, leaving only two attachment sites on the remains of the first muscle fiber. These contact sites grow in size and complexity through 30% and then rapidly extend and branch over the proliferating muscle. Adult muscle differentiation and adult endplates are then elaborated between 60 and 95% of development. (Data from Truman, J.W., Reiss, S.E., 1995. Neuromuscular metamorphosis in the moth *Manduca sexta*: hormonal regulation of synapses loss and remodeling. *J. Neurosci.* 15, 4815–4826; © by the Society for Neuroscience.)

Reiss, 1995). The concentration of steroid provided, though, is critical, as demonstrated by injecting pupae with a high dose of 20E early in adult development, thereby mimicking the rise in 20E that occurs on day P + 8. The early 20E causes the premature extension of the axonal arbor over its muscle target (Truman and Riddiford, 2002). Hence, E or low levels of 20E are associated with growth zone organization, whereas high levels of 20E shift the neuron into branch extension and scaffold formation.

A number of *in vitro* studies with isolated, identified neurons show that neurite outgrowth is a cell-autonomous response that can be elicited by 20E (review: Levine and Weeks, 1996). The earliest study utilized *Manduca* leg motoneurons (Prugh *et al.*, 1992), which were labeled *in vivo* by injecting a retrograde tracer into the leg of the caterpillar. Thoracic ganglia were then dissociated and the leg motoneurons grown in low density cultures. In the absence of 20E, these motoneurons extended an axon and showed long-term survival. When cultured with $1 \mu\text{g } 20\text{E ml}^{-1}$, however, they showed extensive branching growth that mimicked their response to 20E *in vivo*. The 20E treatment had a striking effect on the form and the cytoskeletal composition of the growth cones of these neurons (Matheson and Levine, 1999). Growth cones from 20E-exposed neurons were larger and had an increased number of microtubule-based branches, presumably leading to enhanced formation and retention of higher order branches. Interestingly, if the same neurons were explanted from larval rather than pupal nervous systems, they showed no sprouting but a slight reduction in branching when challenged with steroid. Therefore, the stage specificity of the nature of the response that the cell shows *in vivo*, in terms of steroid-induced pruning versus sprouting, is preserved when the neurons are treated in isolation.

Most *in vitro* studies of identified neurons in both *Manduca* and *Drosophila* (e.g., Prugh *et al.*, 1992; Kraft *et al.*, 1998; Matheson and Levine, 1999) have used a concentration of 20E of $1 \mu\text{g ml}^{-1}$. The study by McGraw *et al.* (1998), though, examined the role of different concentrations of 20E on the pattern of neurite outgrowth from identified neurosecretory cells (Figure 11). 20E levels in the 100 ng ml^{-1} range were ineffective in supporting the growth of secondary and tertiary branches, and concentrations over $1 \mu\text{g ml}^{-1}$ were needed for this higher-order branching. Interestingly, the neurons grew the most elaborate arbors if they were first exposed to 100 ng ml^{-1} for 5 days before the shift to the high steroid concentration. Possibly, the low dosage of

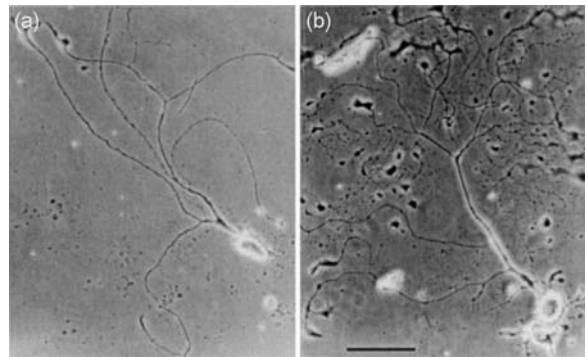


Figure 11 Photomicrographs of single lateral neurosecretory cells (LNCs) *in vitro*. (a) A single LNC in culture exposed to $100 \text{ ng } 20\text{E ml}^{-1}$ for the entire 10-day period of growth. (b) A single LNC exposed to $100 \text{ ng } 20\text{E ml}^{-1}$ for the first 5 days *in vitro* and then shifted to $1.5 \mu\text{g } 20\text{E ml}^{-1}$ for days 6–10. (From McGraw, H.F., Prier, K.R.S., Wiley, J.C., Tublitz, N.J., 1998. Steroid-regulated morphological plasticity in a set of identified peptidergic neurons in the moth, *Manduca sexta*. *J. Exp. Biol.* 201, 2981–2992.)

steroid was having an effect similar to growth zone organization seen *in vivo*, which then resulted in a more extensive arbor, when the cell was shifted to the high hormone conditions.

The ecdysteroid requirements for the last phase, that of synaptogenesis, have not been examined. As with other events that occur during the latter half of adult differentiation (Schwartz and Truman, 1983), it is likely that the withdrawal of 20E may be essential for these events to go to completion.

JH is notable during the outgrowth phase only by its absence. In *Manduca*, the experimental application of JH mimics prior to the ecdysteroid rise results in a severe suppression of outgrowth, and neurons remain in a stunted, pupal-like form (Truman and Reiss, 1995). This ability of early JH treatment to suppress adult differentiation of these motoneurons is not surprising and is consistent with JH causing the repetition of a pupal molt. The more intriguing results are seen with later applications of JH after the neuron has been committed to its adult differentiation. For *Manduca* motoneurons, late treatment with JH has a quantitative effect and results in neurons with reduced adult arbors. The manner by which JH exerts quantitative control over arbor growth is discussed for the remodeling of sensory neurons in Section 4.8.2.

One question concerning the quantitative effects of JH on neuronal form is whether the effect of JH is directly on the neuron or indirect through effects on its targets. This is especially pertinent for the motor arbors, since the JH treatments reduce the size of both the target muscle and the arbor of the neuron that grows over it. Local application

of JH in *Manduca*, though, showed that local treatment to the muscle caused the expected reduction of muscle growth but did not suppress the normal elaboration of the motoneuron's axonal arbor (Truman and Reiss, 1995). Hence, the reduced arbor size seen after systemic treatment with JH is likely due to a direct action on the neuron itself.

4.7.3. Differentiation of Adult-Specific Neurons

4.7.3.1. Differentiation of arrested interneurons At the outset of metamorphosis, the larva possesses two classes of arrested interneurons: a small number of neurons born during embryogenesis and a much larger set born later during larval growth. Studies on tangential visual interneurons in *Drosophila* suggest that these two sets of neurons may differ in their developmental time-tables during metamorphosis (Taghert *et al.*, 2000). The OL2-A and OL3 classes of visual interneurons express the neuropeptide dFMRFamide in the adult. The large OL2-A neurons are born during embryogenesis and already show some morphological differentiation at the start of metamorphosis. Based on a *dFMRFa* reporter, the OL2-A neurons start transcribing the *dFMRFa* gene at the beginning of metamorphosis and start expressing the dFMRFamide peptide soon thereafter. The numerous OL3 neurons are generated postembryonically. They begin transcribing the *dFMRFa* gene slightly later than the OL2-A neurons but delay peptide expression until just before adult ecdysis. At this time, it is not known if the OL neurons present a unique case or if this difference in developmental trajectory is consistent for adult-specific neurons that have embryonic versus postembryonic origins.

Studies in *Manduca* have examined the hormonal requirements needed to initiate differentiation of the arrested imaginal neurons (Booker and Truman, 1987b). Resumption of development occurs during wandering, as the neurons show enlargement of their somata and dendritic and axonal sprouts appear (e.g., **Figure 4**). The *Manduca* studies focus on the abdominal lineages and deal only with cell survival and soma size. In the days following wandering, some of the immature neurons within a cluster degenerate, while others show soma enlargement. When larval abdomens were isolated either before (day 5 + 2) or after (W 0) the wandering pulse of ecdysteroids, both neuronal loss and soma growth were prevented. Infusion of 20E into such abdomens restored the normal pattern of cell loss and soma growth. The effect of JH treatment on cell loss was complex, but treatment with a JH mimic suppressed the growth-promoting effects of 20E, irrespective of whether it was given before or after the wandering peak of ecdysteroids. Therefore, unlike remodeling

larval neurons, these adult-specific neurons do not show "commitment" in response to the wandering peak of ecdysteroids. Their early outgrowth is responsive to the JH that normally appears during the prepupal peak. This action of JH may prevent these neurons from showing precocious outgrowth in response to the prepupal peak of ecdysteroids.

4.7.3.2. Visual interneurons of the optic lobes Although the majority of postembryonic neurons likely deal with new adult sensory information, the sensory neurons play no role in their production. Indeed, neurogenesis in the CNS is completed well before sensory axons reach the CNS. An exception to this generalization, though, occurs in the developing visual system. Rows of ommatidia are added progressively from the posterior to the anterior margin of the eye disc, and the outer layers of the optic lobe, the lamina and medulla, undergo a similar sequential production of visual interneurons. Neuroblasts in an outer proliferation zone (OPZ) generate the neurons for both of these regions. In early larval stages, symmetrical cell divisions within the OPZ expand the population of NBs. In the last larval stage, though, the NBs switch to asymmetrical divisions that produce GMCs, which then make neurons. In *Manduca*, this shift to neurogenesis requires the decline in JH (Monsma and Booker, 1996), and medulla neurons start being born early in the last larval stage. Lamina neurons, however, only appear after wandering, as the the first retinal afferents reach the optic lobe. In both *Manduca* (Monsma and Booker, 1996) and *Drosophila* (Selleck *et al.*, 1992), removal of the afferents prevents the birth of lamina neurons, although medulla neurons continue to be made. Studies on *Drosophila* show that the lamina GMCs undergo a G1 arrest and degenerate, unless they are contacted by the afferent axons, which supply Hedgehog, which drives them through the G1 blockage (Huang and Kunes, 1996).

The birth of lamina neurons has a unique requirement for afferent axons, but all regions of the optic lobe are sensitive to ecdysteroids. The ecdysteroid requirement is absolute in *Manduca* (Champlin and Truman, 1998a). In the absence of E or 20E, the cells in the outer proliferation zone arrest in the G2 phase of the cell cycle. A pulse of 20E, as short as 1.5–2 h, is sufficient to remove the block and send cells into mitosis, but they then arrest at the next G2 phase if no steroid is present. Therefore, during each cell cycle there is a single checkpoint, when steroid concentrations are assessed, and sustained proliferation therefore requires the tonic presence of E or a moderate level of 20E. The control point

is likely the transcription of the *Manduca* homolog of *string/cdc2 phosphatase*. This gene is a prominent regulator of the transition from G2 to M phase (Edgar and O'Farrell, 1989). In G2-arrested neuroblasts *string* transcription is directly and rapidly induced by exposure to 20E (D. Champlin and J.W. Truman, unpublished data).

Besides acting via transcriptional control, ecdysteroids also influence neurogenesis through a rapid response pathway that likely does not involve the nucleus. The neuroblasts in the OPZ respond to threshold 20E concentrations in an all-or-none fashion – either all of the cells are cycling or none of them (Figure 12a; Champlin and Truman, 2000). This all-or-none coordination within a lobe is mediated through local suppression of proliferation caused by nitric oxide (NO). Inhibitors of NO production, such as L-NAME, abolish this coordination and lower the levels of steroid needed to drive proliferation. Under these conditions, though, one now sees a graded response to the concentration of ecdysteroids (Figure 12b and c). The source of NO in the OPZ appears to be the NBs themselves. Treatment with moderate levels of 20E suppresses NO production within 15 min and is independent of RNA synthesis. The effect of NO appears to be downstream of *string* transcription because

treatment with 20E in the presence of NO results in the rapid induction of *string* transcripts but the NBs still remain blocked in G2 (D. Champlin and J.W. Truman, unpublished data). Hence, 20E provides a stimulatory signal to NBs to promote neurogenesis and also acts to remove a local inhibition from the neighboring NBs. While ecdysteroids promote neurogenesis at moderate concentrations in *Manduca*, high levels of 20E terminate neurogenesis by causing the death of the NBs (Champlin and Truman, 1998a).

Drosophila differs from *Manduca* in that there is not an absolute requirement for E or 20E to support neurogenesis in the OL. Moderate levels of 20E do enhance proliferation and, as in *Manduca*, high levels of 20E terminate it. In the absence of 20E, neurogenesis is extended beyond its normal time and the brain overproduces optic lobe neurons (T. Awad and J.W. Truman, unpublished data).

4.8. Metamorphosis of the Sensory System

The transformation from a sedentary larva to an active, flying adult requires a profound change in how the insect senses its world. Most of the larval sensory system is directed towards proximal stimuli,

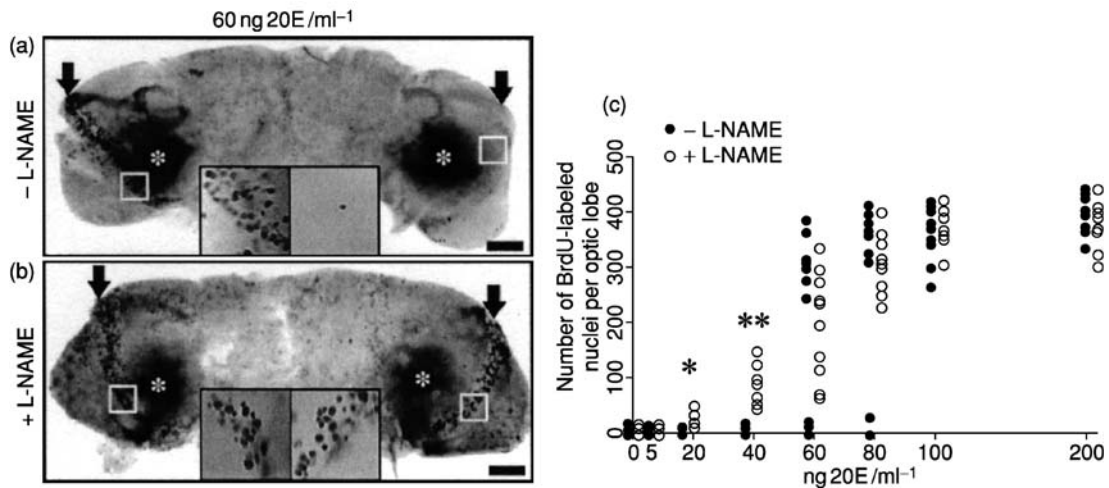


Figure 12 Ecdysteroid-dependent proliferation in the presence and absence of an inhibitor of nitric oxide synthase (NOS). Brains were collected 1 day after pupal ecdysis, desheathed, and cultured for 24 h in various concentrations of 20E. They were then pulsed with Bromodeoxyuridine (BrdU) for 2 h, and the number of cells incorporating BudR was then determined by immunocytochemistry. (a, b) Photomicrographs of brains cultured with 60 ng 20E ml⁻¹. In (b) 1 mM L-NAME was included from the start of the culture. BudR-labeled nuclei are black; arrows point to the outer proliferation zone. * show pigment spots present in each optic lobe. The insets are higher magnification views of the boxed areas in each optic lobe. The brain in (a) illustrates the all-or-none character of the proliferation response seen at threshold concentrations of 20E, with one hemisphere inactive while the other is in full proliferation. With the NOS inhibitor (b), both lobes show similar, moderate levels of proliferation to the same concentration of 20E. (c) The number of cells that labeled with BudR after various treatments. Each point is from a single optic lobe incubated at the indicated 20E concentration with or without L-NAME. The low concentration data sets differ by *, $P < 0.05$, and **, $P < 0.005$. (Data from Champlin, D.T., Truman, J.W., 2000. Ecdysteroid coordinates optic lobe neurogenesis via a nitric oxide signaling pathway. *Development* 127, 3543–3551.)

depending heavily on mechanoreception and having poorly developed visual and olfactory capacities. The adult has elaborate antennae and compound eyes, adapted for perceiving stimuli at distances, and batteries of new external sense organs associated with the adult legs, wings, and genitalia. The extent of carryover of larval sensory receptors into the adult stage varies. In *Drosophila*, all of the larval external sensory neurons degenerate, and adult external sensory neurons arise during the differentiation of imaginal discs and histoblast nests. The few larval sensory neurons that persist through metamorphosis are proprioceptors and some of the da sensory neurons. The axons from these persisting cells provide pathways that are utilized by the axons from adult-specific neurons, as they navigate towards the CNS (Usui-Ishihara *et al.*, 2000; Williams and Shepherd, 2002).

In contrast to *Drosophila*, *Manduca* shows a greater percentage of larval sensory neurons that are carried through metamorphosis. The transition between the larval and adult sensory systems has been best described for the thoracic legs. As the larval leg undergoes degeneration and replacement by the adult leg, most of the larval sensory neurons degenerate but some of the neurons that supplied the external sensilla and the chorditonal organs persist through metamorphosis (Consoulas, 2000). Of special interest are the neurons of larval femoral chorditonal organ. While about half of the original 13 neurons that supplied the larval organ degenerate, the remainder serve to organize the adult structure. They are joined by 45–60 new neurons to make up the chorditonal organs of the adult leg (Consoulas *et al.*, 2000b). While most studies of the hormonal control over the metamorphosis of the peripheral nervous system have focused on differentiation of adult-specific neurons, there is some information on remodeling sensory systems.

4.8.1. Remodeling of Larval Neurons: Pupal-Specific Modifications

The pupa stage has little behavioral relevance for the vast majority of central neurons, but this is not true for the sensory system. Pupae display some specialized behaviors, and a small set of peripheral and central neurons are dedicated to maintain these behaviors as the rest of the nervous system undergoes its metamorphic upheaval. In *Manduca*, these pupal-specific behaviors include abdominal respiratory movements and a pupal-specific defensive reflex, the gin-trap reflex. The neural basis of the latter is best understood (Bate, 1973).

Abdominal segments A4 to A6 of the pupa bear sharp-sided cuticular cavities, the gin traps, that

protect the pupa from small soil arthropods. Stimulation of the sensory hairs in the trap excites an intersegmental interneuron that then excites the motoneurons to the ipsilateral, internal longitudinal muscles causing the rapid closure of the trap, crushing whatever is inside. The sensory hairs within the gin trap are recruited from the larval sensory hairs along the anterior border of the segment. In their transition to their pupal state, the hairs acquire a new morphology, and the sensory neurons expand their axonal projections within the CNS (Figure 13).

The studies on the metamorphosis of the gin-trap system was the first use of JH-induced mosaics to dissect developmental interactions within the CNS (Levine *et al.*, 1986). Local application of JH to larval epidermis, fated to form the gin-trap, resulted in larval cuticle being deposited in that region, while the remainder of the animal became pupal. A normal gin-trap formed on the control side, and its sensory neurons showed their normal axonal expansion and could drive the pupal reflex (Figure 13a). On the treated side, by contrast, the projection pattern of the sensory neurons remained larval, and stimulation of the cells could not evoke the closure reflex.

The pupal-specific sprouting of the sensory neuron requires both an exposure to 20E as well as the absence of JH. The 20E requirement was shown by permanently arresting the development of the larval abdomen by ligating the abdomen after the wandering stage. Under these conditions, the neurons destined for the gin trap permanently retain their larval morphology. Topical application of 20E in undecane to the presumptive gin trap region caused a local pupal molt in the underlying cells, while the remainder of the abdomen, including the CNS, remained arrested. The gin-trap sensory neurons showed their pupal sprouting, despite doing so in a CNS that was permanently larval (Figure 13b; Levine, 1989). These pupal neurons, though, could not initiate a gin-trap reflex, presumably because the interneuron involved in the reflex still was in its larval form. Together, these two sets of experiments show that the growth of the sensory arbor is not induced by the state of the CNS, but rather depends on the hormonal conditions experienced in the periphery, where the cell body resides.

4.8.2. Adult-Specific Remodeling of Sensory Neurons

The da sensory neurons extend complex dendritic arbors over the epidermis of the larva. At metamorphosis neurons like ddaE have their dendrites severely pruned back, the cell bodies migrate dorsally and then elaborate new dendritic trees (Williams and Truman, 2004). During their remodeling, these

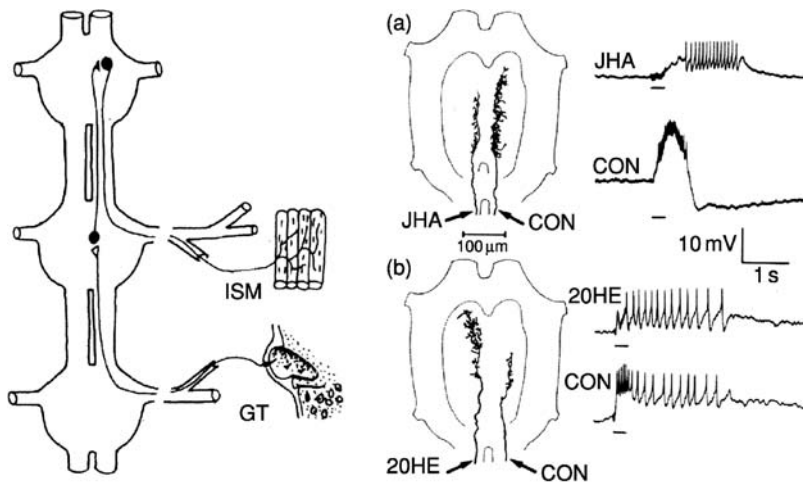


Figure 13 Hormonal control over the larval-pupal transformation of the gin-trap system. (Left) Drawing showing the relationship between the sensory hairs in the gin-trap (GT) and the motoneurons to intersegmental muscles (ISM) that mediate the trap closure. (Right) Motoneuron responses to stimulation of the trap sensory neurons in heterochronic mosaic animals. (a) The A4 ganglion from a mosaic pupa, in which the presumptive gin-trap region of the left fifth segment was treated topically with a JH mimic. The drawing shows that single sensory neurons from the JH treated region retained their sparse larval arbor, whereas the neuron from the control side grew the expanded terminal arbor characteristic of the pupal stage. To the right of the drawing are shown the responses of ipsilateral, pupal ISM motoneurons to stimulation of treated and control sensory neurons. The control neurons evoked the abrupt excitation characteristic of the closure response, whereas the treated neurons evoked a low-level, sustained, larval-like response. (b) The A4 ganglion from a mosaic larva. After abdominal ligation early in the 5th larval stage, the presumptive gin-trap region on one side of the fifth segment was treated with 20E. The contralateral control neurons maintained their larval pattern of branching but the 20E-treated cells grew pupal-like arbor. In both cases, however, the stimulation of the sensory neurons evoked larval-like responses from the ipsilateral motoneurons. ((Right) Data from Levine, R.B., Weeks, J.C., 1990. Hormonally mediated changes in simple reflex circuits during metamorphosis in *Manduca*. *J. Neurobiol.* 21, 1022–1036.)

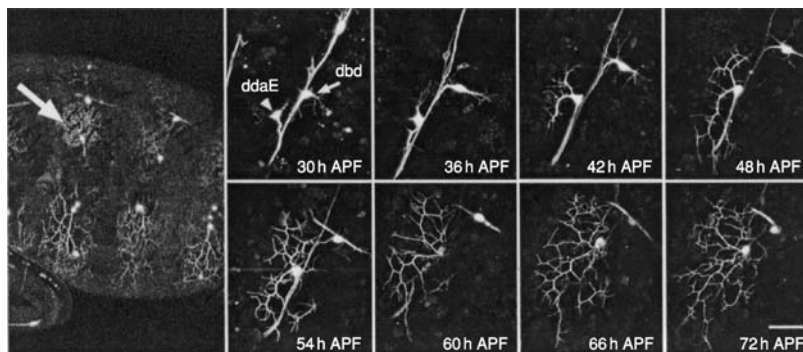


Figure 14 Adult outgrowth of the dendrites of the da sensory neuron ddaE. (Left) Low-power confocal image of the lateral aspect of the abdomen of a live developing adult of *Drosophila* at about 70 h APF (after puparium formation). The da neurons are expressing a membrane-targeted green fluorescent protein (GFP); arrow shows one of the segmentally-repeated ddaE's. (Right) Confocal images of the same set of neurons followed through metamorphosis from 32 to 72 h APF showing the time-course of adult dendrite outgrowth by ddaE. Dbd is a sensory neuron that produces a dorsal stretch receptor. (From Williams, D.W., Truman, J.W., 2004. Mechanisms of dendritic elaboration of sensory neurons in *Drosophila*: insights from in vivo time-lapse. *J. Neurosci.* 24, 1541–1550; © by the Society for Neuroscience.)

sensory neurons progress through similar phases to those seen for central neurons, except that cell body migration occurs as new outgrowth zones are being established. Also, the dendrites of these primary sensory neurons do not receive synaptic inputs.

The growth of the adult dendrites by the da neurons occurs during the surge of 20E that causes adult

differentiation. As evident from the time-lapse images in **Figure 14**, the dendrites show a rapid extension that is also accompanied by occasional branch retractions and the formation of interstitial branches. After the overall form and extent of the arbor is established, the tree then fills in with higher order twigs (Williams and Truman, 2004). The

manner by which 20E directs this outgrowth has not been examined, but the effects of JH are notable. As with other tissues in *Drosophila*, JH treatment does not suppress the larval–pupal transition of these cells – i.e., they prune back normally irrespective of the presence or absence of JH. JH treatment, though, does impair the adult outgrowth of these cells, and they remain in a pupal-like form. Time-lapse studies suggest that the failure of these JH-treated cells to extend branches may be due to the persistence of the strong retraction programs that were part of the pruning process (Williams and Truman, 2004). The effect of JH treatment on the maintenance of retraction programs is more evident for animals treated with JH mimics at about 24 h APF. As with late treatment of motoneurons (see Section 4.7.2.2), da sensory neurons in such animals grow an adult arbor but it is reduced in extent and complexity. These neurons show a normal outgrowth program as measured by the rate of birth of new dendritic branches. The retraction programs, however, are maintained beyond their normal time, thereby reducing the rate of branch extension and the number of branches that finally persist. The manner by which JH prevents the inactivation of retraction programs is unknown.

4.8.3. Control Over Birth of Adult Sensory Neurons

Studies with cultured wing imaginal discs from wandering larvae show that the 20E of the pupariation peak is required for the birth of the sensory neurons on the anterior margin of the wing of *Drosophila*. The effect of this hormone, though, is to relieve a repression imposed by the ecdysone receptor complex (Schubiger and Truman, 2000). This relationship first became evident from experiments, in which clones lacking *Usp* were induced in wing imaginal discs (Schubiger and Truman, 2000). Neuronal birth and axonal outgrowth occurred earlier within such clones as compared to surrounding, wild-type tissues. Similar advancement in development with the lack *Usp* was also seen for the ecdysone-dependent movement of the morphogenetic furrow in the eye disc (Zelhof *et al.*, 1997; Ghbeish *et al.*, 2001). Importantly, when wing discs containing such clones were transferred into culture lacking 20E, they showed the expected arrested development except for the patches that lacked *Usp*. The latter cells progressed into neurogenesis and axonal outgrowth despite the lack of steroid (Figure 15). More recent experiments using RNAi techniques to remove EcR (Roignant *et al.*, 2003) similarly showed that the lack of EcR also brings about the precocious

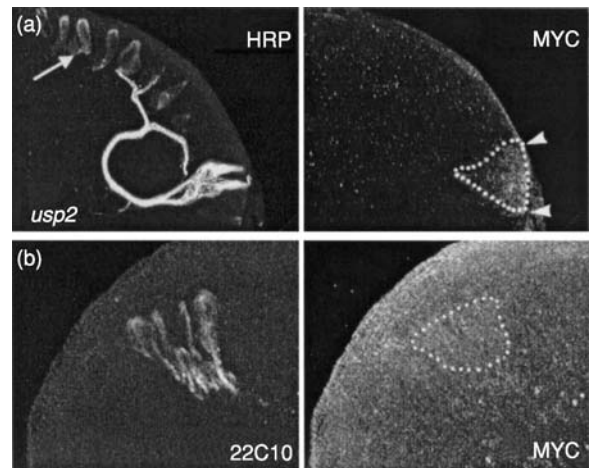


Figure 15 The effect of the lack of *Usp* on the early birth and differentiation of sensory neurons in the wing disc of *Drosophila*. The wing discs bore clonal patches of tissue that lacked a functional *usp* gene and were marked by expression of the MYC protein (right, enclosed by dotted area). Neuronal differentiation was determined by immunostaining for a horseradish peroxidase (HRP) epitope or with the monoclonal antibody 22C10 (left). (a) *In vivo* at pupariation, the sensory neurons along the anterior wing margin (arrow) are just beginning axonal outgrowth and expression of the HRP epitope, but within the *usp* null clone the neurons are well advanced in their development with extensive axonal outgrowth. (b) Wing discs from early wandering larvae that were maintained *in vitro* in the absence of 20E. Metamorphic development failed to occur in the wild-type tissue, but cells within the *usp* null clone showed normal neurogenesis and axonal outgrowth despite the lack of steroid. (Data from Schubiger, M., Truman, J.W., 2000. The RXR-ortholog *Usp* suppresses early metamorphic processes in the absence of ecdysteroids. *Development* 127, 1151–1159.)

birth and axonal outgrowth by wing sensory neurons (M. Schubiger and J.W. Truman, unpublished data). Consequently, in the imaginal discs, the EcR/*Usp* complex functions as a potent repressor of genes involved in the selection and differentiation of sensory neurons. The major role of ecdysteroids is to relieve this repression, thereby allowing development to progress.

Although ecdysteroids are clearly involved in controlling the birth of adult sensory neurons, relatively little is known about how the steroid titer impacts their subsequent differentiation. One case in which we have some insights, though, is in the development of chemosensory sensilla on the antenna of *M. sexta* (Vogt *et al.*, 1993). The final maturation of the chemosensory sensilla occurs during the last 10% of adult development with the appearance of odorant binding proteins (OBPs) and odorant degrading enzymes. In cultured antennae, OBP expression could be induced prematurely if 20E was absent from the culture medium. The addition of

20E then blocked the premature expression of the OBPs. Hence, declining levels of ecdysteroid may have an important function in coordinating the terminal maturation of the sensory organs.

4.8.4. Sensory Systems: The Compound Eye

The building of the compound eye is one of the spectacular achievements of metamorphosis. The organization of the eye imaginal disc begins at the posterior border of the disc and progresses anteriorly as the morphogenetic furrow moves across the disc. In *Manduca* the hormonal control over the organization of the eye disc is relatively simple because the establishment of the furrow occurs after wandering, when metamorphosis has commenced. In *Drosophila*, by contrast, the establishment and early movement of the furrow is advanced to the middle of the last larval stage. The role of hormones in controlling this early patterning of the disc is unclear, but once wandering has begun, the furrow movement then appears to require ecdysteroids (Brennan *et al.*, 2001).

The analysis of the role of ecdysteroids in the formation and patterning of the compound eye is most

detailed for *Manduca* (Figure 16; Champlin and Truman, 1998a). In this insect, the eye imaginal disc does not form until well into the last larval stage. The earlier larval instars have a crescent-shaped eye primordium immediately anterior of the stemmata, but during the larval molts these cells make head capsule cuticle, as do their neighbors. Their developmental potential becomes apparent early in the last larval stage, when they enlarge, detach from the overlying cuticle, and begin proliferating to form the eye imaginal disc. The growing disc rapidly enlarges until the time of wandering, at which time it becomes dependent on ecdysteroid to support further proliferation. The divisions in this latter phase are directed towards the patterning of the eye disc and support the movement of a morphogenetic furrow. In the wake of the furrow, cells differentiate into the photoreceptors and the cone and pigment cells that comprise the ommatidia. The progression of the furrow begins the day after wandering and is completed about 10 days later, well into adult differentiation.

In vitro experiments show that both E and 20E support proliferation and movement of the furrow (Champlin and Truman, 1998b). The levels of

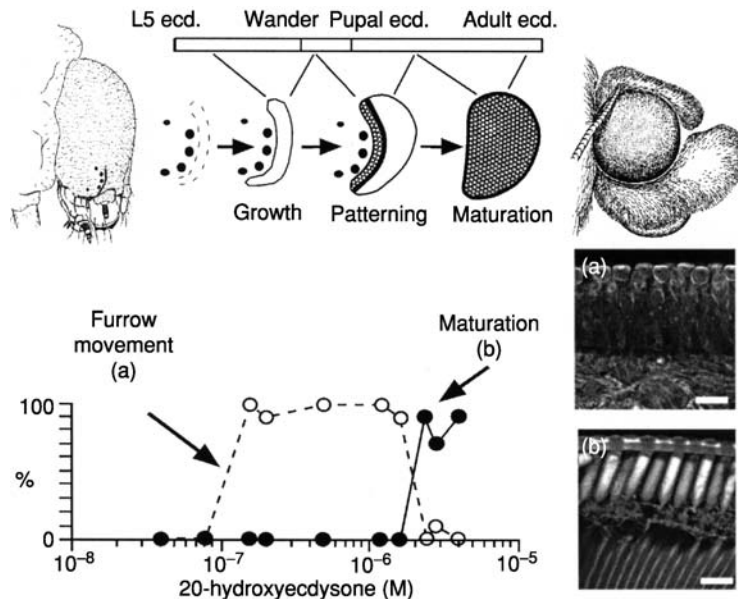


Figure 16 Eye development in *Manduca sexta*. (Top) Time-line of development: at the start of the last larval stage, the crescent-shaped eye primordium (dashed line) lies just anterior to the larval stemmata. These cells detach and begin proliferating by 2 days into the last instar and form the eye imaginal disc. After the larva stops feeding and begins the wandering stage, low levels of ecdysteroid support the movement of the morphogenetic furrow to organize the ommatidia units that then mature in response to high levels of 20E. (Bottom) Summary of the response of the pupal eye imaginal discs to treatment with 20E *in vitro*. (Left) The dose-response relationship showing the 20E concentrations needed to evoke mitosis and movement of the morphogenetic furrow (open circles), and the cellular maturation of the ommatidial units (filled circles). (Right) Confocal optical sections of the region posterior to the furrow. In response to low levels of 20E (a), the ommatidial cell types differentiate and organize into ommatidial clusters. High concentrations of 20E (b) induce terminal maturation as manifest by the synthesis of the screening pigments, cuticular lens, crystalline cones, and elongated rhabdomeres. Scale bars = 25 μ m. (Data from Champlin, D.T., Truman, J.W., 1998b. Ecdysteroids govern two phases of eye development during metamorphosis of the moth, *Manduca sexta*. *Development* 125, 2009–2018.)

20E needed to support proliferation are lower than E, but the latter levels are still in the physiological range seen during early adult differentiation (Warren and Gilbert, 1986). As discussed for the optic lobes above (see Section 4.7.3.2), proliferation and patterning of the eye disc requires the tonic presence of steroid but levels must be maintained in a range between 60 and 1000 ng 20E ml⁻¹. Levels above 1000 ng ml⁻¹ permanently suppress proliferation and furrow movement and cause the cells behind the furrow to undergo maturation and make rhabdoms, crystalline cones, screening pigments etc. (Figure 16). The eye disc cells in front of the furrow, by contrast, are undetermined and are induced to make a naked, adult cuticle (Champlin and Truman, 1998b). The exposure to high levels of 20E initiates a differentiative program that can continue even if the steroid is subsequently withdrawn.

Once the imaginal disc has started ecdysteroid-dependent patterning, the tissue can be shifted into the terminal differentiation at any time by raising the 20E titer above 1000 ng ml⁻¹. Hence, if high levels of 20E are experimentally supplied precociously, then a miniature eye forms (Champlin and Truman, 1998b). One complication with this relationship is that the patterning eye disc is actually exposed to levels of 20E above 1000 ng ml⁻¹ twice; first during the larval pupal transformation and then at about day 8 of adult development. Importantly, though, JH is also present during this first exposure to high levels of 20E. If JH is experimentally removed by allatectomy of the larva, then the high 20E levels of the larval-pupal molt do indeed promote early eye differentiation, and the pupa forms with a miniature adult eye (Kiguchi and Riddiford, 1978; Champlin and Truman, 1998b). *In vitro* experiments on the differentiation of the ventral diaphragm muscle in *Manduca* show that JH does not interfere with responses to moderate levels of 20E but suppresses the new responses that occur when shifting to high levels of 20E (Champlin *et al.*, 1999).

Experiments, in which Usp function was removed from the patches of cells in the eye imaginal disc of *Drosophila*, also illustrate the complex control of the differentiation of this structure (Zelhof *et al.*, 1997; Ghbeish *et al.*, 2001). In clones of cells that lack *usp*, the furrow shows an advancement over that seen in surrounding wild-type tissue. This result is similar to that seen for the wing sensory neurons (see Section 4.8.3) and argues that ecdysteroids regulate furrow progression by removing the repression imposed by EcR/Usp. Later on, Usp also has a role in hormone-dependent activation (Ghbeish *et al.*, 2001).

4.9. Steroid Receptors and the Coordination of the Metamorphic Development

4.9.1. Relationship of Receptor Expression and Cellular Responses

Indications that steroid receptor levels vary through time in the CNS came with autoradiographic studies of the binding of radiolabeled ponasterone. Bidmon *et al.* (1991) reported that very few brain neurons showed ponasterone accumulation early in the last larval stage but that the number increased dramatically shortly after wandering, and then declined as the ecdysteroid peak declined. Substantial ponasterone binding then reappeared after pupal ecdysis. The variation in ponasterone binding at certain times could be correlated with different steroid-dependent fates. For example, at the end of metamorphosis neurons fated to die showed much higher levels of ponasterone binding than did those that would persist in the adult stage (Fahrbach and Truman, 1989).

Studies of receptor distributions became easier with the identification of the EcR gene (Koelle *et al.*, 1991) and with the production of antibodies to detect the receptor protein. The story became complex, though, with the discovery that there were three isoforms of the receptor (Talbot *et al.*, 1993) and that these were expressed in a complex pattern through metamorphosis (Figure 17; Truman *et al.*, 1994; see Chapter 7). The expression patterns of only EcR-A and EcR-B1 are known in detail. That of EcR-B2 is generally assumed to be similar to EcR-B1, but this remains to be established. The pattern of EcR-B1 and -A expression mirrors the diverse origins and cellular fates of the neurons in the CNS. Little EcR expression is detected in neurons through the early larval instars. Also, when EcRs that carry dominant negative mutations (Cherbas *et al.*, 2003) are expressed in larval neurons, they produce no obvious effects on the morphology or functioning of the neurons (J.W. Truman, L. Cherbas, and P. Cherbas, unpublished data). This suggests that the ecdysteroid signaling pathway has little function in most larval neurons prior to metamorphosis.

Detectable EcR first appears in *Drosophila* neurons in the middle of the last larval instar in preparation for metamorphosis. As summarized in Figures 9 and 17, each of the phases in metamorphic remodeling of larval neurons is associated with a different combination of EcR isoforms. EcR-B1 is at its highest levels in larval neurons and in the γ -neurons of the mushroom bodies just before both sets of cells begin pruning back their larval processes (Truman *et al.*, 1994; Lee *et al.*, 2000). All other

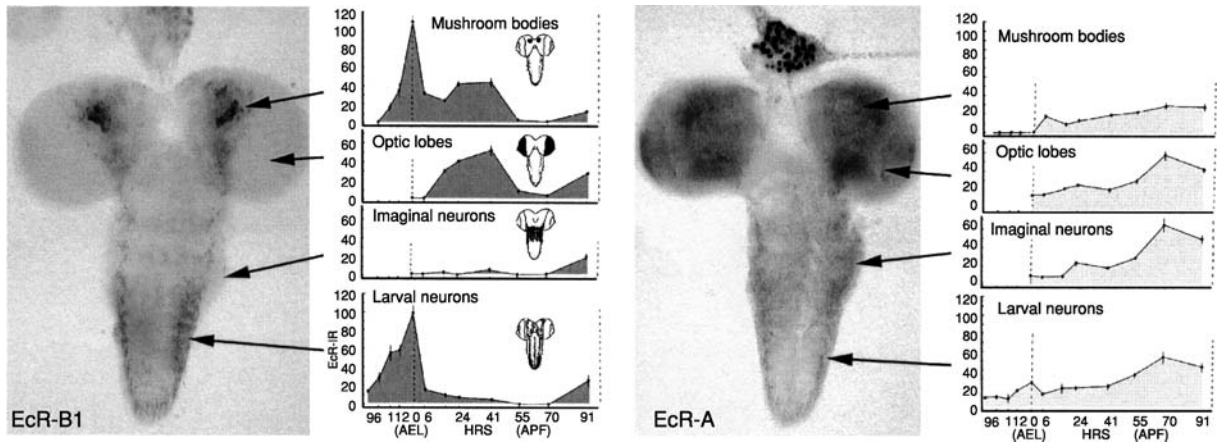


Figure 17 Temporal and spatial variation in the expression of the EcR-B1 (Left) and EcR-A (Right) through metamorphosis. The photomicrograph shows the distribution of both isoforms in the brain lobes and ventral nervous system at the time of puparium formation; the endocrine ring gland is located in front of the brain at the top of the picture. The graphs show the isoform-specific immunoreactivity in various types of neurons as determined by confocal microscopy. Major peaks of ecdysteroid occur at pupariation, and about 24–40 h after puparium formation (APF). Hours 96–112 AEL (after egg-laying) are during the 3rd larval instar. (Modified from Truman, J.W., Talbot, W.S., Fahrbach, S.E., Hogness, D.S., 1994. Ecdysone receptor expression in the CNS correlates with stage-specific responses to ecdysteroids during *Drosophila* and *Manduca* development. *Development* 120, 219–234.)

postembryonically derived neurons, as well as the arrested embryonic neurons (J.W. Truman and D.D. Williams, unpublished data), lack this early EcR-B1 expression. The importance of the EcR-B isoforms to the pruning response was established using *Drosophila* mutants that lacked one or both of the EcR-B isoforms. Most larvae homozygous for such mutations die during larval life but a few survive to the start of metamorphosis (Bender *et al.*, 1998), at which time tissues within the larva desynchronize their development; the larval tissues arrest their development but imaginal tissues continue into metamorphosis. Within the CNS, arrested imaginal neurons begin adult differentiation, but larval neurons (Schubiger *et al.*, 1998) and the γ neurons of the mushroom bodies (Lee *et al.*, 2000) do not prune back their larval processes. Since EcR-B mutants show this early metamorphic arrest, it was possible that the lack of pruning was not the direct result of a lack of EcR-B in the neurons in question. Subsequent experiments expressed each of the EcR isoforms in specific neurons in arrested EcR-B mutants. Both axonal pruning in the mushroom body neurons (Lee *et al.*, 2000) and dendritic pruning in the Tv neurosecretory cells (Schubiger *et al.*, 2003) were induced in arrested mutant larvae, when EcR was expressed in the respective neurons. Importantly, EcR-A was relatively ineffective, EcR-B1 was moderately effective, and EcR-B2 was very effective in causing pruning of the larval branches (Figure 18). Hence, the proper receptor isoform(s) is essential for mediating the steroid-induced pruning of these cells. A question remains, though, as to whether the

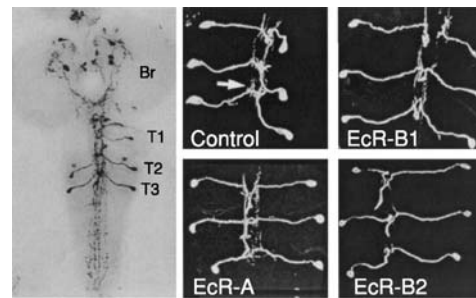


Figure 18 EcR isoform requirements for the pruning of dendritic arbors by the Tv neurosecretory neurons. (Left) Photomicrograph showing the immunostaining of the larval nervous system of *Drosophila* with an antibody against the molluscan neuropeptide SCP_B. The Tv neurosecretory cells in the thoracic neuromeres (T1, T2, T3) show prominent immunostaining (one cell of the Tv pair in T1 is missing). (Right) Confocal images showing the Tv neurons in an EcR-B mutant. The cells were visualized by using the UAS/GAL4 system to express a membrane directed GFP in only the Tv neurons. The fine dendrites at the midline (arrow) are the larval dendrites that fail to be removed in the mutant (Control). For the remaining examples, each of the indicated EcR isoforms was expressed in the Tv neurons in an EcR-B mutant background. Full pruning occurred with expression of the EcR-B2 isoform. (Data from Schubiger, M., Tomita, S., Sung, C., Robinow, S., Truman, J.W., 2003. Isoform specific control of gene activity *in vivo* by the *Drosophila* ecdysone receptor. *Mech. Devel.* 120, 909–918.)

pruning decision is a “binary” one or whether variation in the levels of EcR-B isoforms determines how much of a neuron’s arbor will be removed.

Pruning is the only phase of neuronal development in which EcR isoform requirements have been rigorously established by loss-of-function and gain-of-function experiments. Correlation of isoform

presence with other developmental phases, however, suggests that the EcR-B isoforms may have functions beyond pruning. For example, EcR-B1 persists in remodeling neurons as they organize their growth zone, but then disappears with the beginning of axon elongation and scaffold formation. Perhaps the presence of EcR-B during this phase maintains some branch retraction programs that help in organizing a growth zone, and also prevents the premature extension of the neuron to its final targets.

The neurons of the optic lobes are unique because they do not prune but they show prominent EcR-B1 expression. This expression begins relatively late, starting at 10 h APF, when the last wave of photoreceptor axons arrive in the OL, and lasting until about 45 h APF, when the gradients of development across the optic lobes are finally complete (Meinertzhagen and Hansen, 1993). This EcR-B1 expression in the OL may have a function similar to that proposed above for organization of the new growth zone in remodeling larval neurons, i.e., it may maintain the growth cones of the interneurons in a plastic state until all of neurons across the optic lobe have developed to the state that they can finalize their synaptic partners.

Both remodeling larval neurons and adult-specific neurons express only EcR-A at the time when they extend branches over their target or through the CNS. They all express EcR-B1, however, as they enter into the synaptogenesis phase. The functional roles of these receptor isoforms during this last phase have not been examined experimentally.

A striking aspect of EcR expression is seen in the neurons that will die at the end of metamorphosis. These neurons begin showing high levels of EcR-A early in adult differentiation (18 h APF) and these levels stay elevated until the neurons degenerate at 6–10 h after adult emergence (Robinow *et al.*, 1993). As described above (see Section 4.7.1.2), the disappearance of 20E at the end of metamorphosis is essential to induce *reaper* and to bring about cell death. Since the A/B region of EcR-A appears to have a repressive function (Mouillet *et al.*, 2001), it may be that the protein that represses *reaper* may be EcR-A itself. This repressive function would only be relieved when the steroid titers finally fell at the end of the molt.

4.9.2. Regulation of EcR Isoform Expression

EcR is a direct response gene and treatment with 20E enhances the production of EcR transcripts (see Chapter 7). At least at the start of metamorphosis, the B promoter appears more sensitive than the A promoter, so that rising steroid titers initially favor EcR-B1 expression but then switch to EcR-A

when titers are high enough (e.g., Hiruma *et al.*, 1997). In the developing eye of *Manduca*, for example, levels of 20E that shift the eye from proliferation and patterning to final differentiation also causes a shift in expression from EcR-B1 to EcR-A (Champlin and Truman, 1998b).

The steroid regulation of EcR expression is likely shared by many tissues, but the selection of EcR-isoforms may also depend on cell–cell interactions. For example, in the remodeling musculature of *Manduca*, myoblasts at the site of nerve contact show EcR-B1 expression and elevated mitotic activity. Denervation of the remodeling muscle results in both the rapid loss of EcR-B expression and the suppression of proliferation (Hegstrom *et al.*, 1998). These results suggest that the neuron controls the selection of receptors expressed by the underlying myoblasts. Such interactions also take place in the CNS. Mutations in the TGF- β /activin receptor suppress pruning in the γ -neurons of the mushroom bodies and also blocks the appearance of EcR-B1 in these cells. Importantly, in mutant animals, the selective expression of EcR-B1 in the γ -neurons partially restores the ability of these cells to prune (Zheng *et al.*, 2003).

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References

- Anderson, H., 1978. Postembryonic development of the visual system of the locust, *Schistocerca gregaria*. II. An experimental investigation of the formation of the retina–lamina projection. *J. Embryol. Exp. Morphol.* 46, 147–170.
- Bate, C.M., 1973. The mechanism of the pupal gin-trap. I. Segmental gradients and the connections of triggering sensilla. *J. Exp. Biol.* 59, 95–107.
- Bate, C.M., 1976a. Embryogenesis of an insect nervous system. I. A map of the thoracic and abdominal neuroblasts in *Locusta migratoria*. *J. Embryol. Exp. Morphol.* 35, 107–123.
- Bate, C.M., 1976b. Pioneer neurones in an insect embryo. *Nature* 260, 54–56.
- Bender, M., Imam, F.B., Talbot, W.S., Ganetzky, B., Hogness, D.S., 1998. *Drosophila* ecdysone receptor mutations reveal functional differences among receptor isoforms. *Cell* 91, 777–788.
- Bidmon, H.J., Granger, N.A., Cherbas, P., Maroy, P., Stumpf, W.E., 1991. Ecdysteroid receptors in the

- central nervous system of *Manduca sexta*: their changes in distribution and quantity during larval–pupal development. *J. Comp. Neurol.* 310, 337–355.
- Booker, R., Truman, J.W., 1987a. Postembryonic neurogenesis in the CNS of the tobacco hornworm, *Manduca sexta*. I. Neuroblast arrays and the fate of their progeny during metamorphosis. *J. Comp. Neurol.* 255, 548–559.
- Booker, R., Truman, J.W., 1987b. Postembryonic neurogenesis in the CNS of the tobacco Hornworm, *Manduca sexta*. II. Hormonal control of imaginal nest cell degeneration and differentiation during metamorphosis. *J. Neurosci.* 7, 4107–4114.
- Boyan, G.S., 1983. Postembryonic development in the auditory system of locusts. *J. Comp. Physiol. A* 151, 499–513.
- Brennan, C.A., Li, T.R., Bender, M., Hsiung, F., Moses, K., 2001. Broad-complex, but not ecdysone receptor, is required for progression of the morphogenetic furrow in the *Drosophila* eye. *Development* 128, 1–11.
- Britton, J.S., Edgar, B.A., 1998. Environmental control of the cell cycle in *Drosophila*: nutrition activates mitotic and endoreplicative cells by distinct mechanisms. *Development* 125, 2149–2158.
- Broadus, J., Skeath, J.B., Spana, E.P., Bossing, T., Technau, G., et al., 1995. New neuroblast markers and the origin of the aCC/pCC neurons in the *Drosophila* central nervous system. *Mech. Devel.* 53, 393–402.
- Brody, T., Odenwald, W.F., 2000. Programmed transformations in neuroblast gene expression during *Drosophila* CNS lineage development. *Devel. Biol.* 226, 34–44.
- Brody, T., Odenwald, W.F., 2002. Cellular diversity in the developing nervous system: a temporal view from *Drosophila*. *Development* 129, 3763–3770.
- Caldwell, M., Data, S., 1998. Expression of cyclin E or DP/E2F rescues the G1 arrest of *trol* mutant neuroblasts in the *Drosophila* larval central nervous system. *Mech. Devel.* 79, 121–131.
- Cayre, M., Strambi, C., Charpin, P., Augier, R., Meyer, M.R., et al., 1996. Neurogenesis in adult insect mushroom bodies. *J. Comp. Neurol.* 371, 300–310.
- Champlin, D.T., Truman, J.W., 1998a. Ecdysteroid control of cell proliferation during optic lobe neurogenesis in the moth *Manduca sexta*. *Development* 125, 269–277.
- Champlin, D.T., Truman, J.W., 1998b. Ecdysteroids govern two phases of eye development during metamorphosis of the moth, *Manduca sexta*. *Development* 125, 2009–2018.
- Champlin, D.T., Truman, J.W., 2000. Ecdysteroid coordinates optic lobe neurogenesis via a nitric oxide signaling pathway. *Development* 127, 3543–3551.
- Champlin, D.T., Reiss, S.E., Truman, J.W., 1999. Hormonal control of ventral diaphragm myogenesis during metamorphosis of the moth, *Manduca sexta*. *Devel. Genes Evol.* 209, 265–274.
- Cherbas, L., Hu, X., Zhimulev, I., Belyaeva, E., Cherbas, P., 2003. EcR isoforms in *Drosophila*: testing tissue-specific requirements by targeted blockade and rescue. *Development* 130, 271–284.
- Chiba, A., Shepherd, D., Murphey, R.K., 1988. Synaptic rearrangement during postembryonic development in the cricket. *Science* 240, 901–905.
- Consoulas, C., 2000. Remodeling of the leg sensory system during metamorphosis of the hawkmoth, *Manduca sexta*. *J. Comp. Neurol.* 419, 154–174.
- Consoulas, C., Duch, C., Bayline, R.J., Levine, R.B., 2000a. Behavioral transformations during metamorphosis: remodeling of neural and motor systems. *Brain Res. Bull.* 53, 571–583.
- Consoulas, C., Rose, U., Levine, R.B., 2000b. Remodeling of the femoral chordotonal organ during metamorphosis of the hawkmoth, *Manduca sexta*. *J. Comp. Neurol.* 426, 391–405.
- Consoulas, C., Restifo, L.L., Levine, R.B., 2002. Dendritic remodeling and growth of motoneurons during metamorphosis of *Drosophila melanogaster*. *J. Neurosci.* 22, 4906–4917.
- Datta, S., 1995. Activation of neuroblast proliferation in explant cultures of the *Drosophila* CNS. *Brain Res.* 818, 77–83.
- Doe, C.Q., 1992. Molecular markers for identified neuroblasts and ganglion mother cells in the *Drosophila* central nervous system. *Development* 116, 855–863.
- Duch, C., Bayline, R.J., Levine, R.B., 2000. Postembryonic development of the dorsal longitudinal flight muscle and its innervation in *Manduca sexta*. *J. Comp. Neurol.* 422, 1–17.
- Duch, C., Levine, R.B., 2000. Remodeling of membrane properties and dendritic architecture accompanies the postembryonic conversion of a slow into a fast motoneuron. *J. Neurosci.* 20, 6950–6961.
- Ebens, A.J., Garren, H., Cheyette, B.N., Zipursky, S.L., 1993. The *Drosophila* anachronism locus: a glycoprotein secreted by glia inhibits neuroblast proliferation. *Cell* 74, 15–27.
- Edgar, B.A., O'Farrell, P.H., 1989. Genetic control of cell division patterns in the *Drosophila* embryo. *Cell* 57, 177–187.
- Erezylmaz, D.F., Riddiford, L.M., Truman, J.W., 2004. Juvenile hormone acts at embryonic molts and induces the nymphal cuticle in the direct-developing cricket. *Devel. Genes Evol.* 214, 313–323.
- Fahrbach, S.E., Truman, J.W., 1987. Possible interactions of a steroid hormone and neural inputs in controlling the death of an identified neuron in the moth *Manduca sexta*. *J. Neurobiol.* 18, 497–508.
- Fahrbach, S.E., Truman, J.W., 1989. Autoradiographic identification of ecdysteroid-binding cells in the nervous system of the moth *Manduca sexta*. *J. Neurobiol.* 20, 681–702.
- Fernandes, J.J., Keshishian, H., 1998. Nerve–muscle interactions during flight muscle development in *Drosophila*. *Development* 125, 1769–1779.
- Ghbeish, N., Tsai, C.C., Schubiger, M., Zhou, J.Y., Evans, R.M., et al., 2001. The dual role of ultraspiracle, the *Drosophila* retinoid X receptor, in the ecdysone response. *Proc. Natl Acad. Sci. USA* 98, 3867–3872.

- Gilbert, L.I., Rybczynski, R., Warren, J.T., 2002. Control and biochemical nature of the ecdysteroidogenic pathway. *Annu. Rev. Entomol.* 47, 883–916.
- Grueber, W.B., Truman, J.W., 1999. Development and organization of a nitric-oxide-sensitive peripheral neural plexus in larvae of the moth, *Manduca sexta*. *J. Comp. Neurol.* 404, 127–141.
- Hegstrom, C.D., Truman, J.W., 1996. Synapse loss and axon retraction in response to local muscle degeneration. *J. Neurobiol.* 31, 175–188.
- Hegstrom, C., Riddiford, L.M., Truman, J.W., 1998. Steroid and neuronal regulation of ecdysone receptor expression during metamorphosis of muscle in the moth *Manduca sexta*. *J. Neurosci.* 18, 1786–1794.
- Hiruma, K., Bocking, D., Lafont, R., Riddiford, L.M., 1997. Action of different ecdysteroids on the regulation of mRNAs for the ecdysone receptor, MHR3, dopa decarboxylase, and a larval cuticle protein in the larval epidermis of the tobacco hornworm, *Manduca sexta*. *Gen. Comp. Endocrinol.* 107, 84–97.
- Hu, X., Cherbas, L., Cherbas, P., 2003. Transcription activation by the ecdysone receptor (EcR/Usp): identification of activation functions. *Mol. Endocrinol.* 17, 716–731.
- Huang, Z., Kunes, S., 1996. Hedgehog, transmitted along retinal axons, triggers neurogenesis in the developing visual centers of the *Drosophila* brain. *Cell* 86, 411–422.
- Isshiki, T., Pearson, B., Holbrook, S., Doe, C.Q., 2001. *Drosophila* neuroblasts sequentially express transcription factors which specify the temporal identity of their neuronal progeny. *Cell* 106, 511–521.
- Jacobs, G.A., Weeks, J.C., 1990. Postsynaptic changes at a sensory-to-motoneuron synapse contribute to the developmental loss of a reflex behavior during insect metamorphosis. *J. Neurosci.* 10, 1341–1356.
- Kiguchi, K., Riddiford, L.M., 1978. The role of juvenile hormone in pupal development of the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol.* 24, 673–680.
- Kimura, K.I., Truman, J.W., 1990. Postmetamorphic cell death in the nervous and muscular systems of *Drosophila melanogaster*. *J. Neurosci.* 10, 403–411.
- Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P., et al., 1991. The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* 67, 59–77.
- Kraft, R.N., Levine, R.B., Restifo, L.L., 1998. The steroid hormone 20-hydroxyecdysone enhances neurite growth of *Drosophila* mushroom body neurons isolated during metamorphosis. *J. Neurosci.* 18, 8886–8899.
- Kraus, W.L., Wong, J., 2002. Nuclear receptor-dependent transcription with chromatin. Is it all about enzymes? *Eur. J. Biochem.* 269, 2275–2283.
- Kuwada, J.Y., Goodman, C.S., 1985. Neuronal determination during embryonic development of the grasshopper nervous system. *Devel. Biol.* 110, 114–126.
- Kutsch, W., Hechmann, R., 1995. Homologous structures, exemplified by motoneurons of Mandibulata. In: Breidbach, O., Kutsch, W. (Eds.), *The Nervous Systems of Invertebrates: An Evolutionary and Comparative Approach*. Birkhauser Verlag, Basel, pp. 221–248.
- Lanzrein, B., Gentinetta, V., Abegglen, H., Baker, F.C., Miller, C.A., et al., 1985. Titters of ecdysone, 20-hydroxyecdysone and juvenile hormone III throughout the life cycle of a hemimetabolous insect, the ovoviviparous cockroach *Nauphoeta cinerea*. *Experientia* 41, 913–917.
- Lee, T., Marticke, S., Sung, C., Robinow, S., Luo, L., 2000. Cell-autonomous requirement of the Usp/EcR-B ecdysone receptor for mushroom body neuronal remodeling in *Drosophila*. *Neuron* 28, 807–818.
- Levine, R.B., 1989. Expansion of the central arborizations of persistent sensory neurons during insect metamorphosis: the role of the steroid hormone, 20-hydroxyecdysone. *J. Neurosci.* 9, 1045–1054.
- Levine, R.B., Truman, J.W., 1985. Dendritic reorganization of abdominal motoneurons during metamorphosis of the moth, *Manduca sexta*. *J. Neurosci.* 5, 2424–2431.
- Levine, R.B., Truman, J.W., Linn, D., Bate, C.M., 1986. Endocrine regulation of the form and function of axonal arbors during insect metamorphosis. *J. Neurosci.* 6, 293–299.
- Levine, R.B., Weeks, J.C., 1990. Hormonally mediated changes in simple reflex circuits during metamorphosis in *Manduca*. *J. Neurobiol.* 21, 1022–1036.
- Levine, R.B., Weeks, J.C., 1996. Cell culture approaches to understanding the actions of steroid hormones on the insect nervous system. *Devel. Neurosci.* 18, 73–86.
- Li, T., Bender, M., 2000. A conditional rescue system reveals essential functions for the ecdysone receptor (EcR) gene during molting and metamorphosis in *Drosophila*. *Development* 127, 2897–2905.
- Matheson, S.F., Levine, R.B., 1999. Steroid hormone enhancement of neurite outgrowth in identified insect motor neurons involves specific effects on growth cone form and function. *J. Neurobiol.* 38, 27–45.
- McGraw, H.F., Prier, K.R.S., Wiley, J.C., Tublitz, N.J., 1998. Steroid-regulated morphological plasticity in a set of identified peptidergic neurons in the moth, *Manduca sexta*. *J. Exp. Biol.* 201, 2981–2992.
- Meinertzhagen, I.A., Hanson, T.E., 1993. The development of the optic lobe. In: Bate, M., Martinez Arias, A. (Eds.), *The Development of Drosophila melanogaster*. Cold Spring Harbor Press, Cold Spring Harbor, NY, pp. 1363–1491.
- Meier, T., Chabaud, F., Reichert, H., 1991. Homologous patterns in the embryonic development of the peripheral nervous system in the grasshopper *Schistocerca gregaria* and the fly *Drosophila melanogaster*. *Development* 112, 241–253.
- Monsma, S.A., Booker, R., 1996. Genesis of the adult retina and outer optic lobes of the moth, *Manduca sexta*. II. Effects of differentiation and developmental hormone manipulation. *J. Comp. Neurol.* 367, 21–35.
- Mouillet, J.F., Henrich, V.C., Lezzi, M., Vogtli, M., 2001. Differential control of gene activity by isoforms A, B1 and B2 of the *Drosophila* ecdysone receptor. *Eur. J. Biochem.* 268, 1811–1819.

- Oland, L.A., Tolbert, L.P., 2003. Key interactions between neurons and glial cells during neural development in insects. *Annu. Rev. Entomol.* 48, 89–110.
- Olberg, R.M., 1986. Metamorphosis of identified visual interneurons which steer flight in the dragonfly. *Soc. Neurosci. Abst.* 12, 927.
- Park, Y., Fujioka, M., Kobayashi, M., Jaynes, J.B., Datta, S., 2001. *even skipped* is required to produce a *trans*-acting signal for larval neuroblast proliferation that can be mimicked by ecdysone. *Development* 128, 1899–1909.
- Pfluger, H.J., Witten, J.L., Levine, R.B., 1993. Fate of abdominal ventral unpaired median cells during metamorphosis of the hawkmoth, *Manduca sexta*. *J. Comp. Neurol.* 335, 508–522.
- Postlethwait, J.H., 1974. Juvenile hormone and the adult development of *Drosophila*. *Biol. Bull. (Woods Hole, Mass.)* 147, 119–135.
- Prugh, J., Della Croce, K., Levine, R.B., 1992. Effects of the steroid hormone, 20-hydroxyecdysone, on the growth of neurites by identified insect motoneurons *in vitro*. *Devel. Biol.* 154, 331–347.
- Restifo, L.L., Wilson, T.G., 1998. A juvenile hormone agonist reveals distinct developmental pathways mediated by ecdysone-inducible broad complex transcription factors. *Devel. Genetics* 22, 141–159.
- Riddiford, L.M., 1994. Cellular and molecular actions of juvenile hormone. I. General considerations and metamorphic actions. *Adv. Insect Physiol.* 24, 213–274.
- Riddiford, L.M., Ajami, A.M., 1973. Juvenile hormone: its assay and effects on pupae of *Manduca sexta*. *J. Insect Physiol.* 19, 749–762.
- Riddiford, L.M., Ashburner, M., 1991. Effects of juvenile hormone mimics on larval development and metamorphosis of *Drosophila melanogaster*. *Gen. Comp. Endocrinol.* 82, 172–183.
- Riddiford, L.M., Cherbas, P., Truman, J.W., 2000. Ecdysone receptors and their biological actions. *Vitamins Hormones* 60, 1–73.
- Robinow, S., Talbot, W.S., Hogness, D.S., Truman, J.W., 1993. Programmed cell death in the *Drosophila* CNS is ecdysone-regulated and coupled with a specific ecdysone receptor. *Development* 119, 1251–1259.
- Robinow, S., Draizen, T.A., Truman, J.W., 1997. Genes that induce apoptosis: transcriptional regulation in identified, doomed neurons of the *Drosophila* CNS. *Devel. Biol.* 190, 206–213.
- Roignant, J.Y., Carre, C., Mugat, B., Szymczak, D., Lepesant, J.A., *et al.*, 2003. Absence of transitive and systemic pathways allows cell-specific and isoform-specific RNAi in *Drosophila*. *RNA* 9, 299–308.
- Sandstrom, D.J., Weeks, J.C., 1998. Segment-specific retention of a larval neuromuscular system and its role in a new, rhythmic, pupal motor *pattern* in *Manduca sexta*. *J. Comp. Physiol. [A]* 183, 283–302.
- Schaffer, R., Sanchez, T.V., 1973. Antennal sensory system of the cockroach *Periplaneta americana*: postembryonic development and morphology of sense organs. *J. Comp. Neurol.* 149, 335–354.
- Schubiger, M., Tomita, S., Sung, C., Robinow, S., Truman, J.W., 2003. Isoform specific control of gene activity *in vivo* by the *Drosophila* ecdysone receptor. *Mech. Devel.* 120, 909–918.
- Schubiger, M., Truman, J.W., 2000. The RXR-ortholog Usp suppresses early metamorphic processes in the absence of ecdysteroids. *Development* 127, 1151–1159.
- Schubiger, M., Wade, A.A., Carney, G.E., Truman, J.W., Bender, M., 1998. *Drosophila* EcR-B ecdysone receptor isoforms are required for larval molting and for neuron remodeling during metamorphosis. *Development* 125, 2053–2062.
- Schwartz, L.M., Truman, J.W., 1982. Peptide and steroid regulation of muscle degeneration in an insect. *Science* 215, 1420–1421.
- Schwartz, L.M., Truman, J.W., 1983. Hormonal control of rates of metamorphic development in the tobacco hornworm *Manduca sexta*. *Devel. Biol.* 99, 103–14.
- Selleck, S., Gonzalez, C., Glover, D.M., White, K., 1992. Regulation of the G1-S transition in postembryonic neuronal precursors by axon ingrowth. *Nature* 355, 253–255.
- Shankland, M., Goodman, C.S., 1982. Development of the dendritic branching pattern of the medial giant interneuron in the grasshopper embryo. *Devel. Biol.* 92, 489–506.
- Shepherd, D., Bate, C.M., 1990. Spatial and temporal patterns of neurogenesis in the embryo of the locust (*Schistocerca gregaria*). *J. Comp. Neurol.* 319, 436–453.
- Stevenson, P.A., Kutsch, W., 1988. Demonstration of functional connectivity of the flight motor system in all stages of the locust. *J. Comp. Physiol. A* 162, 247–259.
- Streichert, L.C., Pierce, J.T., Nelson, J.A., Weeks, J.C., 1997. Steroid hormones act directly to trigger segment-specific programmed cell death of identified motoneurons *in vitro*. *Devel. Biol.* 183, 95–107.
- Taghert, P.H., Doe, C.Q., Goodman, C.S., 1984. Cell determination and regulation during development of neuroblasts and neurones in grasshopper embryo. *Nature* 307, 163–165.
- Taghert, P.H., Roberts, M.E., Renn, S.C., Jacobs, P.S., 2000. Metamorphosis of tangential visual system neurons in *Drosophila*. *Devel. Biol.* 222, 471–485.
- Talbot, W.S., Swyryd, E.A., Hogness, D.S., 1993. *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. *Cell* 73, 1323–1337.
- Tessier-Lavigne, M., Goodman, C.S., 1996. The molecular biology of axon guidance. *Science* 274, 1123–1133.
- Thomas, J.B., Bastiani, M.J., Bate, M., Goodman, C.S., 1984. From grasshopper to *Drosophila*: a common plan for neuronal development. *Nature* 310, 203–207.
- Thompson, K.J., 1993. Embryonic neural activity and serial homology underlying grasshopper oviposition. *Soc. Neurosci. Abst.* 19, 348.
- Thompson, K.J., Siegler, M.V., 1993. Development of segment specificity in identified lineages of the grasshopper CNS. *J. Neurosci.* 13, 3309–3318.

- Truman, J.W., 1983. Programmed cell death in the nervous system of an adult insect. *J. Comp. Neurol.* 216, 445–452.
- Truman, J.W., Ball, E.E., 1998. Patterns of embryonic neurogenesis in a primitive wingless insect, the silverfish, *Ctenolepisma longicaudata*: comparison with those seen in flying insects. *Devel. Genes Evol.* 208, 357–368.
- Truman, J.W., Bate, M., 1988. Spatial and temporal patterns of neurogenesis in the central nervous system of *Drosophila melanogaster*. *Devel. Biol.* 125, 145–157.
- Truman, J.W., Reiss, S.E., 1995. Neuromuscular metamorphosis in the moth *Manduca sexta*: hormonal regulation of synapses loss and remodeling. *J. Neurosci.* 15, 4815–4826.
- Truman, J.W., Riddiford, L.M., 2000. The origins of insect metamorphosis. *Nature* 401, 447–452.
- Truman, J.W., Riddiford, L.M., 2002. Insect developmental hormones and their mechanism of action. In: Pfaff, D.W., Arnold, A.P., Etgen, A.M., Fahrbach, S.E., Rubin, R.T. (Eds.), *Hormones, Brain and Behavior*, vol. 2. Academic Press, San Diego, pp. 841–873.
- Truman, J.W., Schwartz, L.M., 1984. Steroid regulation of neuronal death in the moth nervous system. *J. Neurosci.* 4, 274–280.
- Truman, J.W., Talbot, W.S., Fahrbach, S.E., Hogness, D.S., 1994. Ecdysone receptor expression in the CNS correlates with stage-specific responses to ecdysteroids during *Drosophila* and *Manduca* development. *Development* 120, 219–234.
- Tublitz, N.J., Sylwester, A.W., 1990. Postembryonic alteration of transmitter phenotype in individually identified peptidergic neurons. *J. Neurosci.* 10, 161–168.
- Urbach, R., Technau, G.M., 2003. Early steps in building the insect brain: neuropil formation and segmental patterning in the developing brain of different insect species. *Arthropod Struct. Devel.* 32, 103–123.
- Usui-Ishihara, A., Simpson, P., Usui, K., 2000. Larval multidendrite neurons survive metamorphosis and participate in the formation of imaginal sensory axonal pathways in the notum of *Drosophila*. *Devel. Biol.* 225, 357–369.
- Vogt, R.G., Rybczynski, R., Cruz, M., Lerner, M.R., 1993. Ecdysteroid regulation of olfactory protein expression in the developing antenna of the tobacco hawk moth, *Manduca sexta*. *J. Neurobiol.* 24, 581–597.
- Walsh, M.K., Lichtman, J.W., 2003. *In vivo* time-lapse imaging of synaptic takeover associated with naturally occurring synapse elimination. *Neuron* 37, 67–73.
- Warren, J.T., Gilbert, L.I., 1986. Ecdysone metabolism and distribution during the pupal–adult development of *Manduca sexta*. *Insect Biochem.* 16, 65–82.
- Watts, R.J., Hoopfer, E.D., Luo, L., 2003. Axon pruning during *Drosophila* metamorphosis: evidence for local degeneration and requirement of the ubiquitin-proteasome system. *Neuron* 38, 871–885.
- Weeks, J.C., Truman, J.W., 1985. Independent steroid control of the fates of motoneurons and their muscles during insect metamorphosis. *J. Neurosci.* 5, 2290–2300.
- Weeks, J.C., Roberts, W.M., Trimble, D.L., 1992. Hormonal regulation and segmental specificity of motoneuron phenotype during metamorphosis of the tobacco hornworm, *Manduca sexta*. *Devel. Biol.* 149, 185–196.
- Wheeler, D.E., Nijhout, H.F., 2003. A perspective for understanding the modes of juvenile hormone action as a lipid signaling system. *Bioessays* 25, 994–1001.
- White, K., Kankel, D.R., 1978. Patterns of cell division and cell movement in the formation of the imaginal nervous system in *Drosophila melanogaster*. *Devel. Biol.* 65, 296–321.
- Whittington, P.M., Bate, M., Seifert, E., Ridge, K., Goodman, C.S., 1982. Survival and differentiation of identified embryonic neurons in the absence of their target muscles. *Science* 215, 973–975.
- Wigglesworth, V.B., 1940. Local and general factors in the development of ‘pattern’ in *Rhodnius prolixus* (Hemiptera). *J. Exp. Biol.* 17, 180–200.
- Wigglesworth, V.B., 1953. The origin of sensory neurons in an insect, *Rhodnius prolixus* (Hemiptera). *Q. J. Microsc. Sci.* 94, 93–112.
- Williams, C.M., 1961. The juvenile hormone. II. Its role in the endocrine control of molting, pupation, and adult development in the Cecropia Silkworm. *Biol. Bull. (Woods Hole, Mass.)* 116, 323–338.
- Williams, D.W., Shepherd, D., 2002. Persistent larval sensory neurones are required for the normal development of the adult sensory afferent projections in *Drosophila*. *Development* 129, 617–624.
- Williams, D.W., Truman, J.W., 2004. Mechanisms of dendritic elaboration of sensory neurons in *Drosophila*: insights from in vivo time-lapse. *J. Neurosci.* 24, 1541–1550.
- Witten, J.L., Truman, J.W., 1998. Distribution of GABA-like immunoreactive neurons in insects suggests lineage homology. *J. Comp. Neurol.* 398, 515–528.
- Wolfgang, W.J., Riddiford, L.M., 1986. Larval cuticular morphogenesis in the tobacco hornworm, *Manduca sexta*, and its hormonal regulation. *Devel. Biol.* 113, 305–316.
- Zelhof, A.C., Ghbeish, N., Tsai, C., Evans, R.M., McKeown, M., 1997. A role for ultraspiracle, the *Drosophila* RXR, in morphogenetic furrow movement and photoreceptor cluster formation. *Development* 124, 2499–2506.
- Zheng, X., Wang, J., Haerry, T.E., Wu, A.Y., Martin, J., et al., 2003. TGF-beta signaling activates steroid hormone receptor expression during neuronal remodeling in the *Drosophila* brain. *Cell* 112, 303–315.

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5 Programmed Cell Death in Insect Neuromuscular Systems during Metamorphosis

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5.1. Introduction

Programmed cell death (PCD) is a fundamental component of the postembryonic development of the neuromuscular systems of holometabolous insects (see **Chapter 3**). Nerve and muscle cells born during embryogenesis are deleted in a segment- and cell-specific fashion at both the larval-pupal and pupal-adult transitions. Although such deaths presumably occur in all insects with complete metamorphosis, almost all of the information on this topic has been obtained from the study of two species, the fruit fly *Drosophila melanogaster* and the tobacco hawkmoth *Manduca sexta*. The sophistication of the genetic tools available for manipulating and analyzing *Drosophila* has allowed many of the key regulatory events in cell death to be identified in this model. In addition, the success of large-scale genetic screens for embryonic pattern formation as

well as detailed analysis of Hox gene clusters (see **Chapter 1**) has provided tremendous insight into the mechanisms underlying specification of cell populations, elaboration of body axes, and organogenesis. These insights into early development provide a strong base for our understanding of metamorphosis. The small size of fruit flies, however, limits the utility of *Drosophila* for physiological and biochemical studies involving hormone manipulations, tissue transplantation, and surgical interventions. The lepidopteran *M. sexta* is an alternative model that is uniquely suited for these sorts of experimental approaches and serves as the insect equivalent of the laboratory white rat (Fahrbach, 1997).

Most investigations have focused on the regulation of PCD by hormonal signals or the molecular mechanisms of developmental cell death (see **Chapter 4**). In the first sections of this chapter, the

history of this field is reviewed, and then an overview of the *Manduca* and *Drosophila* models is presented. The following sections describe the endocrine control of cell death, and the molecular, physiological, and genetic mechanisms that bring about the controlled loss of these cells. A final section presents unresolved issues. An unusual feature of this chapter will be the parallel presentation of information on neuronal and muscle death during metamorphosis. The aim of this chapter is to highlight the features of PCD common to both tissues without obscuring tissue-specific features.

5.2. PCD, Apoptosis, Autophagy, or Necrosis?

It is now widely recognized that cell death is a normal part of development in all multicellular and some single-celled organisms (Ameisen, 2002). The capacity to remove selected cells during development provides organisms with a plastic response to many developmental contingencies. In vertebrates, PCD has been demonstrated to:

1. Match the sizes of interacting populations of cells, such as oligodendrocytes, to the axons they myelinate (Barres *et al.*, 1992).
2. Remove deleterious cells, such as self-reactive T cells (Smith *et al.*, 1989).
3. Sculpt the body, such as in the loss of interdigital cells in the fetal hand (Zuzarte-Luis and Hurler, 2002).
4. Remove “obsolete” cells, such as the tail of the tadpole (Yoshizato, 1996).

In insects, the best-characterized examples of PCD take place not only during embryogenesis, but also during metamorphosis in holometabolous insects, when larval tissues are destroyed to allow the formation of new structures in the adult. PCD during insect development, therefore, provides specific examples of a widespread phenomenon rather than being unique to Arthropoda.

5.2.1. Apoptosis

In both vertebrates and insects, dying cells can display one of several distinct morphologies. The most widely displayed and best-characterized morphology associated with both developmental and pathological cell death is apoptosis, a term that was coined by Kerr, Currie, and Wyllie (Kerr *et al.*, 1972). As introduced, apoptosis was a purely morphological description that implied neither an underlying mechanism nor a specific developmental context. During the process of apoptosis,

cells shrink and display dramatic plasma membrane zeosis, during which numerous protuberances or “blebs” are formed. Time-lapse photographs of cells undergoing apoptosis in culture look like drops of water skittering on a hot skillet. Under normal conditions *in vivo*, these cells are rapidly phagocytosed either by neighboring cells or by macrophage-like phagocytes (Platt *et al.*, 1998; Savill and Fadok, 2000; Fadok and Chimini, 2001; Henson *et al.*, 2001; Franc, 2002; Geske *et al.*, 2002).

The nucleus of apoptotic cells condenses, and the chromatin in these cells becomes electron dense and margined along the inner aspect of the nuclear envelope. These morphological changes are the physical manifestation of a massive cleavage of genomic DNA that occurs when endogenous nucleases become activated and cleave the linker DNA between individual nucleosomes (Wyllie *et al.*, 1984; Enari *et al.*, 1998). The fragmentation of the genome can be visualized when DNA is extracted and fractionated by size in agarose (Eastman, 1995).

It is sometimes difficult to detect these “apoptotic ladders” because dying cells are usually intermingled with healthy ones. In addition, as tissues are homogenized during DNA isolation, it is impossible to determine which subpopulation of cells within a tissue contributed to the degraded DNA. An alternative strategy for detecting cells with fragmented genomic DNA is to employ *in situ* labeling techniques on tissue sections such as terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling, commonly referred to as the TUNEL method (Gavrieli *et al.*, 1992; Ben-Sasson *et al.*, 1995).

5.2.2. Autophagy

While apoptosis is the morphology that is most commonly observed during PCD, particularly during embryogenesis, it is not the only one. Other suicidal cell deaths are designated as autophagic or type II degeneration (Clarke, 1990; Nixon and Cataldo, 1993; Bursch, 2001). Cells undergoing autophagy lack, in general, an initial condensation of chromatin and are instead characterized by the formation of autophagic bodies within the cytoplasm. Unlike apoptosis, there are no definitive markers visible using light microscopy that define autophagic cell death. This has limited the analysis of autophagy during development.

5.2.3. PCD is an Inclusive Term

The term PCD encompasses both apoptotic and autophagic cell deaths that occur as a normal part of development. The term PCD is used in this

chapter, unless the death of a uniquely identifiable cell at a particular developmental stage has been clearly defined as being either apoptotic or autophagic based on ultrastructural criteria. The distinction is more than semantic, as the evidence argues strongly that there are multiple molecular mechanisms of nonpathological cell deaths (Schwartz *et al.*, 1993; Bursch *et al.*, 2000; Jones and Schwartz, 2001; Thummel, 2001). Occasional confusion has arisen in the literature because autophagy and apoptosis can sometimes share features, such as caspase activation and positive TUNEL staining (Ben-Sasson *et al.*, 1995; Kinch *et al.*, 2003); also, relatively few studies have investigated the ultrastructure of dying insect cells, which is required for the detection of autophagic bodies.

5.2.4. Necrosis

While apoptosis and autophagy are carefully orchestrated developmental decisions, all cells can be induced to die by necrosis, a passive form of cell death that occurs in the absence of normal physiological responses. Necrosis can be induced in any cell following external insult such as heat, salt, abrasion, toxin exposure, etc. (Dive *et al.*, 1992; Kroemer *et al.*, 1998). Necrosis typically follows direct or indirect disruption of plasma membrane integrity, which in turn results in an influx of ions and water and subsequent cell swelling and lysis. In vertebrates, which have an adaptive immune response, necrosis provides a valuable warning to the immune system that focal injury has occurred, because the cellular constituents that are liberated during necrosis are highly inflammatory (MacDonald and Stoodley, 1998; Fadok *et al.*, 2001). In fact, endogenous responses to necrosis are responsible for much of the secondary tissue damage that accompanies injury.

5.3. Historical Overview and Current Trends

The first morphological description of PCD during insect metamorphosis of which we are aware was a description of the death of intersegmental muscles (ISMs) provided in 1936 by Kuwana working with the silkworm *Bombyx mori* (Kuwana, 1936). The phenomenon of ISM death was largely unknown in the West, until it was independently described by Finlayson (1956). With the exception of a modest number of descriptive papers documenting examples of cell death in other insect taxa, little was published about cell loss during arthropod development for most of the subsequent decade.

(A compendium of the early studies of cell death in insects can be found in Glücksmann's (1951) famous but now largely forgotten review of the field of cell death.) A multifaceted analysis of PCD based on analysis of the ISMs was initiated in the mid-1960s by Richard Lockshin and Caroll Williams (Lockshin and Williams, 1964, 1965a, 1965b, 1965c). In fact, it was in these early reports that the term "programmed cell death" was first introduced into the literature (Lockshin, 1963).

The first detailed description of neuronal death during insect metamorphosis, published in 1974, was a description of the postembryonic changes in neuronal populations observed in the abdominal ganglia of *M. sexta* (Taylor and Truman, 1974). James Truman and his colleagues exploited their ability to count the motoneurons in individual ganglia by backfilling the cut ends of peripheral nerves with cobalt; they reported that motoneurons were lost from the abdominal ganglia at both the larval-pupal and pupal-adult transitions. Many of these motoneurons were later identified as specific individuals, permitting the precisely regulated nature of metamorphic neuron death to be established (Truman, 1983; Levine and Truman, 1985).

Studies by Schwartz and Truman subsequently demonstrated that the changes associated with ISM development and death in *Manduca* at the end of metamorphosis are controlled by hormonal signals, particularly by the steroid hormone 20-hydroxyecdysone (20E) (Schwartz and Truman, 1982, 1983) (see Chapter 4). This principle of steroid control of nerve and muscle fate at the metamorphic transitions was extended to the death of abdominal neurons that occurs after adult eclosion in *Manduca* (Truman and Schwartz, 1984) and to the death of *Manduca* proleg motoneurons and their associated muscles at pupation (Weeks and Truman, 1985; Weeks, 1987, 1999). Studies of changes in the pattern of gene expression in dying ISMs pioneered investigations of the molecular mechanisms of PCD in insects (Schwartz *et al.*, 1990a).

Descriptive studies of the death of neurons and muscles after adult eclosion in *D. melanogaster* (Kimura and Truman, 1990) foreshadowed the initiation in the 1990s of an intensive genetic analysis of PCD in fruit flies (reviews: McCall and Steller, 1997; Wing and Nambu, 1998a; Abrams, 1999; Bangs and White, 2000; Vernooij *et al.*, 2000). Efforts have focused primarily on the elucidation of the intracellular machinery of cellular suicide and on extracellular signals that trigger death in specific target cells. Two important current trends are the use of genomic approaches to identify genes associated with different forms of PCD, and the

development of *Drosophila* models of human neurodegenerative disease (Mutsuddi and Nambu, 1998; Gorski and Marra, 2002; Muqit and Feany, 2002; Driscoll and Gerstbrein, 2003; Lee *et al.*, 2003). The underpinning of this work is the growing consensus within the field of cell death studies that mechanisms of cell death are broadly conserved across phylogeny (Yuan *et al.*, 1993; Steller, 1995; Jacobson *et al.*, 1997; Tittel and Steller, 2000; Vernooy *et al.*, 2000) (see Section 5.5.4.1). Studies of muscle and nerve cell death in insects are, therefore, well positioned to address specific questions concerning metamorphosis, basic questions in cell biology, and topics of direct relevance to human health.

5.3.1. Earlier Reviews of PCD Relevant to Insects

The following previously published reviews provide essential background on PCD of insect nerve and muscle cells during metamorphosis: Truman *et al.* (1990, 1992), Schwartz (1992), and Weeks (1999). PCD in other insect tissues during development is reviewed in other chapters in this volume. Reviews covering the discovery of the first genes associated with PCD in *D. melanogaster* are found in McCall and Steller (1997), Wing and Nambu (1998a), Abrams (1999), Bangs and White (2000), and Vernooy *et al.* (2000). Autophagic and apoptotic cell deaths during *Drosophila* metamorphosis are compared in Thummel (2001); and a recent review of *Drosophila* as a model system for the study of PCD is found in Richardson and Kumar (2002). Overviews of PCD during animal development in general are provided in Milligan and Schwartz (1997), Jacobson *et al.* (1997), Meier *et al.* (2000), and Baehrecke (2002).

5.4. The *Manduca* Model

5.4.1. Choice of *Manduca sexta* as a Model System for the Study of PCD of Nerve and Muscle Cells

Insect endocrinologists and neurobiologists have long favored *M. sexta* (Lepidoptera: Sphingidae) because of its large size and ease of rearing in culture on artificial diet (Bell and Joachim, 1976; Arnett, 1993; Fahrback, 1997). This species has a facultative rather than an obligatory diapause and, therefore, all life stages can be produced in the laboratory at any time of year. Development from egg to adult requires roughly 40 days. There are five successive larval stages (each referred to as an instar, so that the final larval stage is the fifth instar) and a single

pupal stage. The adult lives approximately 10 days. The relatively large size of this insect in all life stages permits extensive surgical and endocrine manipulations. The central nervous system (CNS) consists of a dorsal brain and a ventral nerve cord. The abdominal portion of the ventral nerve cord retains its segmental organization of discrete thoracic and abdominal ganglia throughout the postembryonic period, a feature that facilitates anatomical, electrophysiological, and functional analyses.

The thoracic and abdominal musculature of *M. sexta* has also been fully described (Eaton, 1988). Importantly, many aspects of neuromuscular metamorphosis first described in *Manduca* have now also been identified in *Drosophila* (e.g., Kimura and Truman, 1990; Truman *et al.*, 1994), despite evidence that the insect orders of Lepidoptera and Diptera have been evolving independently for at least 200 and, possibly, 300 million years (Hoy, 1994). This suggests that many aspects of neuromuscular metamorphosis, including PCD, are ancient in origin and highly conserved. This relationship permits phenomena first observed in *Manduca* to be subjected to indirect molecular genetic analysis by shifting to *Drosophila* for follow-up studies (e.g., Robinow *et al.*, 1993). Conversely, orthologs of genes first identified in *Drosophila* can be cloned in *Manduca* and their developmental expression patterns determined (e.g., Nagy *et al.*, 1991; Kraft and Jäckle, 1994).

The striking changes in the organization of the nervous and muscular systems that accompany lepidopteran metamorphosis result from a combination of postembryonic cell proliferation, modification of structures formed initially in the embryo, and PCD. While cell death is typically restricted to small populations of cells in the CNS, wholesale loss of entire bundles of muscle fibers in the periphery is more usual (Schwartz, 1992; Bayline *et al.*, 1998). In fact, the coordinated loss of the ISMs following adult eclosion in *Manduca* offers a nearly ideal system for the study of PCD, because these cells are exceptionally large, easily accessible throughout the entire process of degeneration, and uncontaminated by persisting muscle fibers (Figure 1).

The ISMs of *Manduca* are composed of giant, syncytial fibers approximately 5 mm long and 1 mm in diameter. These embryonically derived cells are used by the larva for locomotion and by the pupa for defensive and respiratory behaviors. The ISMs also perform the major abdominal movements required for the eclosion behavior of the adult moth. The ISMs begin to atrophy at 3 days prior to adult eclosion, which results in a loss of 40% of muscle mass, although the ability to contract is maintained during this early phase (Schwartz and

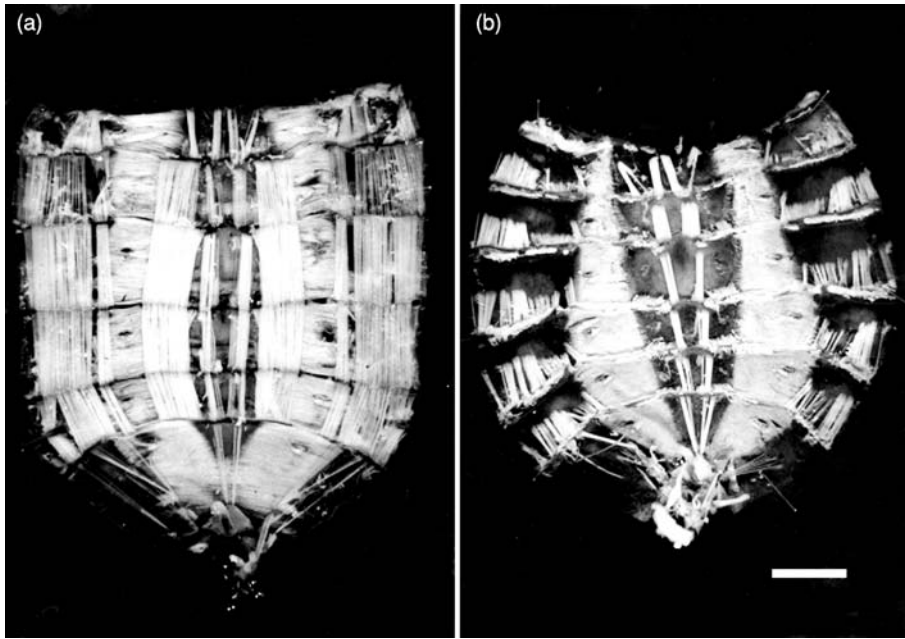


Figure 1 Loss of the intersegmental muscles (ISMs) following adult eclosion in *Manduca sexta*. These photographs display the abdominal musculature from animals (a) before and (b) 36 h after adult eclosion. The ISMs are the large vertical muscle fibers seen prior to eclosion. The small oblique muscles present after eclosion are external muscles that form during adult development and persist for the life span of the adult. Scale bar = 5 mm. (Adapted from Schwartz, L.M. 1992. Insect muscle as a model for programmed cell death. *J. Neurobiol.* 23, 1312–1326.)

Ruff, 2002). Once the ISMs have participated in eclosion, they are no longer required for any adult behaviors and die during the subsequent 30 h (Schwartz, 1992). As described below (see Section 5.4.5), studies of the ISM death of *Manduca* have provided numerous insights into the cellular and molecular mechanisms of PCD.

5.4.2. Hormonal Regulation of Metamorphosis

As in all insects, metamorphosis in *Manduca* is regulated by two categories of developmental hormones, ecdysteroids and juvenoids (Truman and Riddiford, 2002; Nijhout, 1994) (see Chapters 7 and 8). The ecdysteroids are steroid hormones that exert their primary actions through members of the nuclear hormone receptor superfamily of proteins (Robinson-Rechavi *et al.*, 2003) (see Chapter 7). The juvenile hormones are terpenoids (Nijhout, 1994) (see Chapter 8). At present, the cellular mode of action of juvenile hormones in metamorphosis remains to be defined and possibly involves more than one signaling pathway (Gilbert *et al.*, 2000; Jones and Jones, 2000). Experiments on the hormonal regulation of neuromuscular metamorphosis in *Manduca* have clearly established that the changes in cell populations and connectivity that occur during neuromuscular metamorphosis are controlled by the direct actions of ecdysteroids and juvenile hormones on neurons, glia, and muscle

(Bennett and Truman, 1985; Streichert *et al.*, 1997) (see Chapter 4). Receptors for ecdysteroids have been localized to neuronal, glial, and muscle cell nuclei in *Manduca* (Bidmon and Koolman, 1989; Fahrbach and Truman, 1989; Bidmon and Sliter, 1990; Bidmon *et al.*, 1992; Hegstrom *et al.*, 1998; Fahrbach, 1992) (see Chapter 4).

5.4.3. PCD of Neurons during Metamorphosis

5.4.3.1. Background and overview Neuronal death during metamorphosis in *Manduca* involves motoneurons, interneurons, and identified peptidergic neurons. Most studies have focused on the death of motoneurons not only because of the greater ease of identification of specific neurons across individuals, but also because the highly visible degeneration of muscles during postembryonic development often suggests that innervating motoneurons will be lost, so that investigators in effect “know where to look.” The presence of sexually dimorphic structures in the adult, such as the oviduct, also often suggests where PCD will be found in the CNS (Giebultowicz and Truman, 1984; Thorn and Truman, 1989). Dying peptidergic neurons can be identified after the detection of stage-specific patterns of antibody staining (Ewer *et al.*, 1998). Cell counts and the presence of small pycnotic (shrunken) profiles often provide the only anatomical clues that interneurons have died, although the

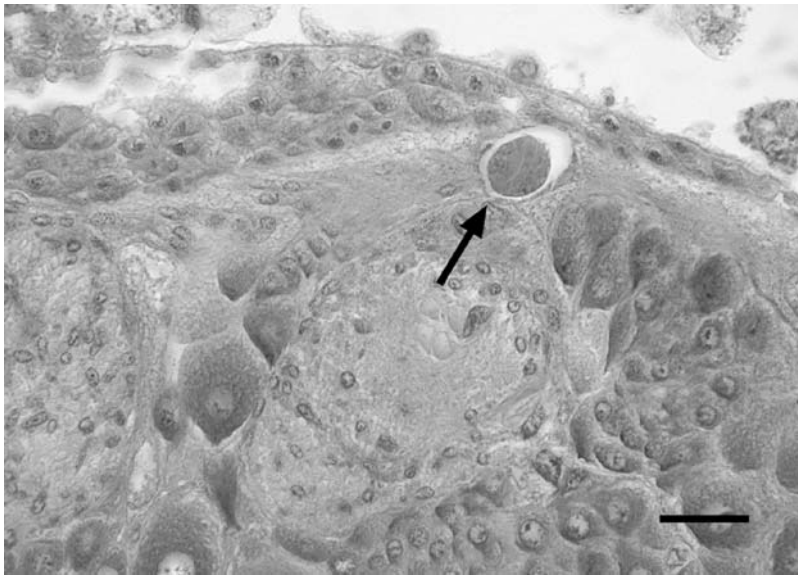


Figure 2 Transverse section (8 μm) through the first thoracic (prothoracic or T1) ganglion of the ventral nerve cord of *Manduca sexta* fixed in alcoholic Bouin's 72 h after pupal ecdysis. After fixation, the ganglion was dehydrated, embedded in a paraffin-based medium, sectioned, and stained with hematoxylin and eosin. The arrow points to the shrunken, condensed profile of a motoneuron that has undergone programmed cell death (PCD). Because of the absence of phagocytosis in the ventral nerve cord during metamorphosis, the persisting fragments of such neurons are easily detected in insect nervous tissue prepared using routine histological techniques. Scale bar = 30 μm .

impact of such deaths on the function of neural circuits must be profound (Truman, 1983). The observation that the corpses of many dying neurons are not phagocytosed and therefore persist in the ganglia facilitates the identification and analysis of uniquely identified cells or populations well after the initiation of the PCD program.

Patterns of neuronal death during metamorphosis in *Manduca* have been mostly fully described for the motoneurons of the abdominal ganglia, although histological surveys of thoracic ganglia reveal that the PCD of neurons is also found in this tissue at the metamorphic transitions (S.E. Fahrback, unpublished data) (Figure 2). At the larval–pupal transition, the best-studied examples of neuronal death are the proleg motoneurons (Weeks and Truman, 1985; Weeks, 1999). Studies of neuronal death associated with adult eclosion have focused primarily on the death of motoneurons (including the death of proleg motoneurons that persist after pupation) in the unfused abdominal ganglia, A3 through A5. In addition to the deaths of fully differentiated motoneurons and peptidergic interneurons, deaths of undifferentiated neurons occur in the imaginal nests of the segmental ganglia, both as the larvae feed and at the onset of metamorphosis (Giebultowicz and Truman, 1984; Booker and Truman, 1987a; Thorn and Truman, 1989; Booker *et al.*, 1996). The PCD of neuroblasts that are active during postembryonic life terminates proliferation

in specific lineages in both the segmental ganglia and the brain: in *Manduca*, the death of neuroblasts has been most comprehensively studied in the segmental ganglia (Booker and Truman, 1987a; Booker *et al.*, 1996).

The emphasis on PCD of neurons at the larval–pupal and pupal–adult transitions in *Manduca* should not obscure the fact that the majority of larval neurons survive metamorphosis and become integral components of the adult nervous system, where they interact with other neurons derived from embryonic and postembryonic neurogenesis.

5.4.3.2. PCD of abdominal motoneurons during metamorphosis in *M. sexta* With rare exceptions (see Section 5.3.1), the abdominal motoneurons of *Manduca* are born during embryonic life. At both the larval–pupal and the pupal–adult metamorphic transitions, some of the larval motoneurons lose their muscle targets as a consequence of changes in the organization of the skeletal musculature (Levine and Truman, 1985). At the larval–pupal transition, some of these now targetless motoneurons die. Examples of motoneurons that undergo PCD at this time include some of the motoneurons that innervate muscles associated with the five pairs of abdominal prolegs, the locomotory appendages of the caterpillar that disappear at pupation (Weeks and Truman, 1985). In some segments however, the proleg motoneurons survive this round of PCD,

and after a period of dendritic regression, acquire new muscle targets in the developing adult (Weeks and Ernst-Uttschneider, 1989; Weeks *et al.*, 1992). Targetless motoneurons, can therefore, either undergo PCD or become respecified. Experimental evidence suggests that the death of these motoneurons is triggered by endocrine cues independent of contact with target muscles (Bennett and Truman, 1985; Weeks and Truman, 1985). Abdominal motoneurons that lose their muscle targets after adult eclosion all die within 48 h of emergence, as the short (approximately 10 day) life of the adult apparently does not provide sufficient opportunity for respecification. It should be noted that unpublished observations from studies on the giant silkworm *Antheraea pernyi* (J.W. Truman) and *Hyalophora cecropia* (S.E. Fahrbach) suggest that, in these species, motoneurons persist well after the death of the ISMs and, therefore, neither respecify nor die. This suggests that these motoneurons do not play out a hormonally triggered program of PCD and that, in contrast to many vertebrate motoneurons, their survival is not dependent on target-derived trophic signals. Comparative studies on a broader range of insect taxa are needed to complete our understanding of neuronal PCD following adult eclosion.

5.4.3.3. Sex-specific PCD of abdominal motoneurons during metamorphosis The populations of abdominal motoneurons are the same in male and female larvae, but during metamorphosis the genital segments undergo sex-specific morphological changes that are accompanied by a wave of sex-specific PCD in the ganglia that innervate these segments (Giebultowicz and Truman, 1984; Thorn and Truman, 1989). This is an example of equal opportunity sexual differentiation, as some motoneurons persist in males but degenerate in females, while others show the opposite pattern (Thorn and Truman, 1989). There is also a difference in the timing of sex-specific PCD relative to pupation, with most neurons in females dying during the first 2 days following pupation, while most neurons in males die during the third to the sixth day after pupation (Thorn and Truman, 1994a).

A particularly interesting example of sex-specific neuronal death in *Manduca* is seen in the case of some of the imaginal midline neurons (IMNs), which are highly unusual motoneurons, because they are born postembryonically during the fourth (penultimate) larval instar (Thorn and Truman, 1994a). These motoneurons innervate visceral rather than skeletal musculature, and some of them can be tracked during the larval-pupal transition, because they are immunoreactive with an antibody directed

against molluscan small cardioactive peptide b (Thorn and Truman, 1994b). IMNs that innervate the sperm duct in males are absent from the terminal ganglia of females, while IMNs that innervate the oviduct in females are absent from the terminal ganglia of males. There is some evidence that contact with an appropriate target enhances the survival of the IMNs that innervate the sperm duct in males, another quite atypical aspect of their physiology (Thorn and Truman, 1994b).

5.4.3.4. PCD of identified peptidergic neurons during metamorphosis Two identified interneurons (INs) that contain crustacean cardioactive peptide (CCAP), cell 27 and IN 704, have been shown to undergo PCD within 36 h of adult eclosion (Ewer *et al.*, 1998). Both cell 27 and IN 704 display increases in cGMP immunoreactivity during larval ecdyses, and cell 27 also shows this response at pupal ecdysis and at adult eclosion (Ewer *et al.*, 1994; Ewer and Truman, 1997). Because application of CCAP to the isolated CNS can trigger the motor patterns of ecdysis, this set of neurons appears to be central to the control of molting behavior (Gammie and Truman, 1997; Mesce and Fahrbach, 2002). The death of peptidergic neurons involved in the control of ecdysis behavior, after the moth's molting career is finished, supports the hypothesis that obsolescent neurons are actively eliminated from the *Manduca* nervous system.

5.4.3.5. PCD in the brain during metamorphosis Detailed studies of specific regions of the *Manduca* brain have clearly demonstrated that newly generated neurons die during adult development. PCD is a prominent feature of the development of both the medulla and lamina cortices in the optic lobes (Monsma and Booker, 1996a). Both developmental hormones and retinal afferents appear to regulate this process (Monsma and Booker, 1996b). The extent of PCD in other regions of the developing *Manduca* brain is largely unstudied. A general role for ingrowing sensory afferents in the regulation of neuronal survival is suggested by the sex differences that arise in populations of antennal lobe interneurons, as a result of sexual dimorphisms in the antennae (Schneiderman *et al.*, 1982).

5.4.3.6. PCD of imaginal nest cells during metamorphosis Postembryonic neurogenesis is a major source of adult populations of neurons in holometabolous insects, yet a curious aspect of this phenomenon is that many neurons born during the postembryonic period die before they can differentiate. New neurons, which accumulate in nests

around their parent neuroblast, die in a nest- and segment-specific pattern during the feeding larval stages and the onset of metamorphosis (Booker *et al.*, 1996). Studies using bromodeoxyuridine as a marker for mitosis have produced estimates that between a third and 70% of imaginal neurons die within 24 h of their birth (Booker *et al.*, 1996). There is some evidence that the death of imaginal neurons and their associated neuroblasts in *Manduca* is regulated by developmental hormones (Booker and Truman, 1987b).

5.4.4. Regulation of PCD in the Nervous System during Metamorphosis

Neuronal death during insect metamorphosis has been described as hormone dependent, segment specific, and target independent (Truman *et al.*, 1992). Considerable experimental evidence supports each of these claims, although, in some special cases, a role for cell–cell interactions is implicit in the data.

The ecdysteroids are important regulators of PCD in the nervous system, acting to couple neuronal death with other metamorphic changes. It is important to note that the ecdysteroid cue for triggering metamorphic neuronal death can be either a rising or a falling titer, depending upon developmental stage. For example, the decline in circulating levels of 20E that occurs at the end of adult development prior to adult eclosion is the cue for the death of abdominal motoneurons at this time, and treatment with exogenous ecdysteroid at this time blocks the initiation of PCD (Truman and Schwartz, 1984). By contrast, it is the prepupal rise in circulating ecdysteroids that is responsible for the larval–pupal transition death of the motoneurons in abdominal ganglia A5 and A6, which innervate the accessory plant retractor muscle of the prolegs (Weeks and Ernst-Utzschneider, 1989; Weeks *et al.*, 1992; Zee and Weeks, 2001). The response of these proleg motoneurons to the steroid signal, however, is segment specific. Homologous neurons in abdominal ganglia A3 and A4 persist through the pupal stage and adult development, but then undergo PCD within 24 h of adult eclosion (Zee and Weeks, 2001). These responses to 20E, as well as the segment specificity of the response at different stages in development, are retained when individual proleg motoneurons are cultured *in vitro*, providing strong evidence for the cell-autonomous, target-independent nature of these PCDs (Streichert *et al.*, 1997; Hoffman and Weeks, 1998).

It is to be assumed that ecdysteroids exert their control on the timing of PCD by means of transcriptional regulation, but the target genes for steroid hormone action have not been identified in *Manduca*. In

addition, the tissues of *Manduca* and other insects express several different isoforms of the ecdysone receptor, which may have varying relationships to cell fate. In *Drosophila*, the expression of the A isoform of the ecdysone receptor (EcR-A) has been directly correlated with the occurrence of post-eclosion neuronal death in the CNS (Robinow *et al.*, 1993). Whether or not a similar relationship prevails in *Manduca* neurons remains to be determined, although autoradiographic evidence has demonstrated that *Manduca* motoneurons fated to die at the start of adult life display nuclear concentration of radiolabeled ecdysteroids (Fahrbach and Truman, 1989).

Evidence that other signals may fine-tune the timing of the death of neurons during metamorphosis comes from several sources. Adult *M. sexta* emerge from their pupal cuticle in an underground pupation chamber, and must then dig to the surface before inflating their wings. Adult moths forced to continue digging hours beyond the time this behavior would normally cease, exhibited delayed death of abdominal motoneurons (Truman, 1983). In addition, transection of the ventral nerve cord, prior to adult eclosion, blocks the death of specific motoneurons after adult eclosion in ganglia posterior to the point of transection, even in moths in which the levels of 20E undergo their normal decline (Fahrbach and Truman, 1987).

A well-studied example of a spared abdominal motoneuron is MN-12 (Figure 3). Subsequent to ventral nerve cord transection, this supernumerary member of the adult abdominal ganglion maintains both its normal central arborizations and electrophysiological properties, implying that the cell death program has been completely blocked in the absence of a descending signal (Fahrbach *et al.*, 1995; DeLorme and Mesce, 1999). Treatment of cultured abdominal ganglia with extracts prepared from ventral nerve cord restores the normal pattern of cell death to MN-12, but the active factor in the extracts remains to be identified (Choi and Fahrbach, 1995). Other examples of motoneuron death, such as the death of the accessory planta refractors (APRs) at the larval–pupal transition, however, are unaffected by cutting of the connectives prior to the normal time of death (Weeks and Davidson, 1994). This suggests that the phenomenon of interganglionic cell death signaling affects only a limited set of cells.

Because of the scattered, episodic nature of neuronal death during metamorphosis, and the unavailability of transgenic *Manduca* for analysis, relatively little is known about the molecular mechanisms of neuronal death in this species. Hormone-dependent neuronal death is blocked by treatment with inhibitors of transcription or translation,

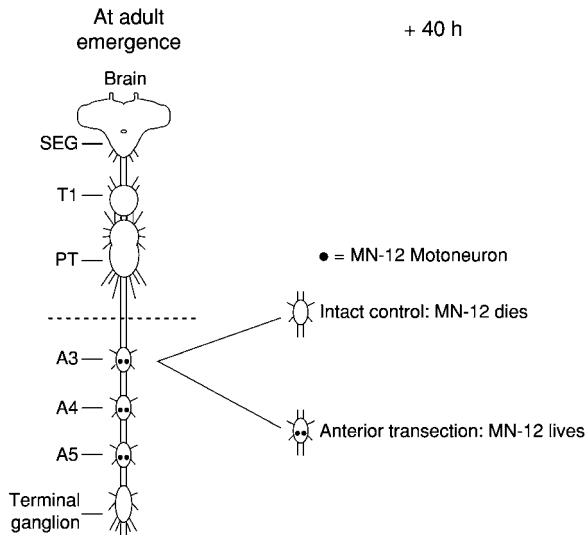


Figure 3 Schematic diagram of the central nervous system of an adult *Manduca sexta* moth. The MN-12 motoneurons in the abdominal ganglia normally undergo programmed cell death (PCD) by 40 h after adult emergence (eclosion), but are spared this fate if connections to the anterior nervous system are severed just posterior to the pterothoracic ganglion (Fahrbach and Truman, 1987). The dashed line indicates the point of anterior nerve cord transection. A3, A4, and A5, abdominal ganglia; PT, pterothoracic ganglion; SEG, subesophageal ganglion; T1, prothoracic ganglion.

suggesting the existence of an active gene-mediated cell death program (Weeks *et al.*, 1993; Fahrbach *et al.*, 1994; Ewer *et al.*, 1998; Hoffman and Weeks, 1998). In support of the hypothesis that PCD of *Manduca* neurons requires protein synthesis, a two-dimensional polyacrylamide gel electrophoresis analysis of protein patterns in the *Manduca* ventral nerve cord revealed changes associated with programmed neuronal death at adult eclosion (Montemayor *et al.*, 1990). Mitochondrial involvement in the death of APRs has been shown by studies in which inhibition of caspase activity blocked the death of cultured proleg motoneurons (Hoffman and Weeks, 2001). Ultrastructural studies of dying *Manduca* neurons are uncommon, but studies of the motoneurons that innervate the ISMs and of APR motoneurons indicate that neuron death in *Manduca* during metamorphosis is autophagic rather than apoptotic (Stocker *et al.*, 1978; Kinch *et al.*, 2003).

Immunostaining studies, in which the distribution of several death-associated gene products (initially identified from a screen of dying moth muscle; see Section 5.5.4.3) was examined in the segmental ganglia, failed to provide a reliable correlation of enhanced ubiquitin- or multicatalytic proteinase-immunoreactivity within dying neurons (Fahrbach and Schwartz, 1994; Hashimoto *et al.*,

1996), despite an association of these gene products with PCD in insect skeletal muscles (Jones *et al.*, 1995; Haas *et al.*, 1995). This discrepancy in gene expression during PCD in neurons and muscles remains unexplained. By contrast, one gene product, expressed by both dying neurons and degenerating muscles in *Manduca*, is apolipoprotein III, a finding that suggests that this molecule has functions in addition to its role in lipid transport (Sun *et al.*, 1995).

5.4.5. PCD of Muscles during Metamorphosis

The ISMs of *Manduca* (Figure 1) are the major abdominal muscles of the larva, pupa, and pharate adult. The ISMs are divided into three separate pairs of bilaterally symmetric bundles, each of which attaches to the cuticle at the intersegmental boundaries. These muscles initially form in the embryo and span eight of the abdominal segments in the larva. The ISMs provide the major propulsive force for both hatching and subsequent larval locomotion. Following pupation, the muscles in the first two and last two abdominal segments die and rapidly disappear. The muscles in the middle four segments persist throughout metamorphosis and are used for the defensive and respiratory movements of the pupa. Following adult eclosion, these remaining ISMs undergo PCD and are lost during the subsequent 30 h. While the molecular basis for this segmental fate determination has not been examined, presumably it is established early in embryogenesis due to the actions of gap, pair-rule, and segment-polarity genes (Bejsovec and Wieschaus, 1993; DiNardo *et al.*, 1994; French, 2001; Sanson, 2001).

The nuclear changes that accompany ISM death display none of the features of apoptosis (Schwartz *et al.*, 1993) (Figure 4). The chromatin does not become electron dense but remains dispersed throughout the nucleoplasm. In addition, agarose gel electrophoresis of ISM genomic DNA fails to reveal apoptotic ladders, although activation of DNases may occur as a very late event, after membrane integrity has been lost. Ultrastructurally, there is an increase in autophagic vesicles, and these cells are thought to die by autophagy (Lockshin and Beaulaton, 1974, 1979).

Following PCD in most organisms, the cell corpse is phagocytosed by neighboring cells or circulating macrophage-like cells (see Section 5.2.1). A classic example of this phenomenon is found in amphibian metamorphosis, where the massive tail musculature is lost during the short transition from larva to adult (Weber, 1964; Watanabe and Sasaki, 1974). During this process, the muscle fibers become

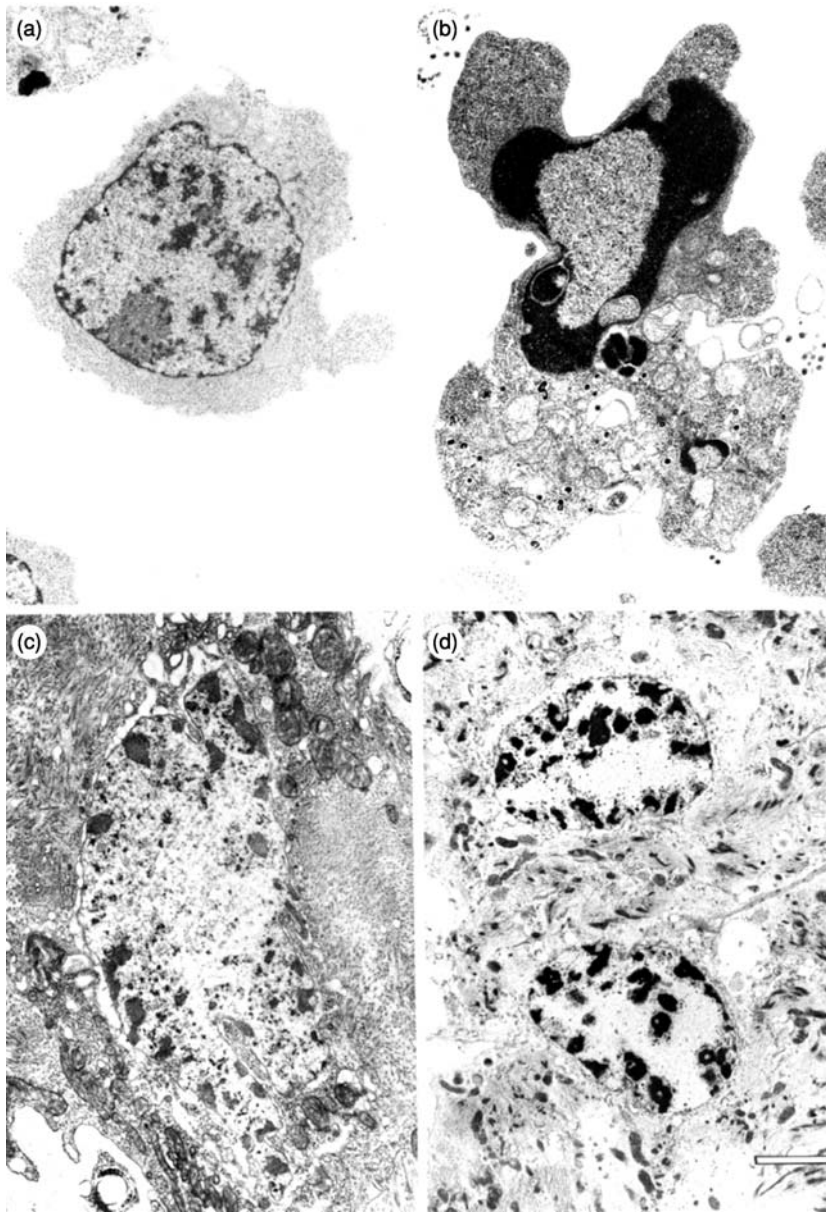


Figure 4 Transmission electron micrographs comparing the morphology of mouse lymphocyte apoptosis (a and b) with the autophagic death of the ISMs of *Manduca* (c and d). The mouse T cell hybrid cell line D011.10 was induced to die with the glucocorticoid dexamethasone, and examined either at time zero (a) or 16 h after exposure (b). These cells die by apoptosis and display many of the classical features of the program, including membrane blebbing and the deposition of electron-dense chromatin along the inner surface of the nuclear envelope. These images contrast those of normal *Manduca* ISMs (in a day 16 pupa; c) and dying ISMs (at 18 h posteclosion; d). During autophagy, the nuclei round up and the chromatin remains dispersed in the nucleoplasm.

decorated with macrophages that, in turn, contain identifiable remnants of skeletal muscle debris (Metchnikoff, 1892; Nishikawa *et al.*, 1998).

While dying muscles in insects are often phagocytosed by hemocytes (Crossley, 1968), this is not universally the case (Jones *et al.*, 1978). In particular, the death of the ISMs, following adult eclosion in moths, does not attract macrophage-like cells or rely on phagocytosis for resolution

(Beaulaton and Lockshin, 1977). Although this cannot be accounted for solely by trivial reasons, such as an absence of circulating macrophages capable of phagocytosing foreign bodies (Jones and Schwartz, 2001), estimates of ISM volumes and hemocyte numbers in adult *Manduca* suggest that animals would require at least an order of magnitude greater number of phagocytic cells than has been shown to reside in the hemolymph (Jones and Schwartz,

2001). While the ISMs of adult animals are not phagocytosed, some of the ISMs that die following pupation in *Manduca* appear to behave differently. Rheuben examined the death of mesothoracic muscles in pupae, and observed an intimate association between phagocytic hemocytes and the sarcolemma (Rheuben, 1992). The phagocytes were well spaced along the fibers and appeared to specifically degrade the basal lamina. One difference between the ISMs and the mesothoracic fibers is that the latter are not completely degraded during development. Instead, they act as scaffolds for myoblasts that remodel the fibers during the formation of adult muscle fibers. Phagocytes may play a more significant role in facilitating tissue remodeling, as opposed to cell death.

5.4.5.1. Endocrine control of ISM death The timing of ISM death must be precisely coordinated with other metamorphic events or the animal might suffer disastrous consequences. For example, premature loss of the ISMs in moths would leave the animal trapped within the pupal cuticle and locked in either a cocoon or an underground chamber. Delays in ISM death might also have deleterious consequences, possibly depriving the adult of essential nutrients required for gametogenesis.

As described above (see Section 5.4.4), the titer of ecdysteroids serves as an endogenous developmental time reference, that can be used by the different organs of the pupa to coordinate developmental decisions. Sequential declines in the ecdysteroid titer serve to coordinate developmental changes in the tissues, including the timing of ISM death (Schwartz and Truman, 1983).

Early reports identified the cessation of motor neuron activity as the proximal trigger for ISM death (Lockshin and Williams, 1965b). This hypothesis was subsequently proven incorrect, when it was found that normal timing of ISM death in *A. polyphemus* was not altered either by silencing motoneuron activity with the highly specific sodium channel blocker tetrodotoxin or by the removal of the entire ventral nerve cord (Schwartz and Truman, 1983, 1984b). Instead, it was shown that the peptide eclosion hormone (EH) acts to trigger ISM death in this species (Schwartz and Truman, 1984b; 1984a). The EH acts via cGMP, and the description of its role in ISM death represented the first study demonstrating that cGMP met all of Earl Sutherland's requirements for identifying a second messenger for a hormone action (Sutherland, 1972; Schwartz and Truman, 1984a). The capacity of EH to act on the ISMs is itself under the control of circulating ecdysteroids, as a decline in circulating

20E is required to determine both the timing of EH release and the developmental capacity of the muscles to respond to this trigger (Truman, 1984; Schwartz and Truman, 1984b).

5.4.5.2. Physiology of ISM death The size of the ISMs and the coordinated nature of the developmental changes that take place in this tissue during metamorphosis facilitate the examination of the physiological changes that accompany naturally occurring muscle atrophy and death. Under laboratory conditions, metamorphosis in *Manduca* takes 18 days, with adult eclosion taking place late on day 18. On day 15 of adult development, the mass of the ISMs begins to decline and, during the subsequent 3 days, ISMs lose 40% of their mass. This pre-eclosion program of atrophy is nonpathological, and the muscles retain virtually all of their normal physiological responses, including force/cross-sectional area and sensitivity to calcium ions in skinned fiber preparations. This suggests that the reduction in muscle mass, observed during the atrophy phase, reflects a generalized enhancement of protein turnover rather than a selective destruction of specific contractile proteins. Ultrastructural studies by Lockshin and Beaulaton (1979) have shown that entire contractile bundles are lost during this phase.

In *Manduca*, the ISMs begin the death program coincident with adult eclosion, late on day 18 after pupation, with the muscles beginning to lose mass at a rate of approximately $4\% \text{ h}^{-1}$ (Schwartz and Truman, 1982). Concurrently, there is a slow, progressive depolarization of the fibers at a rate of $\sim 2.5 \text{ mV h}^{-1}$ (Runion and Pipa, 1970; Schwartz and Ruff, 2002). By 24 h posteclosion, reliable resting potentials can no longer be recorded. The retention of a resting potential during the active phase of ISM death verifies that the cells are not undergoing a necrotic process. While there is little change in the organization of the contractile apparatus during the atrophy phase, the postemergence period is marked by profound sarcomere disruption (Lockshin and Beaulaton, 1979). Whole filaments disappear rapidly, with a preferential loss of thick filaments relative to thin filaments (Beaulaton and Lockshin, 1977). During this same period, mitochondria are lost, autophagic vacuoles form, and the T tubule system swells.

Not surprisingly, there are physiological consequences that accompany these dramatic changes in ISM structure. The fibers rapidly weaken, even when force is normalized to cross-sectional area. This is true for twitches, and for tetanus and caffeine-induced contractions (Schwartz and Ruff, 2002). There are also defects in the ability of the

contractile apparatus to respond to free calcium in either intact muscles or skinned fiber preparations.

5.4.5.3. Patterns of gene expression during PCD of ISMs While ISM atrophy appears to involve an increase in protein catabolism over synthesis (Haas *et al.*, 1995), there is incontrovertible evidence that the posteclosion death of these cells involves the implementation of a new developmental program. Insights into the molecular mechanisms of ISM death were provided in 1969, when Lockshin reported that the death of silkworm ISMs is blocked by inhibitors of RNA or protein synthesis (Lockshin, 1969). These results are similar to those reported for PCD in other insect tissues, including the nervous system (see Section 5.4.4), as well as other taxa such as amphibians (Weber, 1965). Taken together, these data provided some of the earliest evidence that the ability of cells to die required *de novo* gene expression.

To confirm and extend this hypothesis, Schwartz and colleagues made cDNA libraries from condemned, day 18 ISM RNA, and screened them for differentially expressed genes. While the vast majority of ISM genes were found to be constitutively

expressed, a small number were observed to be either repressed or induced when the muscles became committed to die. The cloning of these new sequences from day 18 ISMs represented the first published report of death-associated gene expression changes from any organism (Schwartz *et al.*, 1990a). Subsequent characterization of these sequences has provided new information about the molecular mechanisms of PCD.

Among the genes that are repressed, when the ISMs become committed to die, are actin and myosin heavy chain (Schwartz *et al.*, 1993) (Figure 5). These transcripts are among the most abundant in the muscle throughout metamorphosis, but they begin to disappear late on the day before adult eclosion (day 17), coincident with the commitment to die. By the time of adult eclosion, they are almost undetectable on Northern blots. A single injection of 25 µg of 20E on day 17 is sufficient to delay both the ISM death on day 18 and the loss of these transcripts, thus linking this change in gene expression to the commitment of the muscles to die.

Given that most muscle genes are constitutively expressed during ISM atrophy and death, there must be some advantage to reducing selectively the

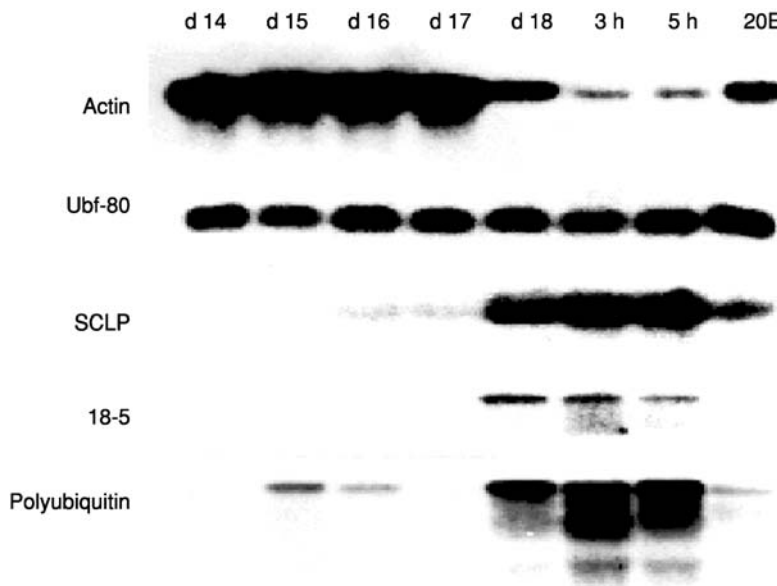


Figure 5 Changes in the patterns of gene expression during the atrophy and death of the intersegmental muscles in *Manduca*. RNA was isolated from the ISMs at the time indicated and fractionated by size in agarose. After transfer to a nylon membrane, the Northern blots were hybridized with different cDNAs isolated from *Manduca*. The expression of some genes, like actin and myosin heavy chain (not shown), is repressed when the ISMs become committed to die on day 18. When animals are injected with 20-hydroxyecdysone (20E) on day 17 of pupal–adult development and examined 5 h after the normal time of eclosion, the loss of these transcripts is reduced. The majority of ISM genes are constitutively expressed throughout the period of ISM development examined, as shown here for the *ubiquitin fusion-80* (*Ubf-80*) gene. A final class of genes is induced when the ISMs become committed to die, including: *SCLP* (small cytoplasmic leucine-rich repeat protein), *polyubiquitin*, and the as yet uncharacterized *18-5* gene. Pretreatment with 20E delays the accumulation of these induced transcripts. d, day of pupal–adult development; h, hours – postadult eclosion.

abundance of actin and myosin heavy chain mRNA. One possible explanation is that it allows the muscles to change, in a dramatic fashion, their developmental program from one of homeostasis to another of death, by allowing newly expressed transcripts to accumulate and effectively compete for the cellular translational machinery.

One mechanism for reducing transcript abundance is transcriptional repression. A complementary mechanism is to enhance transcript degradation. In fact, it was found that there is a transient increase in endogenous ISM RNase activity on day 17, which facilitates the removal of all transcripts prior to the induction of new gene expression (Cascone and Schwartz, 2001). This coordinated control of transcription and degradation may allow the muscles to shift developmental programs extremely rapidly. It has yet to be determined whether a similar strategy is used by other organisms during development.

In addition to transcript loss, several newly expressed death-associated cDNAs have been cloned and characterized from the ISMs (Schwartz *et al.*, 1990a). While the majority of these cDNAs encoded previously uncharacterized genes, the first one to be sequenced was shown to be the product of the polyubiquitin gene (Schwartz *et al.*, 1990b). Ubiquitin is a 76 amino acid peptide that is the most highly conserved protein across phylogeny. At the protein level, insect and human ubiquitins are identical (review: Rechsteiner, 1988). The posttranslational covalent attachment of ubiquitin to selected lysine residues on substrate proteins serves as a molecular tag, to help target proteins to specific fates within the cell (Murphey and Godenschwege, 2002; Schwartz and Hochstrasser, 2003). The addition of single ubiquitin moieties serves to target proteins to specific subcellular locations, while the addition of multiple head-to-tail ubiquitin chains promotes binding to the 26S proteasome. This multisubunit protease then releases the ubiquitin, unfolds the substrate and rapidly degrades it to small peptides.

In *Manduca* ISMs, expression of polyubiquitin mRNA increases transiently on days 15 and 16 of adult development, and then accumulates to prodigious levels on day 18, the day of adult eclosion. The early expression of ubiquitin is associated with the increased turnover of contractile proteins that accompanies the atrophy program that begins on day 15 and a transient increase in ubiquitin-dependent proteolysis (Haas *et al.*, 1995). On day 18, there is a coordinated exponential increase in the expression of the entire ubiquitin-proteasome pathway, including ubiquitin conjugation, ubiquitin-dependent proteolysis, and proteasome subunit expression. In fact, it has been found that there is a coordinated

exponential increase in the expression of both 20S and 26S proteasome subunit proteins, at both the mRNA and protein levels (Schwartz *et al.*, 1990b; Dawson *et al.*, 1995; Haas *et al.*, 1995; Jones *et al.*, 1995; Takayanagi *et al.*, 1996; Low *et al.*, 1997). Placed in a broader context, this enhancement in ubiquitin-dependent proteolysis is presumably adaptive, because the ISMs are not phagocytosed and, therefore, the liberation of cellular constituents requires a cell-autonomous mechanism.

Other previously described transcripts were also identified in the condemned ISMs, but their relationship to PCD remains unknown. For example, the abundance of apolipoprotein III (apoLp-III) is dramatically induced, at both the RNA and protein levels, in both the ISMs and in certain dying interneurons (Sun *et al.*, 1995). ApoLp-III is synthesized predominantly in the fat body and normally associates with lipophorin in the hemolymph to facilitate lipid transport. The role of apolipoprotein in PCD is mysterious, particularly since the ISMs do not express lipophorin.

The majority of cDNAs isolated in the ISM screen encoded novel proteins of unknown function. For example, one of these cDNAs encodes a small protein that is composed of multiple leucine-rich repeat protein-protein interaction motifs (Kuelzer *et al.*, 1999). Termed *SCLP* (small cytoplasmic leucine-rich repeat protein), this gene is dramatically induced at both the RNA and protein levels. Exploiting a paradigm for the study of genes initially isolated in moths, transgenic flies were generated that express *Manduca SCLP* under the control of the Gal4 protein upstream activating sequence (UAS) using the *P* element system that allows gene expression to be targeted to specific tissues in the fly. Ectopic expression of *SCLP* in a variety of tissues failed to reveal an overt phenotype, suggesting that overexpression of *SCLP* is nonpathological (Kuelzer *et al.*, 1999). Studies employing loss-of-function mutations will be needed to define the role of *SCLP* in development and death.

In contrast to *SCLP*, other novel death-associated transcripts from *Manduca* ISMs have been shown to play key roles in PCD. The characterization of two such genes has provided insights into the development and death of muscle in mammals as well as insects. The first of these genes is *DALP* (death-associated LIM-only protein). In contrast to the genes described above, *DALP* is induced on day 17, well in advance of the other death-associated cDNAs from *Manduca* ISMs (Hu *et al.*, 1999). Expression of the *DALP* protein is restricted to the ISMs and is not detected in the flight muscle, fat body, Malpighian tubules, ovary/eggs or male

sexual accessory gland. The most significant structural feature of the DALP protein is the presence of one perfect and two imperfect LIM domains, structural motifs that consist of paired zinc fingers, which participate in protein–protein interactions (Leon and Roth, 2000; Laity *et al.*, 2001). While many LIM proteins also possess a homeodomain, DALP belongs to the subfamily of LIM-only proteins (LMO) that lack this DNA binding motif.

As with SCLP, the function of *Manduca* DALP was explored using transgenic flies (Hu *et al.*, 1999). Ectopic expression of *Manduca* DALP in the abdominal intersegmental muscles of fly pupae resulted in the disorganization of the contractile apparatus and subsequent muscle atrophy (Figure 6). Targeted mutations in the LIM domain blocked muscle atrophy, suggesting that the observed effects of ectopic DALP expression were dependent on expression of the intact functional protein.

Further insights into the function of DALP were gained by examining the effects of expressing *Manduca* DALP in the mouse myoblast C₂C₁₂ cell line (Hu *et al.*, 1999). This muscle satellite cell line has been extensively used as a model for examining

skeletal muscle differentiation in mammals. C₂C₁₂ cells can be maintained as a stable, nontransformed line that ceases cycling and differentiates into multinucleated myotubes following incubation in a low serum differentiation medium (Yaffe and Saxel, 1977). Expression of DALP was able to block the ability of C₂C₁₂ cells to differentiate into myotubes by blocking the induction of MyoD, a basic helix–loop–helix muscle transcription factor required for differentiation. These effects of DALP were overcome by cotransfecting the cells with an expression vector driving ectopic MyoD. In addition to blocking differentiation, DALP greatly enhanced the probability of cell death. Identical data were obtained when C₂C₁₂ cells were forced to express ectopic *Hic-5* (hydrogen peroxide-inducible clone-5), the mammalian ortholog of DALP. Taken together, these data demonstrate that DALP and *Hic-5* are phylogenetically conserved proteins that function as negative regulators of muscle differentiation and survival in both insect and mammalian cells. These results also validate the view that general conclusions about PCD and differentiation may be drawn from the study of insect metamorphosis.

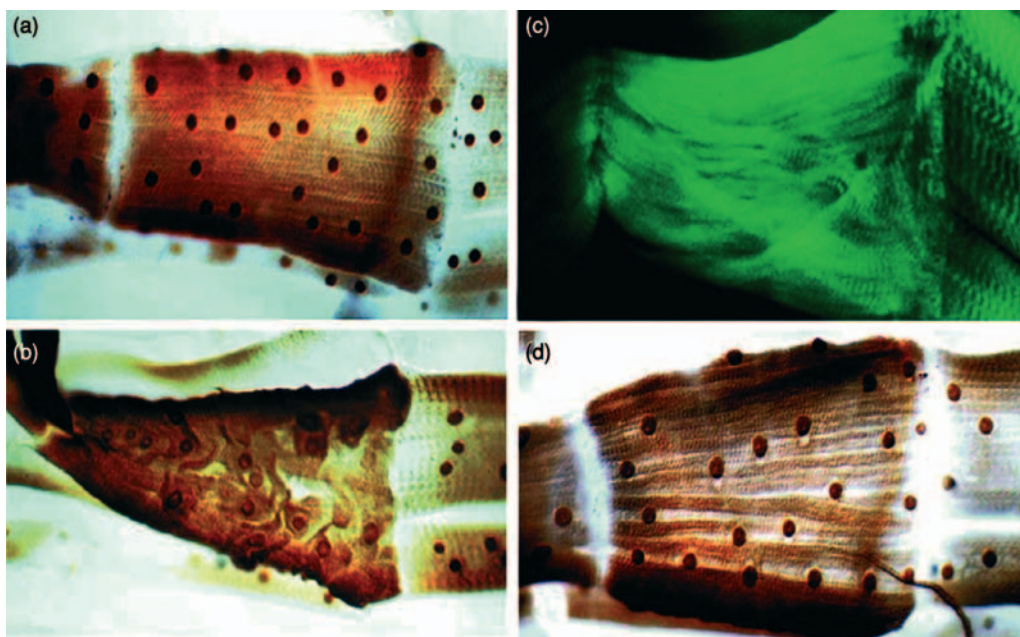


Figure 6 The effects of ectopic DALP expression in *Drosophila* skeletal muscle. (a) Bacterial β -galactosidase was expressed in *Drosophila* ISMs using the Gal4/UAS expression system. Expression (nuclear as well as cytoplasmic) was monitored via anti- β -galactosidase immunocytochemistry. (b) The same Gal-4 enhancer trap line was used to coexpress β -galactosidase and moth DALP in the ISMs. DALP expression was independently shown to parallel that of β -galactosidase, monitored in this panel by immunohistochemistry. (c) The organization of the contractile apparatus was visualized using fluorescein isothiocyanate (FITC)-labeled phalloidin in separate animals expressing ectopic DALP. In this panel, the disorganization of the contractile apparatus is evidenced by the loss of the sarcomeres that are obvious in the two fibers at right. (d) The WHPEHF motif was deleted from DALP and coexpressed with β -galactosidase in the ISMs. Muscles retained normal appearance and were contractile. Note that the triangular muscle in these assays is muscle 31 and is about 260 μ m long. (Reproduced from Hu, Y., Cascone, P., Cheng, L., Sun, D., Nambu, J.R., Schwartz, L.M. 1999. Lepidopteran DALP, and its mammalian ortholog *Hic-5*, function as negative regulators of muscle differentiation. *Proc. Natl Acad. Sci. USA* 96, 10218–10223.)

The last gene of the moth ISMs to be cloned and analyzed to date encodes a protein named Acheron (Ach; after the river that must be crossed to reach to the realm of the dead in Greek mythology). Acheron is undetectable in ISMs until early on day 18, when it begins to accumulate to high levels (Wang, Valavanis, Sun, and Schwartz, unpublished data). This increase in *Acheron* expression on day 18 can be blocked with a single intrathoracic injection of 25 µg of 20E on day 17. *Acheron* expression is not restricted to the ISMs, however, because Northern blot analysis revealed low levels of expression in the fat body and flight muscle but not in Malpighian tubules, ovaries, or the male sexual accessory gland. Because the ovary is composed predominantly of unfertilized oocytes, these data suggest that *Acheron* is not a maternal transcript.

Database analysis revealed a human expressed sequence tag (EST) that shares 59% identity and 68% similarity over 86 amino acids with *Manduca Acheron* (Wang, Valavanis, Sun, and Schwartz, unpublished data). Using this sequence as a probe, full-length human and mouse homologs of Acheron were cloned. It was found that they display about 31% identity and 40% similarity to *Manduca Acheron*.

Mammalian *Acheron* expression has also been studied in the mouse C₂C₁₂ line (Wang, Valavanis, Sun, and Schwartz, unpublished data). It appears that *Acheron* acts near the apex of the genetic hierarchy that regulates myoblast survival and differentiation, because it is required for myotube formation and, when its activity is blocked with either antisense or a dominant-negative form of the protein, differentiation is blocked. Acheron was also found to block the expression of the anti-apoptotic protein Bcl-2 (B cell lymphoma 2), which, in turn, enhances the death of cells. These data suggest that Acheron, like DALP/Hic-5, acts to control key developmental decisions in muscle cells, including the initiation of PCD.

5.5. The *Drosophila* Model

5.5.1. Choice of *Drosophila melanogaster* as a Model System for the Study of PCD of Nerve and Muscle Cells

The successful application of genetics to define the molecular controls of PCD in the nematode *Caenorhabditis elegans* provided impetus to apply this approach to the study of PCD in the fruit fly, *D. melanogaster* (Diptera: Drosophilidae). Kimura and Truman (1990) extended earlier observations of muscle death during *Drosophila* metamorphosis by

systematically documenting the accompanying post-eclosion neuronal death in the fused ventral ganglia. Injection of living flies with a toluidine blue solution revealed numerous examples of dying neurons in the dorsal and lateral regions of the abdominal and metathoracic neuromeres (Kimura and Truman, 1990). These investigators also mapped changes in both the head and abdominal musculature, before and after eclosion, by using polarized light microscopy to monitor the loss of birefringent muscle fibers. Several studies described below (see Section 5.5.3.1) have made profitable use of these baseline observations to explore ecdysteroid regulation and molecular mechanisms of PCD in the nervous system of adult flies (Robinow *et al.*, 1993, 1997; Draizen *et al.*, 1999).

However, the first identification of novel cell death genes in *Drosophila* was not based on the study of metamorphosis, but instead resulted from a screen for aberrant patterns of cell death in embryos carrying homozygous chromosomal deletions, carried out in the laboratory of Hermann Steller (White *et al.*, 1994). Embryo staining with the vital dye acridine orange was used to compare wild-type embryos with 129 different deletion strains. Chromosomal deletions that remove a small region at 75C on chromosome 3L, such as Df(3L)H99, were found to exhibit a blockade of both developmental and X-irradiation induced apoptosis. Subsequent studies led to the identification of four related proapoptotic genes that reside within a 300 kb interval in this region, *reaper* (*rpr*), *head involution defective* (*hid*), *grim*, and *sickle* (*skl*) (White *et al.*, 1994; Grether *et al.*, 1995; Chen *et al.*, 1996; Christich *et al.*, 2002; Srinivasula *et al.*, 2002; Wing *et al.*, 2002) (Figure 7). These linked genes are all transcribed in the same direction and are expressed predominantly in doomed and dying cells. As will be described below, proper regulation of cell survival in *Drosophila* involves precise transcriptional control of this gene complex.

Reaper, Grim, and Sickle are all small proteins (65, 138, and 108 amino acids respectively), while Hid is substantially larger at 410 amino acids. Each of these proteins possesses a related 14 amino acid region at the N-terminus, the RHG (Reaper, Hid, Grim) motif or IBM (inhibitor of apoptosis (IAP)-binding-motif) that has potent proapoptotic activities. The RHG and IBM motifs of Reaper and Grim are very similar: they share 71% identity, and three of the four amino acid differences are conservative substitutions. Surprisingly, despite this similarity, *in vivo* studies have shown that these domains possess distinct death-inducing activities (Wing *et al.*, 1998b). Reaper, Grim, and Sickle also share a

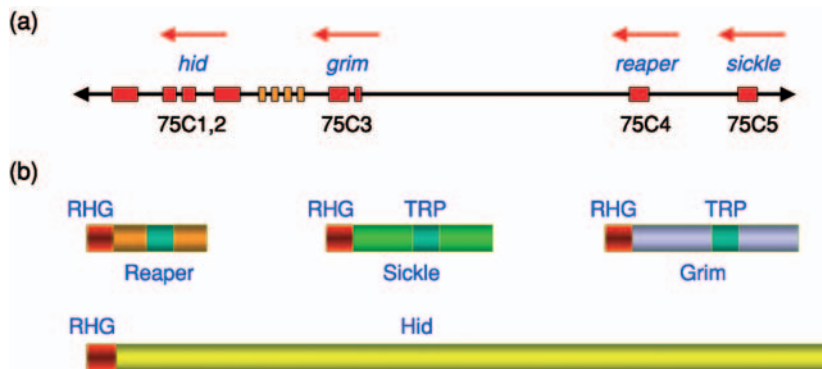


Figure 7 The *grim-reaper* genes encode related proapoptotic proteins. (a) The *hid*, *grim*, *reaper*, and *sickle* genes all reside within a 300 kb interval in the 75C region of chromosome 3L of *Drosophila melanogaster*. The four genes are transcribed in the same direction (arrows above genes) and are expressed predominantly in doomed and dying cells. No genes are predicted to reside between *grim* and *reaper*, or *reaper* and *sickle*, while four unrelated genes (orange blocks) reside between *hid* and *grim*. (b) *reaper*, *sickle*, and *grim* encode small proteins that contain a related N-terminal RHG motif/IBM as well as a C-terminal Trp block/GH3 domain. *Hid* is a substantially larger protein that also contains an RHG motif but does not exhibit strong sequence similarity to the Trp block/GH3 domain.

second region of sequence similarity, the 15 amino acid Trp block or Grim Helix 1, 2, and 3 (GH3) domain (Figure 7), which also has proapoptotic functions (Wing *et al.*, 2001; Claveria *et al.*, 2002). While *Hid* possesses four regions that resemble a Trp block/GH3 domain, the level of similarity is much lower. Together, the *reaper*, *hid*, *grim*, and *sickle* genes should be considered a genetic complex that controls apoptosis.

The considerable insights into the molecular mechanisms of PCD gained by further studies of this complex during *Drosophila* embryonic development and metamorphosis are described in detail below (see Section 5.5.4). In addition, it should be noted that targeted ectopic expression of these genes in cells that are normally fated to live, provides a valuable tool for producing cell-specific lesions of the CNS (McNabb *et al.*, 1997; Renn *et al.*, 1999; Rulifson *et al.*, 2002; Park *et al.*, 2003).

5.5.2. Hormonal Regulation of Metamorphosis in *Drosophila melanogaster*

Hormonal regulation of metamorphosis in *Drosophila* is similar to that described for other insects, with postembryonic development wholly dependent upon exposure of tissues to coordinated pulses of 20E, which, in turn, produce a coordinated cascade of gene expression (Riddiford, 1993; Truman and Riddiford, 2002). Cloning of the *Drosophila* ecdysone receptor (*EcR*) gene permitted the undertaking of studies of expression patterns of the receptor in tissues, including neurons and muscles (Koelle *et al.*, 1991; Robinow *et al.*, 1993; Talbot *et al.*, 1993; Truman *et al.*, 1994). Consistent with the results from *Manduca*, these studies have reinforced the view that ecdysteroid regulation of PCD in insects

is typically a result of direct action of the steroid on the cells that are fated to die (Robinow *et al.*, 1993).

5.5.3. PCD of Neurons during Early Development and Metamorphosis

In *Drosophila*, there are four periods of cell death in the nervous system: during mid to late embryogenesis, in late third instar larvae, in pupae during metamorphosis, and in the newly eclosed adult. The cell death that occurs during these stages permits the generation of a functional larval and adult nervous system by eliminating cells that are produced in excess, such as the embryonic midline glia, or cells with transient functions, such as larval abdominal ganglion neuroblasts (Kimura and Truman, 1990; Truman *et al.*, 1993; Sonnenfeld and Jacobs, 1995a; Zhou *et al.*, 1995). As in mammalian development, the extent of cell death in the developing *Drosophila* nervous system is profound: approximately two-thirds of all cells born within the embryonic CNS die before larval hatching (Abrams *et al.*, 1993; White *et al.*, 1994) (Figure 8).

The great extent of cell death in the *Drosophila* embryos suggests that this process is critical for early nervous system development. In this regard, mutations in several genes that are important for cell death result in hypertrophy of the embryonic, larval, or adult CNS, and these mutants can exhibit stage-specific lethality or sterility (Grether *et al.*, 1995; Song *et al.*, 1997; Zhou *et al.*, 1997; Peterson *et al.*, 2002). Interestingly, the removal of dead cells within the CNS is also apparently important for nervous system development, as mutants lacking functional macrophages exhibit disruptions in the normal architecture of the embryonic axon scaffold (Sears *et al.*, 2003).

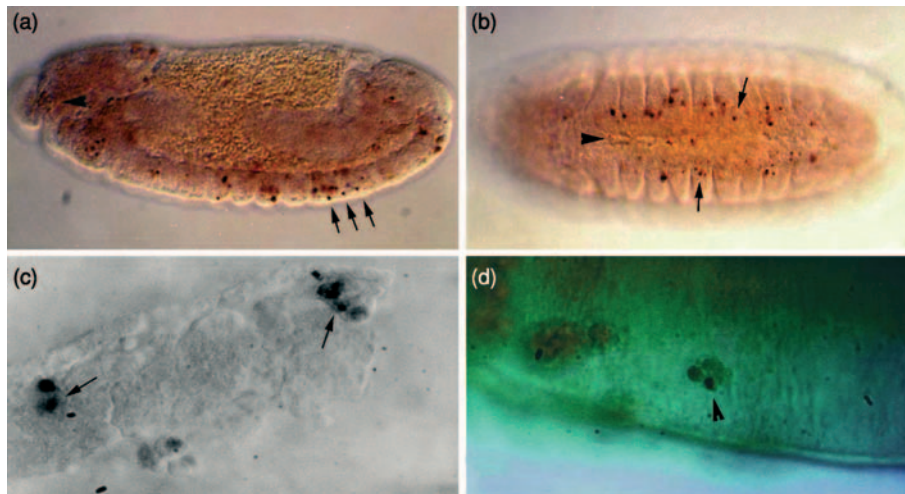


Figure 8 Detection of dying cells in *Drosophila* embryos. (a) Sagittal view of a stage 12 wild-type embryo analyzed via *in situ* nick translation to detect fragmented chromatin in dying cells. Note the prominent cell death in the retracting germ band (arrows). Significant cell death is also detected in the cephalic region of the embryo (arrowhead). Anterior is left and dorsal is up. (b) Ventral view of a stage 12 embryo labeled via *in situ* nick translation. Note the asymmetric pattern of dying cells in the neuroectoderm (arrows) on both sides of the CNS midline (arrowhead). Anterior is to left. (c) Section of tissue showing multiple labeled apoptotic bodies within phagocytic macrophages (arrows). (d) Ventral region of stage 16 embryo indicating an apoptotic body (arrowhead) engulfed by a circulating macrophage.

In contrast to the situation in the embryo, the proportion of neurons that die during the postembryonic period is much lower, and the widespread degeneration of larval tissues, characteristic of metamorphosis in flies, does not extend to the CNS. As in the *Manduca* model described above (see Section 5.4.3.1), most neurons of the larva persist rather than die, and the adult nervous system is, therefore, a composite structure of persisting neurons, born during embryogenesis, and adult-specific neurons. After pupariation, however, many dying neurons are observed in the ventral CNS in both thoracic and abdominal neuromeres (Truman *et al.*, 1993). The identity of most of these neurons has not been determined, with the exception of several motoneurons identified by retrograde fills of the T2 mesothoracic nerve (Consoulas *et al.*, 2002). The population of neurons that die at this metamorphic transition likely includes motoneurons that have lost their muscle targets, and interneurons in circuits for larval-specific behaviors. In addition, PCD of neuroblasts after pupariation marks the termination of postembryonic neurogenesis in the ventral nervous system (Truman and Bate, 1988; Truman *et al.*, 1993).

The death of neurons after adult eclosion has received more in-depth study than the neuronal deaths associated with pupariation (Kimura and Truman, 1990). Neurons dying at this time presumably include the motoneurons that innervate the abdominal muscles used during ecdysis and wing

expansion (Truman *et al.*, 1993). Many features of posteclosion neuronal death in *Drosophila* mirror phenomena previously described in the *Manduca* model. For example, neuronal death is delayed in newly eclosed flies that are forced to continue ecdysis behavior beyond the normal period (Kimura and Truman, 1990), as is the death of motoneurons in the abdominal ganglia of moths forced to dig after eclosion (Truman, 1983). Decapitation of flies at the time of eclosion results in inhibition of neuronal death in the ventral nervous system, similar to the sparing of MN-12 in *Manduca* by nerve cord transection (Kimura and Truman, 1990) (see Section 5.4.4).

Another major feature shared by the *Manduca* and *Drosophila* models of posteclosion neuronal death is its regulation by ecdysteroids. Treatment of both newly emerged flies and moths with ecdysteroids delays the PCD of neurons: only in the fly, however, can the molecular basis of this phenomenon be readily investigated (e.g., Baehrecke, 2000). In fact, the powerful molecular genetic approaches available in *Drosophila* make it possible to explore in detail the specific mechanisms that regulate cell survival in the developing nervous system. In addition to the continuing study of the posteclosion neuronal deaths initially described by Kimura and Truman (1990), this issue has been addressed using several distinct cellular systems, including the embryonic CNS midline, as well as embryonic and larval neuroblasts. In the following sections these

examples will be considered and the specific contributions that each has made toward the understanding of cell death regulation will be discussed.

5.5.3.1. Role of EcR in posteclosion neuronal death The *EcR* gene of *Drosophila* encodes three ecdysone receptor subunits: EcR-A, EcR-B1, and EcR-B2 (Talbot *et al.*, 1993). These receptor subunits share common DNA and ligand-binding domains, but have different N-terminal regions. EcR-A and EcR-B1 can be distinguished by monoclonal antibodies. The use of these antibodies to examine the expression of EcR isoforms in the metamorphosing *Drosophila* CNS revealed that a high level of expression of EcR-A is correlated with posteclosion PCD (Robinow *et al.*, 1993). Nearly 300 neurons, whose neuronal status was confirmed by double labeling with antibodies against EcR-A and ELAV (embryonic lethal, abnormal vision, a neuron-specific protein) in the ventral ganglia, displayed high levels of EcR-A-immunoreactivity. With rare exceptions, all EcR-A/ELAV-immunopositive neurons were absent from the ganglion 24 h after adult eclosion. Confocal microscopy of EcR-A-immunopositive neurons during day 1 of adult life revealed that these cells underwent PCD during this time. This study implies that EcR-A expression during the period of ecdysteroid decline, which accompanies the end of metamorphosis, is critical to the decision for these neurons to commit suicide.

Because nuclear hormone receptors such as EcR act as transcription factors, an important follow-up to the aforementioned studies is to determine the identity of genes that are regulated by ecdysteroids via the EcR-A. Obvious candidates are the *reaper*, *grim*, and *hid* genes, previously shown to be involved in the apoptotic pathway, now known to be indirect regulators of caspase activity by means of their ability to bind to inhibitors of apoptosis proteins (IAPs) (review: Hay, 2000) (see Section 5.5.4.1). When newly emerged adult flies were injected with 20E within 20 min of eclosion, the presence of the steroid blocked both PCD and the accumulation of *reaper* transcripts, normally seen in EcR-A-immunopositive neurons at this time (Robinow *et al.*, 1997). Neurons destined for death at this time were also demonstrated to accumulate *grim* but not *hid* transcripts.

The relationship of EcR-A to other examples of PCD in *Drosophila* and in insects, generally, remains to be determined. It is known that EcR-A and EcR-B1 have different tissue distributions during *Drosophila* metamorphosis (Talbot *et al.*, 1993), and that not all cells that express EcR-A will undergo PCD. However, at present there is no model to explain the tissue-specific distributions of the

different isoforms. Expression of individual isoforms in a line of dominant-negative EcR mutants showed that EcR-A can support metamorphosis in numerous tissues, including the fat body, eye discs, salivary glands, and wing discs, but revealed no links that could be interpreted in relationship to PCD (Cherbas *et al.*, 2003).

5.5.4. Molecular Mechanisms of Neuronal Death

Most of the insights into the genetics of PCD in *Drosophila* have come from studies of embryonic development rather than metamorphosis. The key insights from studies on embryos, as they form the foundation for understanding control of post-embryonic PCD are presented here.

5.5.4.1. The *Drosophila* cell death “machinery” Flies utilize a conserved cell death machinery that includes caspases, a Ced (cell death abnormal)-4/Apaf (apoptosome associated factor)-1 ortholog, and Ced-9/Bcl-2 protein family members (Tittel and Steller, 2000; Vernooy *et al.*, 2000) (Table 1). These proteins all have critical functions in apoptosis that were initially defined by studies in *C. elegans* (Horvitz *et al.*, 1994; Liu and Hengartner, 1999). In addition, however, several novel cell death regulators and cell survival mechanisms have been identified in flies.

Caspases are specialized members of the cysteine protease family that cleave aspartate or glutamate residues within enzymatic or structural substrate proteins to promote the dismantling of a cell (Hengartner, 2000). In *Drosophila*, there are seven caspases (Table 1), including three long prodomain initiator caspases, such as Dronc (*Drosophila* *nedd2*-like caspase; similar to caspase-9), and four

Table 1 *Drosophila* cell death regulators

Family	Protein
Caspases	Dronc
	Dcp-1
	Drice
	Dredd
	Strica/Dream
	Decay
	Damm
Caspase inhibitor	DIAP1/Thread
	DIAP2
Bcl-2 family	Drob-1/Debcl/Dborg-1/Dbok
	Buffy/Dborg-2
IAP inhibitors	Reaper
	Hid
	Grim
	Sickle
	Jafrac2
	Apaf-1/Ced 4 family

short prodomain effector caspases, such as drICE (similar to caspase-3) and DCP-1 (death caspase1; similar to caspase-7). Loss-of-function mutations in *dcp-1* result in larval lethality and formation of melanotic tumors (Song *et al.*, 1997). The *Drosophila* Ced-4/Apaf-1 homolog is known as Dark (Drosophila apaf-1 related Killer)/HAC (homolog of apaf-1 and Ced-4)-1/Dapaf-1 and, like its worm and mammalian counterparts, associates with and promotes activation of long prodomain caspases (Kanuka *et al.*, 1999; Rodriguez *et al.*, 1999; Zhou *et al.*, 1999). Dark is expressed in dying cells and, interestingly, resembles its mammalian but not worm counterpart in that it contains WD repeats that can bind cytochrome *c*, and *dark* mutants exhibit decreased cell death in the embryonic CNS, hypertrophy of the larval CNS, and the formation of melanotic tumors.

Flies possess two Bcl-2 family members, *drob* (*Drosophila* ortholog of the Bcl-2 family)-1/*debcl* (death executioner Bcl-2 homolog)/*Dborg* (*Drosophila* Bcl-2 ortholog)-1/*dbok* and *Buffy/Dborg-2*, which both possess Bcl-2 homology (BH) domains BH1, BH2, and BH3 (Brachmann *et al.*, 2000; Colussi *et al.*, 2000; Igaki *et al.*, 2000; Zhang *et al.*, 2000). Both of these proteins resemble the proapoptotic mammalian Bok protein and act to promote apoptosis. Thus, unlike *C. elegans* and mammals, *Drosophila* does not appear to possess antiapoptotic Bcl-2 family members. This suggests that, both in terms of molecular and cellular complexity, *Drosophila* may prove to be a very useful system for bridging the gap that exists between the relatively simple nematode *C. elegans* and the more complex mammals. For example, in *C. elegans*, cell death eliminates approximately 10% of all cells, is governed largely by cell lineage, and is not required for organismal viability or fertility (Horvitz *et al.*, 1994; Liu and Hengartner, 1999). In contrast, in flies and mammals a much larger proportion of cells die, their survival is often governed by extrinsic signals, and normal progression of cell death programs is required for viability.

5.5.4.2. Role of IAP family proteins in PCD The Grim-Reaper proteins, initially identified in the first molecular genetic studies of PCD in *Drosophila*, all act upstream of caspases and their proapoptotic activities are blocked by the baculovirus caspase inhibitor, p35 (Grether *et al.*, 1995; Chen *et al.*, 1996; White *et al.*, 1996; Christich *et al.*, 2002; Srinivasula *et al.*, 2002; Wing *et al.*, 2002). Because the Grim-Reaper proteins do not share extensive homology to other proteins of known function, the mechanism through which they promote cell death

was not immediately apparent. Elucidation of their actions was greatly advanced by the identification and analysis of DIAP1 and DIAP2, *Drosophila* members of the IAP family (Hay *et al.*, 1995). IAPs were first identified from the *Cydia pomonella* granulosis virus (Crook *et al.*, 1993); since then, a large number of viral and cellular IAPs have been identified that are potent death inhibitors (Deveraux and Reed, 1999; Miller, 1999; Hay, 2000). IAPs all contain one or more copies of a 70 amino acid baculovirus IAP repeat (BIR) domain, typically located in the central or N-terminal region of the protein. In addition, they also contain a 50 amino acid RING (Really Interesting New Gene) domain, generally situated towards the C-terminal region of the protein. The sequences of the BIR and RING domains resemble zinc fingers, and both serve as protein-protein interaction domains. The BIR domains and associated linker regions are required to block caspase activities, and association between the linker domain and caspases is strengthened by the BIR domain (Huang *et al.*, 2001). The RING domain possesses ubiquitin E3 ligase activity that can target bound caspases for polyubiquitination and degradation via the 26S proteasome (Suzuki *et al.*, 2001b; Wilson *et al.*, 2002). In addition, IAPs bound to caspases are also targeted themselves for proteasome-dependent proteolysis via the N-end rule pathway of protein turnover (Ditzel *et al.*, 2003). The turnover of caspases via the ubiquitin-proteasome pathway affords an efficient mechanism for the IAPs to reduce caspase levels, and the interplay between caspases and IAPs is a key determinant of cell survival. The levels of IAPs themselves are also under rigid regulation, as proapoptotic stimuli can result in IAP autoubiquitination and proteasome-dependent degradation, thus promoting cell death (Yang *et al.*, 2000; Yoo *et al.*, 2002). In *Drosophila*, DIAPs play a particularly critical role in cell survival; loss of *diap1* results in early embryonic lethality associated with massive ectopic cell death (Wang *et al.*, 1999; Goyal *et al.*, 2000; Lisi *et al.*, 2000).

Significantly, the BIR domains of DIAP1 bind both caspases and the Grim-Reaper proteins. In particular, the BIR2 domain of DIAP1 associates with the RHG motif/IBM of the Grim-Reaper proteins. Hid, Grim, and, presumably, Reaper utilize a conserved 7 amino acid stretch of the RHG motif/IBM (amino acids 2–8) to directly contact residues within the BIR2 domain (Wu *et al.*, 2001). Sickie also binds the BIR2 domain, but its RHG motif/IBM differs from that of Reaper, Hid, and Grim and only the first four residues make direct contacts (Srinivasula *et al.*, 2002). This association with Grim-Reaper proteins promotes cell death by

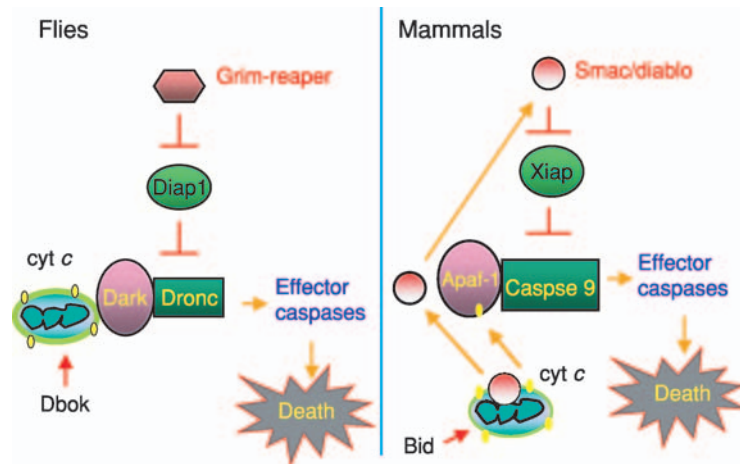


Figure 9 IAP inhibition is a conserved mechanism of regulation of apoptosis. In *Drosophila*, expression of the *grim-reaper* genes is activated in doomed cells, and the corresponding proteins bind to DIAP1 and block its ability to inhibit caspases. This results in active caspases that degrade cellular proteins to mediate apoptosis. In mammals, proapoptotic stimuli induce the release of Smac/Diablo as well as other proapoptotic factors, including cytochrome *c*, from the mitochondria. Cytoplasmic Smac/Diablo binds XIAP and represses its caspase-inhibitory actions, thereby promoting apoptosis. In both flies and mammals, the orthologous Dark and Apaf-1 proteins as well as proapoptotic members of the Ced-9/Bcl-2 family promote activation of initiator caspases.

blocking the ability of DIAP1 to bind and repress caspases. Thus, cell survival in *Drosophila* is controlled by a “double-inhibition” mechanism (Figure 9). In surviving cells, DIAP1 binds and inhibits caspase activities. In contrast, in cells signaled to die, the Grim-Reaper proteins are expressed and compete with caspases for DIAP1 binding. The displacement of caspases from DIAP1 results in proteolytically active molecules that promote cell death. Cell survival decisions are, therefore, determined to a large extent by the interactions between DIAP1, Grim-Reaper proteins, and caspases. Not surprisingly, the relative levels of these proteins within a cell are critical, and the effect of ectopic expression of these genes is highly dosage sensitive.

IAP inhibition is a conserved mechanism for regulating cell survival (Figure 9; review: Vaux and Silke, 2003). Thus, in mammals Smac (second mitochondria-derived activator of caspase)/Diablo is a mitochondrial protein that is released along with cytochrome *c* in dying cells (Du *et al.*, 2000; Verhagen *et al.*, 2000). Cytoplasmic Smac/Diablo associates with XIAP to prevent its binding and inhibition of caspase-9 (Chai *et al.*, 2000; Liu *et al.*, 2000). Strikingly, the binding of Smac/Diablo to IAPs is mediated through an N-terminal tetrapeptide sequence that is conserved in the Grim-Reaper RHG motif, indicating conserved modes of action for the fly and mammalian proteins (Chai *et al.*, 2000; Srinivasula *et al.*, 2000, 2002). Another mammalian IAP-inhibitor protein is the mitochondrial serine protease, Omi/HtrA2 (high temperature requirement protein A2), which also contains the conserved

BIR-binding tetrapeptide at its N-terminus (Suzuki *et al.*, 2001a; Hegde *et al.*, 2002; Martins *et al.*, 2002; van Loo *et al.*, 2002; Verhagen *et al.*, 2002). These similarities indicate that analysis of IAP inhibition mechanisms will provide insights into cell survival pathways in a wide range of species.

5.5.4.3. Evolutionary scenarios for the *grim-reaper* gene complex The small Reaper, Grim, and Sickie proteins are clearly ancestrally related, and the much larger Hid protein is also likely to share a common progenitor with them. Why are four related proteins needed to control DIAP1 inhibition and apoptosis, and how similar are the functions of these proteins? What proapoptotic activities, in addition to IAP inhibition, do the Grim-Reaper proteins share? Gene complexes can provide enhanced capabilities for a cell to finely control specific biological processes by providing gene products that exhibit overlapping yet distinct expression patterns and functions. Thus, multiple Hox proteins, for example, act together to permit elaboration of profound as well as subtle distinctions in cell and tissue types, and morphogenesis, along a major body axis (see Chapter 1). Presumably, the existence of the *reaper*, *hid*, *grim*, and *sickle* genes enables cells to control apoptosis with the exquisite specificity and efficiency that are required for developmental and physiological processes. The Grim-Reaper proteins do exhibit differences in their cell death-inducing abilities in developing tissues (Chen *et al.*, 1996; Wing *et al.*, 1998b, 2002), suggesting that they may have distinct abilities to interact with DIAPs

and promote apoptosis. In addition, gene expression studies as well as analysis of loss-of-function and gain-of-function mutants have indicated that these genes exhibit overlapping yet distinct patterns of expression and nonredundant cell killing activities (Grether *et al.*, 1995; Robinow *et al.*, 1997; Zhou *et al.*, 1997; Wing *et al.*, 1998b, 2002; Draizen *et al.*, 1999). Presumably, each individual Grim-Reaper protein provides a unique capability to promote apoptosis and, together, these proteins permit a more complex control of apoptosis patterns than would be afforded by a single protein.

Interestingly, while the *grim-reaper* genes are essential cell death activators in *Drosophila*, *bona fide* structural homologs have so far been identified only in other *Drosophila* species. Thus, a *reaper* ortholog was described in *D. simulans* (White *et al.*, 1994), and each *grim-reaper* gene is conserved in *D. pseudoobscura*. However, clear cut *grim-reaper* orthologs have not been identified from any other species, including the mosquito *Anopheles gambiae*. Therefore, the proapoptotic properties of Grim-Reaper proteins may have evolved only during divergence within the insect lineage. Finally, while the RHG motif/IBM has well-defined functions in IAP inhibition, the activities of the conserved Trp block/GH3 domain are less clear. The Trp block/GH3 domain appears to be important for mitochondrial actions of Grim and Reaper (Claveria *et al.*, 2002; Olson *et al.*, 2003), and may also be involved in interactions with other apoptosis regulators, such as Scythe, a large protein with a ubiquitin-like domain at its N-terminus (Thress *et al.*, 1998). Alternatively, the Trp block/GH3 domain could be important for Reaper and Grim to act as general translational inhibitors (Holley *et al.*, 2002; Yoo *et al.*, 2002), and may promote cell death by favoring the accumulation of long-lived caspases versus less stable IAPs. Interestingly, the Trp block/GH3 domain, which shares sequence similarity to a nonstructural protein from some insect bunyaviruses (J.R. Nambu unpublished data), may also act to repress translation and promote cell death (Bridgen *et al.*, 2001).

5.5.4.4. Regulation of embryonic glial cell survival One important mechanism for regulating the survival of neurons and glia within the developing vertebrate nervous system involves the actions of trophic factors (Raff *et al.*, 1993). These prosurvival molecules are synthesized in restricted amounts by target tissues, and permit winnowing of innervating cells to ensure appropriate matching of interacting cell populations. Both neuron and glial cell types can either provide or respond to trophic signaling.

In *Drosophila*, interactions between neurons and glia are also important for cell survival (Booth *et al.*, 2000; Kinrade *et al.*, 2001), and recent studies have revealed that trophic mechanisms regulate cell survival in the developing CNS. Specifically, members of the epidermal growth factor (EGF) family of proteins have been shown to act as neuron-derived glial survival factors (Hidalgo *et al.*, 2001; Bergmann *et al.*, 2002).

Approximately 10% of all cells within the *Drosophila* nervous system are glia, and these cells provide critical functions in neuronal homeostasis, axonogenesis, and removal of cellular debris (Jones, 2001; Klambt *et al.*, 2001). Among the best-characterized glial cells are the embryonic longitudinal and midline glia of the CNS. In both these lineages, cells undergo extensive apoptosis during embryogenesis (Sonnenfeld and Jacobs, 1995a; Zhou *et al.*, 1995; Kinrade *et al.*, 2001) (Figure 10). The selective survival of subsets of these cell populations is governed by trophic actions of EGF-related ligands and activation of the Ras/MAP (rat sarcoma/mitogen activated protein) kinase pathway via the EGF receptor homolog (EGFR).

During embryogenesis, a single, laterally positioned glioblast precursor gives rise to approximately 10 longitudinal glia in each hemisegment of the ventral nerve cord. These glial cells play a critical role in the formation of the longitudinal axon bundles; they migrate medially, contact pioneer longitudinal axons, and ultimately ensheath the longitudinal nerve bundles (Hidalgo and Booth, 2000) (see Chapter 3). Coinciding with the onset of axon/glial contact, many of the glia undergo apoptosis (Kinrade *et al.*, 2001), suggesting that, as the glia contact the axons, they become dependent upon them for survival. Consistent with this notion, ablation of pioneer and other neurons results in a decrease in longitudinal glia (Hidalgo *et al.*, 2001). Thus, apoptosis determines the final numbers of longitudinal glia during embryogenesis, and axon-derived factors are required for longitudinal glial survival. One of these factors is Vein (Vn), a *Drosophila* neuregulin homolog that contains both an IgG domain and EGF domain (Schnepf *et al.*, 1996). The *vein* gene is expressed in a small subset of neurons within the embryonic CNS, including the midline precursor 2 (MP2) pioneer neurons as well as the Ventral Unpaired Median (VUM) neurons of the CNS midline (Hidalgo *et al.*, 2001) (see Chapter 3). Gene *vein* mutants exhibit ectopic apoptosis of longitudinal glial cells, and ectopic apoptosis also results from RNAi-mediated loss of *vein* function in either all neurons or the MP2 neurons (Hidalgo *et al.*, 2001).

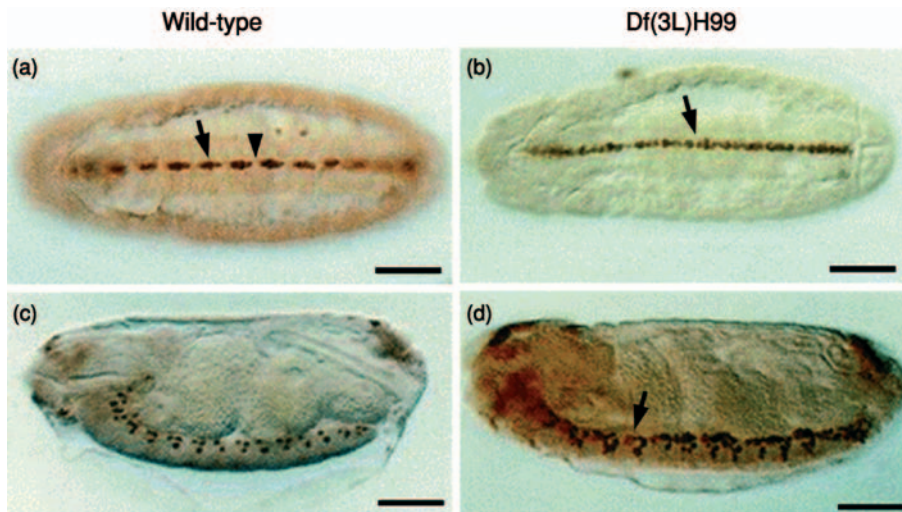


Figure 10 The embryonic CNS midline glia undergo developmental apoptosis. Embryos carrying a P[1.0slit-lacZ] transgene were immunostained with anti- β -galactosidase to detect the CNS midline glia. (a, c) In wild-type embryos (stages 15 and 16, respectively), there are approximately three midline glia per segment within the ventral nerve cord (arrow in a). The arrowhead in (a) points to the separation between two adjacent segmental clusters of midline glia that are eliminated with advanced development (c). (b, d) In Df(3L)H99 mutant embryos (stages 15 and 16, respectively), which lack *reaper*, *hid*, and *grim*, there is a blockade of apoptosis and accumulation of excess midline glia (arrow in b). The rescued midline glial cells accumulate at the dorsal surface of the ventral nerve cord (arrow in d).

Vein is a secreted ligand for the *Drosophila* EGFR receptor homolog (EGFR), and EGFR-mediated activation of the Ras/MAP kinase pathway is essential for longitudinal glial cell survival (Hidalgo *et al.*, 2001). The EGFR is transiently expressed in a subset of longitudinal glia, suggesting that Vein is secreted from axons to promote survival of longitudinal glial cells. Thus, Vein functions similarly to vertebrate neuregulins by providing axon-derived trophic support for developing glial cells via activation of receptor tyrosine kinase signaling pathways.

Another EGF family member, the transforming growth factor alpha (TGF- α) homolog Spitz (Spi), acts as a trophic factor to promote survival of the CNS midline glia (Bergmann *et al.*, 2002).

The midline glia are essential for proper formation of the axon scaffold, as migrating midline glia contact, separate, and ultimately ensheath the anterior and posterior axon commissures (Klammt *et al.*, 1991). They synthesize several axon guidance factors such as D-Netrin (Net), Commissureless (Comm), and Slit (Sli) that control the crossing and recrossing of commissural axons (Jacobs, 2000; Kaprielian *et al.*, 2000).

Ultrastructural and genetic analyses indicate that the midline glia undergo apoptosis, which reduces their number from an initial set of nine cells to three cells in each segment of the mature ventral nerve cord (Sonnenfeld and Jacobs, 1995a; Zhou *et al.*, 1995). This apoptosis is dependent upon caspases and the actions of multiple Grim-Reaper proteins;

loss of *reaper*, *hid*, and *grim* blocks all midline glial cell death and results in the survival of the nine midline glia per segment. The loss of *hid* and *grim* results in approximately seven to eight midline glia per segment, while the loss of *hid* alone results in six midline glia (Zhou *et al.*, 1997; Bergmann *et al.*, 2002). Thus, *hid* is required for the death of three midline glia and *reaper* and *grim* are together essential for the death of the other three midline glia.

Hid activities in the midline glia are regulated by proteins in the EGF signaling pathway, such as Spitz and EGFR (Bergmann *et al.*, 1998, 2002; Kurada and White, 1998). Both Spitz and EGFR are required for midline glial differentiation and survival, which, in turn, are essential for proper elaboration of the anterior and posterior commissures (Klammt *et al.*, 1991; Dong and Jacobs, 1997; Bergmann *et al.*, 2002). The loss of midline glia in *spitz* mutants is rescued in *spitz*; *hid* double mutants, implying that the proapoptotic functions of Hid are normally opposed by the prosurvival functions of Spitz.

The midline glia are normally in close contact with commissural axons, suggesting that glial-axon contact may be important for midline glial cell survival. Consistent with this notion, in *commissureless* mutants where the commissures fail to form, the isolated midline glial cells that fail to contact axons undergo apoptosis (Sonnenfeld and Jacobs, 1995a). The Spitz protein appears to be the axon-derived trophic factor required for midline

glial survival (Bergmann *et al.*, 2002). The loss of midline glia in *spitz* mutants is rescued by targeted expression of the transmembrane Spitz precursor protein in commissural neurons, and the precise number of surviving midline glia can be modulated by controlling the levels of Spitz activity (Bergmann *et al.*, 2002). The model that has emerged is that axon-derived Spitz protein signals the midline glia via EGFR, resulting in activation of the Ras/MAP kinase pathway. Hid activity is subsequently down-regulated via phosphorylation, which permits midline glial survival. Taken together, the actions of Vein in the longitudinal glia and Spitz in the midline glia indicate that trophic survival mechanisms are utilized to match the sizes of interacting populations of neurons and glia in invertebrates as well as vertebrates.

5.5.4.5. Regulation of cell survival by cell intrinsic mechanisms in sensory neuron lineages In *C. elegans*, cell lineage controls the pattern of cell survival, and the identity of each doomed cell can be determined by location, birth date, and gene expression patterns (Horvitz *et al.*, 1994; Liu and Hengartner, 1999). In flies, stereotyped lineages within the developing CNS also undergo cell death (Bossing *et al.*, 1996; Schmidt *et al.*, 1997), and the question of how cell lineage controls cell survival decisions has been addressed in the developing peripheral nervous system (Figure 11). In particular, the ventral multidendritic sensory neuron 1a (vmd1a), which expresses the Cut (Ct) protein, is present in a cluster of five multidendritic (md) neurons in the ventral region of abdominal segments A1–A7

(Orgogozo *et al.*, 2002). The vmd1a neuron is produced by two asymmetric divisions of a single sensory organ primary precursor cell (pI). The pI cell divides to generate a pIIa and pIIb progeny, one of which, pIIa, undergoes apoptosis. The pIIb cell divides again to generate the md neuron and a pIIIb cell, which also dies. Interestingly, the Numb protein, which is a cell fate determinant (Hirata *et al.*, 1995; Knobloch *et al.*, 1995; Spana *et al.*, 1995), segregates asymmetrically into the daughter cells during both of these divisions. In both cases, Numb is detected in the surviving cell and is not present in the daughter cell that undergoes apoptosis. In addition, during the asymmetric division of the pIIb cell, the Prospero (Pros) protein also segregates into the surviving daughter cell. In contrast, the cell death activators Reaper and Grim, but not Hid, are expressed in the pIIa and pIIIb cells, which die. In embryos where cell death is blocked, an ectopic sensory (es) organ is detected, which contains four Cut-expressing cells. These include an ectopic shaft/socket and neuron/sheath cell pairs that are similar to the md–es lineage, where the pIIa and pIIIb cells do not die. Thus, in the absence of cell death, the vmd1a lineage is transformed into an md–es lineage. Cell death is, therefore, used to refine the sensory organ structures by eliminating cells that would otherwise give rise to additional cell types and morphological structures.

Mutants of the *numb* gene exhibit ectopic *reaper* or *grim* gene transcription in the progeny of the vmd1a pI daughter cells, and both daughter cells undergo apoptosis without giving rise to any Cut-expressing progeny. In contrast, ectopic Numb expression in the pI daughter cells results in duplication of md neuron and pIIIb cells. The Numb protein contains a zinc finger and a phosphotyrosine binding domain. Numb directly associates with Notch (N) and interferes with Notch signaling (Guo *et al.*, 1996; Spana and Doe, 1996). This suggests that the death of cells in the vmd1a lineage is promoted by Notch, and that Numb acts to block Notch proapoptotic signaling (Figure 11). Consistent with this notion, ectopic expression of activated Notch, Nintra (Notch intracellular), was shown to promote the death of the pIIb cell when expressed during the period of pI cell division. Ectopic expression of *reaper* and *grim* was also detected, indicating that the effects of Nintra were similar to those observed for *numb* mutants. Therefore, in the vmd1a lineage, the distribution of Numb protein is critical for determining the survival of progeny cells. Daughter cells that inherit Numb are able to repress Notch-mediated activation of *reaper* and *grim* transcription, and subsequent apoptosis (Figure 11). This

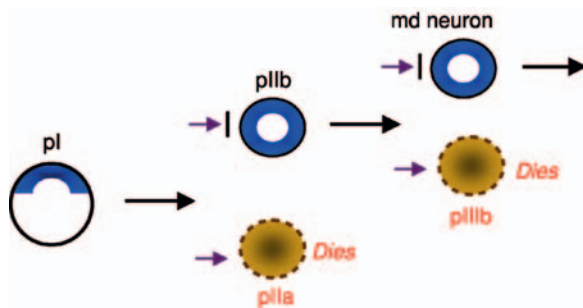


Figure 11 A model for the control of cell survival in the *Drosophila* vmd1 sensory neuron lineage. The pI precursor cell exhibits asymmetric distribution of the Numb protein (blue). Upon division (black arrow) of pI, Numb becomes partitioned into the pIIb but not the pIIa daughter cell. Numb inhibits (black bar) the proapoptotic effects of Notch signaling (purple arrows) in the pIIb cell, which survives and divides to give rise to a Numb-positive multidendritic (md) neuron and a pIIIb daughter cell. As a result of Notch signaling that occurs in Numb-negative pIIa and pIIIb cells, the apoptosis activators Reaper and Grim are expressed (orange), resulting in caspase activation and apoptosis (brown and dashed line).

appears to be a conserved mechanism of cell death during neural differentiation, as asymmetric distribution of the Numb protein also governs survival of the progeny from the embryonic neuroblast NB7-3 by inhibiting the proapoptotic actions of Notch signaling (Lundell *et al.*, 2003).

5.5.4.6. Control of neuron number by neuroblast apoptosis In the *Drosophila* embryonic CNS, approximately 30 neuroblasts are formed in each segment that divide asymmetrically to generate a smaller ganglion mother cell, which divides once to give rise to two post-mitotic neurons (Goodman and Doe, 1993) (see **Chapter 3**). The neurons that form during embryogenesis give rise to the larval nervous system. Generation of the adult nervous system requires the proliferation and differentiation of larval neuroblasts. Strikingly, the number of larval neuroblasts varies dramatically along the anterior–posterior axis. Thus, thoracic segments retain about 23 of the 30 neuroblasts generated during embryogenesis, while the abdominal segments retain only three embryonic neuroblasts (Truman *et al.*, 1993). Abdominal and thoracic neuroblasts also differ in their proliferative properties, as thoracic neuroblasts divide for 4 days and produce approximately 100 cells, while abdominal neuroblasts divide for only 30 h and generate 4–12 cells.

The rapid cessation of abdominal neuroblast cell division is brought about by their elimination via apoptosis midway through the third larval instar. In contrast, the thoracic neuroblasts do not undergo a stage-specific cell death. Abdominal neuroblast apoptosis requires the actions of *reaper*, *hid*, and *grim* and, in Df(3L)H99 mutants, there is an accumulation of ectopic abdominal neurons (Bello *et al.*, 2003). Interestingly, the rescued neuroblasts continue to proliferate and give rise to ectopic neural progeny. *Reaper* appears to be a key player in this process as *reaper* mutants also result in a survival of normally doomed abdominal neuroblasts, and *reaper* mutant flies exhibit a hypertrophied abdominal ganglia and male infertility (Peterson *et al.*, 2002). Thus, the programmed death of abdominal neuroblasts directly controls their reproductive capacity and number of progeny.

The segment-specific differences in neuroblast survival are controlled by the homeotic selector protein, Abdominal A (AbdA), which, along with Ultrabithorax (Ubx) and AbdB, govern abdominal segment identities (Lewis, 1978; Sanchez-Herrero *et al.*, 1985). Loss of AbdA function results in increased numbers of abdominal neuroblast progeny, and the mutant abdominal neuroblasts also survive

longer than the wild type (Bello *et al.*, 2003). In addition, ectopic expression of AbdA is sufficient to induce premature cell death of thoracic neuroblasts. This death requires the *grim-reaper* genes and is blocked in a Df(3L)H99 mutant background. Interestingly, expression of either the Antennapedia (Antp) or Ubx homeodomain proteins also induces thoracic neuroblast apoptosis, implying that a very precise control of homeodomain protein expression is necessary for appropriate cell survival patterns. Indeed, while postmitotic progeny of thoracic neuroblasts do express Ubx or Antp, the neuroblasts themselves do not.

The death of the three abdominal neuroblasts occurs in a highly synchronized fashion, and this timing appears to be the direct result of a very tight burst of AbdA expression specifically within the neuroblasts, but not in the ganglion mother cells or neurons, during the third larval instar (Bello *et al.*, 2003). Thus, the timing of AbdA expression is a critical determinant of cell survival. Indeed, precocious AbdA expression results in a smaller number of neural progeny and early death of the neuroblasts.

One important issue that remains unresolved is how the transient and specific expression of AbdA is controlled in abdominal neuroblasts. Is there a transient extrinsic signal that cues the abdominal neuroblasts to activate AbdA expression, or do the neuroblasts possess an intrinsic counting mechanism that permits them to keep track of the number of divisions they undergo? Elucidation of these questions will provide a molecular mechanism to directly link Hox gene actions and segment-specific differences in the nervous system.

5.5.4.7. Removal of dying cells is important for proper organization of the embryonic CNS The role of phagocytic cells in PCD is described above (see Section 5.2.1). In *Drosophila*, specialized macrophages differentiate from hemocyte precursors that are derived from head mesoderm tissue (Tepass *et al.*, 1994). Macrophages migrate throughout the body cavity, along several stereotypic routes, and rely on guidance cues that include PVR (PDGF and VEGF receptor-related), a member of the platelet-derived and vascular endothelial growth factor (PDGF/VEGF) receptor tyrosine kinase family (Cho *et al.*, 2002). While macrophages are excluded from the CNS itself, they engulf dying cells that are expelled from the nervous system (Sonnenfeld and Jacobs, 1995b).

Because of their intimate association with the CNS, it was of interest to determine if macrophage functions might be important for nervous

system development. Mutations that disrupt macrophage development, migration, or engulfment abilities produce mutants exhibiting disruptions in the normal architecture of the axon scaffold (Sears *et al.*, 2003). Specifically, the junctures between the longitudinal axon connectives and anterior and posterior commissures in each segment exhibit reduced separation and a rounded appearance. In addition, many CNS glia were misplaced and accumulated along the ventral midline. Thus, the efficient removal of dead and dying cells from the CNS is important for the elaboration of the axon scaffold and the glial cell positions within the CNS. In contrast, mutants that lack macrophages still exhibit essentially normal patterns of cell death, indicating that, although macrophages sculpt the CNS, they do not have a major role in promoting apoptosis (Tepass *et al.*, 1994; Sears *et al.*, 2003).

5.5.5. PCD of Muscles during Metamorphosis

As is the case in *Manduca*, fruit flies undergo extensive death of abdominal muscles after adult eclosion (Miller, 1950); the muscles that die at this time appear to be larval muscles that persisted during metamorphosis (Kimura and Truman, 1990). These persisting larval muscles are a very small subset of all larval muscles, the majority of which undergo PCD at the onset of metamorphosis (Currie and Bate, 1991). In the abdomen, only a few muscles survive; although some of the persisting muscles continue to function during pupal life and adult emergence, others persist only to provide a template for adult myogenesis (Broadie and Bate, 1991; Bate, 1993). Some of these new muscles, including the male-specific muscle, may be dependent upon innervation for differentiation and survival (Bate, 1993). Phagocytosis of degenerated (also referred to as histolyzed) muscle is a feature of the larval–pupal transition in *Drosophila* (Crossley, 1978; Bate, 1993), but phagocytic cells do not appear to be associated with dying muscles after adult eclosion, as has also been reported for *Manduca* ISMs (Kimura and Truman, 1990; Jones and Schwartz, 2001).

In contrast to the situation in *Manduca*, PCD of muscles during metamorphosis in *Drosophila* has not been a focus of research, and at present it is not possible to say more than that, most likely, the typical complement of cell death genes is expressed in dying muscle in a steroid-regulated manner. As described above (see Section 5.4.5.3), muscle abnormalities have been observed in transgenic *Drosophila* created to study genes identified in a screen of dying moth ISMs. Interest in the study of PCD of muscle in *Drosophila* may be stimulated by the

development of fly muscle models for human diseases, such as spinal muscular atrophy (Chan *et al.*, 2003) and Parkinson's disease (Greene *et al.*, 2003). The latter studies are particularly interesting, because flies, with loss of function mutations in the *parkin* (*park*) gene, which encodes a ubiquitin E3 ligase, are associated with mitochondrial abnormalities: the involvement of ubiquitin regulation of proteolysis and the mitochondrial abnormalities suggests an important relationship between pathology and PCD in normal development (Haas *et al.*, 1995).

5.6. Future Directions

Many unanswered questions remain concerning PCD of the insect nerve and muscle during metamorphosis. A partial list includes the following:

- Are all deaths of neurons during insect metamorphosis controlled by ecdysteroids?
- What deaths of insect neurons during metamorphosis are apoptotic, if any? Why do different cell populations (or the same cell populations studied at different stages of development) display different forms of PCD?
- What is the relationship between apoptosis in the insect embryo and PCD during metamorphosis?
- Faced with evidence that some genes associated with death in insect muscles are not associated with death in insect neurons, can we conclude that there are tissue-specific death genes?
- What is the source of the segment specificity of PCD at the metamorphic transitions? Can we generalize some of the mechanisms that account for segment specificity in neuroblast apoptosis to postembryonic cell deaths?
- Why is phagocytosis not evident in many examples of metamorphic PCD?
- Since death-associated transcripts accumulate in the ISMs hours before their proteins can be detected or death begins, what molecular event triggers translation?

Answers to these questions are likely to expand our understanding of developmental PCD over a broad taxonomic range.

References

- Abrams, J.M., 1999. An emerging blueprint for apoptosis in *Drosophila*. *Trends Cell Biol.* 9, 435–440.
- Abrams, J.M., White, K., Fessler, L.I., Steller, H., 1993. Programmed cell death during *Drosophila* embryogenesis. *Development* 117, 29–43.
- Ameisen, J.C., 2002. On the origin, evolution, and nature of programmed cell death: a timeline of four billion years. *Cell Death Differ.* 9, 367–393.

- Arnett, R.H.J., 1993. American Insects: A Handbook of the Insects of America North of Mexico. Sandhill Crane Press, Gainesville, FL.
- Baehrecke, E.H., 2000. Steroid regulation of programmed cell death during *Drosophila* development. *Cell Death Differ.* 7, 1057–1062.
- Baehrecke, E.H., 2002. How death shapes life during development. *Nature Rev. Mol. Cell Biol.* 3, 779–787.
- Bangs, P., White, K., 2000. Regulation and execution of apoptosis during *Drosophila* development. *Devel. Dynam.* 218, 68–79.
- Baker, R.T., Board, P.G., 1987. The human ubiquitin gene family: structure of a gene and pseudogenes from the Ub B subfamily. *Nucl. Acids Res.* 15, 443–463.
- Barres, B.A., Hart, I.K., Coles, H.S., Burne, J.F., Voyvodic, J.T., et al., 1992. Cell death and control of cell survival in the oligodendrocyte lineage. *Cell* 70, 31–46.
- Bate, M., 1993. The mesoderm and its derivatives. In: Bate, M., Arias, A.M. (Eds.), *The Development of Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1013–1090.
- Bayline, R.J., Khoo, A.B., Booker, R., 1998. Innervation regulates the metamorphic fates of larval abdominal muscles in the moth, *Manduca sexta*. *Devel. Genes Evol.* 208, 369–381.
- Beaulaton, J., Lockshin, R.A., 1977. Ultrastructural study of the normal degeneration of the intersegmental muscles of *Antheraea polyphemus* and *Manduca sexta* (Insecta, Lepidoptera) with particular reference to autophagy. *J. Morphol.* 154, 39–58.
- Bejsovsec, A., Wieschaus, E., 1993. Segment polarity gene interactions modulate epidermal patterning in *Drosophila* embryos. *Development* 119, 501–517.
- Bell, R.A., Joachim, F.G., 1976. Techniques for rearing laboratory cultures of tobacco hornworms and pink bollworms. *Ann. Entomol. Soc. America* 69, 365–373.
- Bello, B.C., Hirth, F., Gould, A.P., 2003. A pulse of the *Drosophila* Hox protein Abdominal-A schedules the end of neural proliferation via neuroblast apoptosis. *Neuron* 37, 209–219.
- Ben-Sasson, A.A., Sherman, Y., Gavrieli, Y., 1995. Identification of dying cells: *in situ* staining. In: Schwartz, L.M., Osborne, B.A. (Eds.), *Cell Death*. Academic Press, New York, pp. 29–39.
- Bennett, K.L., Truman, J.W., 1985. Steroid-dependent survival of identifiable neurons in cultured ganglia of the moth *Manduca sexta*. *Science* 229, 58–60.
- Bergmann, A., Agapite, J., McCall, K., Steller, H., 1998. The *Drosophila* gene *hid* is a direct molecular target of Ras-dependent survival signaling. *Cell* 95, 331–341.
- Bergmann, A., Tugentman, M., Shilo, B.Z., Steller, H., 2002. Regulation of cell number by MAPK-dependent control of apoptosis: a mechanism for trophic survival signaling. *Devel. Cell* 2, 159–170.
- Bidmon, H.-J., Koolman, J., 1989. Ecdysteroid receptors located in the central nervous system of an insect. *Experientia* 45, 106–109.
- Bidmon, H.-J., Sliter, T.J., 1990. The ecdysteroid receptor. *Invertebr. Reprod. Devel.* 18, 13–27.
- Bidmon, H.-J., Stumpf, W.E., Granger, N.A., 1992. Ecdysteroid receptors in the neuroendocrine–endocrine axis of a moth. *Experientia* 48, 42–47.
- Booker, R., Babashak, J., Kim, J.B., 1996. Postembryonic neurogenesis in the central nervous system of the tobacco hornworm, *Manduca sexta*. 3. Spatial and temporal patterns of proliferation. *J. Neurobiol.* 29, 233–248.
- Booker, R., Truman, J.W., 1987a. Postembryonic neurogenesis in the CNS of the tobacco hornworm, *Manduca sexta*. 1. Neuroblast arrays and the fate of their progeny during metamorphosis. *J. Comp. Neurol.* 255, 548–559.
- Booker, R., Truman, J.W., 1987b. Postembryonic neurogenesis in the CNS of the tobacco hornworm, *Manduca sexta*. 2. Hormonal control of imaginal nest cell differentiation during metamorphosis. *J. Neurosci.* 7, 4107–4114.
- Booth, G.E., Kinrade, E.F., Hidalgo, A., 2000. Glia maintain follower neuron survival during *Drosophila* CNS development. *Development* 127, 237–244.
- Bossing, T., Udolph, G., Doe, C.Q., Technau, G.M., 1996. The embryonic central nervous system lineages of *Drosophila melanogaster*. 1. Neuroblast lineages derived from the ventral half of the neuroectoderm. *Devel. Biol.* 179, 41–64.
- Brachmann, C.B., Jassim, O.W., Wachsmuth, B.D., Cagan, R.L., 2000. The *Drosophila* bcl-2 family member dBorg-1 functions in the apoptotic response to UV-irradiation. *Curr. Biol.* 10, 547–550.
- Bridgen, A., Weber, F., Fazakerley, J.K., Elliott, R.M., 2001. *Bunyamwera bunyavirus* nonstructural protein NSs is a nonessential gene product that contributes to viral pathogenesis. *Proc. Natl Acad. Sci. USA* 98, 664–669.
- Broadie, K.S., Bate, M., 1991. Development of adult muscles in *Drosophila*: ablation of identified muscle precursor cells. *Development* 113, 103–118.
- Bursch, W., 2001. The autophagosomal–lysosomal compartment in programmed cell death. *Cell Death Differ.* 8, 569–581.
- Bursch, W., Ellinger, A., Gerner, C., Frohwein, U., Schulte-Hermann, R., 2000. Programmed cell death (PCD): apoptosis, autophagic PCD, or others? *Ann. New York Acad. Sci.* 926, 1–12.
- Cascone, P.J., Schwartz, L.M., 2001. Role of the 3' UTR in regulating the stability and translatability of death-associated mRNAs in moth skeletal muscle. *Devel. Genes Evol.* 211, 397–405.
- Chai, J., Du, C., Wu, J.W., Kyin, S., Wang, X., et al., 2000. Structural and biochemical basis of apoptotic activation by Smac/DIABLO. *Nature* 406, 855–862.
- Chan, Y.B., Miguel-Aliaga, I., Franks, C., Thomas, N., Trulzsch, B., et al., 2003. Neuromuscular defects in a *Drosophila* survival motor neuron gene mutant. *Hum. Mol. Genet.* 12, 1367–1376.
- Chen, P., Nordstrom, W., Gish, B., Abrams, J.M., 1996. *grim*, a novel cell death gene in *Drosophila*. *Genes Devel.* 10, 1773–1882.
- Cherbas, L., Hu, X., Zhimulev, I., Belyaeva, E., Cherbas, P., 2003. EcR isoforms in *Drosophila*: testing tissue-specific requirements by targeted blockade and rescue. *Development* 130, 271–284.

- Cho, N.K., Keyes, L., Johnson, E., Heller, J., Ryner, L., *et al.*, 2002. Developmental control of blood cell migration by the *Drosophila* VEGF pathway. *Cell* 108, 865–876.
- Choi, M.K., Fahrbach, S.E., 1995. Evidence for an endogenous neurocidin in the *Manduca sexta* ventral nerve cord. *Arch. Insect Biochem. Physiol.* 28, 273–289.
- Christich, A., Kauppila, S., Chen, P., Sogame, N., Ho, S.I., *et al.*, 2002. The damage-responsive *Drosophila* gene *sickle* encodes a novel IAP binding protein similar to but distinct from *reaper*, *grim*, and *hid*. *Curr. Biol.* 12, 137–140.
- Ciechanover, A., 1998. The ubiquitin–proteasome pathway: on protein death and cell life. *EMBO J.* 17, 7151–7160.
- Clarke, P.G.H., 1990. Developmental cell death: morphological diversity and multiple mechanisms. *Anat. Embryol.* 181, 195–213.
- Claveria, C., Caminero, E., Martinez, A.C., Campuzano, S., Torres, M., 2002. GH3, a novel proapoptotic domain in *Drosophila grim*, promotes a mitochondrial death pathway. *EMBO J.* 21, 3327–3336.
- Colussi, P.A., Quinn, L.M., Huang, D.C., Coombe, M., Read, S.H., *et al.*, 2000. Debcl, a proapoptotic Bcl-2 homologue, is a component of the *Drosophila melanogaster* cell death machinery. *J. Cell Biol.* 148, 703–714.
- Consoulas, C., Restifo, L.L., Levine, R.B., 2002. Dendritic remodeling and growth of motoneurons during metamorphosis of *Drosophila melanogaster*. *J. Neurosci.* 22, 4906–4917.
- Crook, N.E., Clem, R.J., Miller, L.K., 1993. An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. *J. Virol.* 67, 2168–2174.
- Crossley, A.C., 1968. The fine structure and mechanism of breakdown of larval intersegmental muscles in the blowfly *Calliphora erythrocephala*. *J. Insect Physiol.* 14, 1389–1407.
- Crossley, A.C., 1978. The morphology and development of the *Drosophila* muscular system. In: Ashburner, M., Wright, T. (Eds.), *The Genetics and Biology of Drosophila*. Academic Press, New York, pp. 499–560.
- Currie, D., Bate, M., 1991. Development of adult abdominal muscles in *Drosophila*: adult myoblasts express *twist* and are associated with nerves. *Development* 113, 91–102.
- Dawson, S.P., Arnold, J.E., Mayer, N.J., Reynolds, S.E., Billett, M.A., *et al.*, 1995. Developmental changes of the 26S proteasome in abdominal intersegmental muscles of *Manduca sexta* during programmed cell death. *J. Biol. Chem.* 270, 1850–1858.
- DeLorme, A.W., Mesce, K.A., 1999. Programmed cell death of an identified motoneuron examined *in vivo*: electrophysiological and morphological correlates. *J. Neurobiol.* 39, 307–322.
- Deveraux, Q.L., Reed, J.C., 1999. IAP family proteins: suppressors of apoptosis. *Genes Devel.* 13, 239–252.
- DiNardo, S., Heemskerk, J., Dougan, S., O'Farrell, P.H., 1994. The making of a maggot: patterning the *Drosophila* embryonic epidermis. *Curr. Opin. Genet. Devel.* 4, 529–534.
- Ditzel, M., Wilson, R., Tenev, T., Zachariou, A., Paul, A., *et al.*, 2003. Degradation of DIAP1 by the N-end rule pathway is essential for regulating apoptosis. *Nature Cell Biol.* 5, 467–473.
- Dive, C., Gregory, C.D., Phipps, D.J., Evans, D.L., Milner, A.E., *et al.*, 1992. Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry. *Biochim. Biophys. Acta* 1133, 275–285.
- Dong, R., Jacobs, J.R., 1997. Origin and differentiation of supernumerary midline glia in *Drosophila* embryos deficient for apoptosis. *Devel. Biol.* 190, 165–177.
- Draizen, T.A., Ewer, J., Robinow, S., 1999. Genetic and hormonal regulation of the death of peptidergic neurons in the *Drosophila* central nervous system. *J. Neurobiol.* 38, 455–465.
- Driscoll, M., Gerstbrein, B., 2003. Dying for a cause: invertebrate genetics takes on human neurodegeneration. *Nature Rev. Genet.* 4, 181–194.
- Du, C., Fang, M., Li, Y., Li, L., Wang, X., 2000. Smac, a mitochondrial protein that promotes cytochrome *c*-dependent caspase activation by eliminating IAP inhibition. *Cell* 102, 33–42.
- Eastman, A., 1995. Assays for DNA fragmentation, endonucleases, and intracellular pH and Ca²⁺ associated with apoptosis. In: Schwartz, L.M., Osborne, B.A. (Eds.), *Cell Death*. Academic Press, New York, pp. 41–55.
- Eaton, J.L., 1988. *Lepidopteran Anatomy*. John Wiley, New York.
- Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., *et al.*, 1998. A caspase activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* 391, 43–50.
- Ewer, J., De Vente, J., Truman, J.W., 1994. Neuropeptide induction of cyclic GMP increases in the insect CNS: resolution at the level of single identifiable neurons. *J. Neurosci.* 14, 7704–7712.
- Ewer, J., Truman, J.W., 1997. Invariant association of ecdysis with increases in cyclic 3',5'-guanosine monophosphate immunoreactivity in a small network of peptidergic neurons in the hornworm, *Manduca sexta*. *J. Comp. Physiol. A* 181, 319–330.
- Ewer, J., Wang, C.M., Klukas, K.A., Mesce, K.A., Truman, J.W., *et al.*, 1998. Programmed cell death of identified peptidergic neurons involved in ecdysis behavior in the moth, *Manduca sexta*. *J. Neurobiol.* 37, 265–280.
- Fadok, V.A., Bratton, D.L., Guthrie, L., Henson, P.M., 2001. Differential effects of apoptotic versus lysed cells on macrophage production of cytokines: role of proteases. *J. Immunol.* 166, 6847–6854.
- Fadok, V.A., Chimini, G., 2001. The phagocytosis of apoptotic cells. *Semin. Immunol.* 13, 365–372.
- Fahrbach, S.E., 1992. Developmental regulation of ecdysteroid receptors in the nervous system of *Manduca sexta*. *J. Exp. Zool.* 261, 245–253.
- Fahrbach, S.E., 1997. The regulation of neuronal death during insect metamorphosis. *BioScience* 47, 77–85.

- Fahrbach, S.E., Choi, M.K., Truman, J.W., 1994. Inhibitory effects of actinomycin D and cycloheximide on neuronal death in adult *Manduca sexta*. *J. Neurobiol.* 25, 59–69.
- Fahrbach, S.E., DeLorme, A.W., Klukas, K.A., Mesce, K.A., 1995. A motoneuron spared from steroid-activated developmental death by removal of descending neural inputs exhibits stable electrophysiological properties and morphology. *J. Neurobiol.* 26, 511–522.
- Fahrbach, S.E., Schwartz, L.M., 1994. Localization of immunoreactive ubiquitin in the nervous system of the *Manduca sexta* moth. *J. Comp. Neurol.* 343, 464–482.
- Fahrbach, S.E., Truman, J.W., 1987. Possible interactions of a steroid hormone and neural inputs in controlling the death of an identified neuron in the moth *Manduca sexta*. *J. Neurobiol.* 18, 497–508.
- Fahrbach, S.E., Truman, J.W., 1989. Autoradiographic identification of ecdysteroid-binding cells in the nervous system of the moth *Manduca sexta*. *J. Neurobiol.* 20, 681–702.
- Fichelson, P., Ghosh, M., 2003. The glial cell undergoes apoptosis in the microchaete lineage of *Drosophila*. *Development* 130, 123–133.
- Finlayson, L.H., 1956. Normal and induced degeneration of abdominal muscles during metamorphosis in the Lepidoptera. *Q. J. Microsc. Sci.* 97, 215–233.
- Franc, N.C., 2002. Phagocytosis of apoptotic cells in mammals, *Caenorhabditis elegans*, and *Drosophila melanogaster*: molecular mechanisms and physiological consequences. *Frontiers Biosci.* 1, 1298–1313.
- French, V., 2001. Insect segmentation: genes, stripes and segments in “Hoppers.” *Curr. Biol.* 11, R910–R913.
- Gammie, S.C., Truman, J.W., 1997. Neuropeptide hierarchies and the activation of sequential motor behaviors in the hawkmoth, *Manduca sexta*. *J. Neurosci.* 17, 4389–4397.
- Gavrieli, Y., Sherman, Y., Ben-Sasson, S.A., 1992. Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* 119, 493–501.
- Geske, F.J., Monks, J., Lehman, L., Fadok, V.A., 2002. The role of the macrophage in apoptosis: hunter, gatherer, and regulator. *Int. J. Hematol.* 76, 16–26.
- Giebultowicz, J.M., Truman, J.W., 1984. Sexual differentiation in the terminal ganglion of the moth *Manduca sexta*: role of sex-specific neuronal death. *J. Comp. Neurol.* 226, 87–95.
- Gilbert, L.I., Granger, N.A., Roe, R.M., 2000. The juvenile hormones: historical facts and speculations on future research directions. *Insect Biochem. Mol. Biol.* 30, 617–644.
- Glücksman, A., 1951. Cell deaths in normal vertebrate ontogeny. *Biol. Rev.* 26, 59–86.
- Goodman, C.S., Doe, C.Q., 1993. Embryonic development of the *Drosophila* central nervous system. In: Bate, M., Arias, A.M. (Eds.), *The Development of Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1131–1206.
- Gorski, S., Marra, M., 2002. Programmed cell death takes flight: genetic and genomic approaches to gene discovery in *Drosophila*. *Physiol. Genom.* 9, 59–69.
- Goyal, L., McCall, K., Agapite, J., Hartwig, E., Steller, H., 2000. Inhibition of apoptosis by *Drosophila reaper*, *hid*, and *grim* through inhibition of IAP function. *EMBO J.* 19, 589–597.
- Greene, J.C., Whitworth, A.J., Kuo, I., Andrews, L.A., Feany, M.B., et al., 2003. Mitochondrial pathology and apoptotic muscle degeneration in *Drosophila* parkin mutants. *Proc. Natl Acad. Sci. USA* 100, 4078–4083.
- Grether, M.E., Abrams, J.M., Agapite, J., White, K., Steller, H., 1995. The *head involution defective* gene of *Drosophila melanogaster* functions in programmed cell death. *Genes Devel.* 9, 1694–1708.
- Guo, M., Jan, L.Y., Jan, Y.N., 1996. Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. *Neuron* 17, 27–41.
- Haas, A.L., Baboshina, O., Williams, B., Schwartz, L.M., 1995. Coordinated induction of the ubiquitin pathway accompanies the developmentally programmed death of insect skeletal muscle. *J. Biol. Chem.* 270, 9407–9412.
- Hashimoto, M.K., Mykles, D.L., Schwartz, L.M., Fahrbach, S.E., 1996. Imaginal cell-specific accumulation of the multicatalytic proteinase complex (proteasome) during post-embryonic development in the tobacco hornworm, *Manduca sexta*. *J. Comp. Neurol.* 365, 329–341.
- Hay, B.A., 2000. Understanding IAP function and regulation: a view from *Drosophila*. *Cell Death Differ.* 7, 1045–1056.
- Hay, B.A., Wassarman, D.A., Rubin, G.M., 1995. *Drosophila* homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. *Cell* 83, 1253–1262.
- Hegde, R., Srinivasula, S.M., Zhang, Z., Wassell, R., Mukattash, R., et al., 2002. Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. *J. Biol. Chem.* 277, 432–438.
- Hegstrom, C.D., Riddiford, L.M., Truman, J.W., 1998. Steroid and neuronal regulation of ecdysone receptor expression during metamorphosis of muscle in the moth, *Manduca sexta*. *J. Neurosci.* 18, 1786–1794.
- Hengartner, M.O., 2000. The biochemistry of apoptosis. *Nature* 407, 770–776.
- Henson, P.M., Bratton, D.L., Fadok, V.A., 2001. Apoptotic cell removal. *Curr. Biol.* 11, R795–R805.
- Hershko, A., Ciechanover, A., 1998. The ubiquitin system. *Annu. Rev. Biochem.* 67, 425–479.
- Hidalgo, A., Booth, G.E., 2000. Glia dictate pioneer axon trajectories in the *Drosophila* embryonic CNS. *Development* 127, 393–402.
- Hidalgo, A., Kinrade, F., Georgiou, M., 2001. The *Drosophila* neuregulin *vein* maintains glial survival during axon guidance in the CNS. *Devel. Cell* 1, 679–690.
- Hirata, J., Nakagoshi, H., Nabeshima, Y., Matsuzaki, F., 1995. Asymmetric segregation of the homeodomain protein Prospero during *Drosophila* development. *Nature* 377, 627–630.

- Hoffman, K.L., Weeks, J.C., 1998. Programmed cell death of an identified motoneuron *in vitro*: temporal requirements for steroid exposure and protein synthesis. *J. Neurobiol.* 35, 300–322.
- Hoffman, K.L., Weeks, J.C., 2001. Steroid-mediated programmed cell death of motoneurons: required role of caspases and mitochondrial events. *Devel. Biol.* 229, 517–536.
- Holley, C.L., Olson, M.R., Colon-Ramos, D.A., Kornbluth, S., 2002. Reaper eliminates IAP proteins through stimulated IAP degradation and generalized translational inhibition. *Nature Cell Biol.* 4, 439–444.
- Horvitz, H.R., Shaham, S., Hengartner, M.O., 1994. The genetics of programmed cell death in the nematode *Caenorhabditis elegans*. *Cold Spring Harbor Symp. Quant. Biol.* 59, 377–385.
- Hoy, M.A., 1994. Insect Molecular Genetics. Academic Press, New York.
- Hu, Y., Cascone, P., Cheng, L., Sun, D., Nambu, J.R., et al., 1999. Lepidopteran DALP, and its mammalian ortholog Hic-5, function as negative regulators of muscle differentiation. *Proc. Natl Acad. Sci. USA* 96, 10218–10223.
- Huang, Y., Park, Y.C., Rich, R.L., Segal, D., Myszka, D.G., et al., 2001. Structural basis of caspase inhibition by XIAP: differential roles of the linker versus the BIR domain. *Cell* 104, 781–790.
- Igaki, T., Kanuka, H., Inohara, N., Sawamoto, K., Nunez, G., et al., 2000. Drob-1, a *Drosophila* member of the Bcl-2/CED-9 family that promotes cell death. *Proc. Natl Acad. Sci. USA* 97, 662–667.
- Jacobs, J.R., 2000. The midline glia of *Drosophila*: a molecular genetic model for the developmental functions of glia. *Progr. Neurobiol.* 62, 475–508.
- Jacobson, M.D., Weil, M., Raff, M.C., 1997. Programmed cell death in animal development. *Cell* 88, 347–354.
- Jones, B.W., 2001. Glial cell development in the *Drosophila* embryo. *BioEssays* 23, 877–887.
- Jones, G., Jones, D., 2000. Considerations on the structural evidence of a ligand-binding function of *ultraspiracle*, an insect homolog of vertebrate RXR. *Insect Biochem. Mol. Biol.* 30, 671–679.
- Jones, M.E.E., Schwartz, L.M., 2001. Not all muscles meet the same fate when they die. *Cell Biol. Int.* 25, 539–545.
- Jones, M.E.E., Haire, M.F., Kloetzel, P.M., Mykles, D.L., Schwartz, L.M., 1995. Changes in the structure and function of the multicatalytic proteinase (proteasome) during programmed cell death in the intersegmental muscles of the hawkmoth, *Manduca sexta*. *Devel. Biol.* 169, 437–447.
- Jones, R.G., Davis, W.L., Hung, A.C., Vinson, S.B., 1978. Insemination-induced histolysis of the flight musculature in fire ants (*Solenopsis* spp.). *Am. J. Anat.* 151, 603–610.
- Kanuka, H., Sawamoto, K., Inohara, N., Matsuno, K., Okano, H., et al., 1999. Control of the cell death pathway by Dapaf-1, a *Drosophila* Apaf-1/CED-4-related caspase activator. *Mol. Cell* 4, 757–769.
- Kaprielian, Z., Imondi, R., Runko, E., 2000. Axon guidance at the midline of the developing CNS. *Anat. Rec.* 261, 176–197.
- Kerr, J.F.R., Wyllie, A.H., Currie, A.R., 1972. Apoptosis: a basic biological process with wide-ranging implications in tissue kinetics. *Br. J. Cancer* 26, 239–257.
- Kimura, K.-I., Truman, J.W., 1990. Postmetamorphic cell death in the nervous and muscular systems of *Drosophila melanogaster*. *J. Neurosci.* 10, 403–411.
- Kinch, G., Hoffman, K.L., Rodrigues, E.M., Zee, M.C., Weeks, J.C., 2003. Steroid-triggered programmed cell death of a motoneuron is autophagic and involves structural changes in mitochondria. *J. Comp. Neurol.* 457, 384–403.
- Kinrade, E.F., Brates, T., Tear, G., Hidalgo, A., 2001. Roundabout signalling, cell contact and trophic support confine longitudinal glia and axons in the *Drosophila* CNS. *Development* 128, 207–216.
- Klambt, C., Hummel, T., Granderath, S., Schimmelpfeng, K., 2001. Glial cell development in *Drosophila*. *Int. J. Devel. Neurosci.* 19, 373–378.
- Klambt, C., Jacobs, J.R., Goodman, C.S., 1991. The midline of the *Drosophila* central nervous system: a model for the genetic analysis of cell fate, cell migration, and growth cone guidance. *Cell* 64, 801–815.
- Knoblich, J.A., Jan, L.Y., Jan, Y.N., 1995. Asymmetric segregation of Numb and Prospero during cell division. *Nature* 377, 624–627.
- Koelle, M.R., Talbot, W.S., Seagraves, W.A., Bender, M.T., Cherbas, P., et al., 1991. The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* 67, 59–77.
- Kraft, R., Jäckle, H., 1994. *Drosophila* mode of metamorphosis in the embryogenesis of the lepidopteran insect, *Manduca sexta*. *Proc. Natl Acad. Sci. USA* 91, 6634–6638.
- Kroemer, G., Dallaporta, B., Resche-Rigon, M., 1998. The mitochondrial death/life regulator in apoptosis and necrosis. *Annu. Rev. Physiol.* 60, 619–642.
- Kuelzer, F., Kuah, K., Bishoff, S.J., Cheng, L., Nambu, J., et al., 1999. SCLP (small cytoplasmic leucine-rich repeat protein) encodes a novel protein that is dramatically up-regulated during the programmed death of moth skeletal muscle. *J. Neurobiol.* 41, 482–494.
- Kurada, P., White, K., 1998. Ras promotes cell survival in *Drosophila* by downregulating Hid expression. *Cell* 95, 319–329.
- Kuwana, 1936. Degeneration of muscles in the silkworm moth. *Zool. Mag. Tokyo* 48, 881–884.
- Laity, J.H., Lee, B.M., Wright, P.E., 2001. Zinc finger proteins: new insights into structural and functional diversity. *Curr. Opin. Struct. Biol.* 11, 39–46.
- Lee, C.-Y., Clough, E.A., Yellon, P., Teslovich, T.M., Stephan, D.A., et al., 2003. Genome-wide analyses of steroid- and radiation-triggered programmed cell death in *Drosophila*. *Curr. Biol.* 13, 350–357.
- Lee, C.Y., Baehrecke, E.H., 2001. Steroid regulation of autophagic programmed cell death during development. *Development* 128, 1443–1455.

- Lehmann, M., Jiang, C., Ip, Y.T., Thummel, C.S., 2002. AP-1, but not NF-kappa B, is required for efficient steroid-triggered cell death in *Drosophila*. *Cell Death Differ.* 9, 581–590.
- Leon, O., Roth, M., 2000. Zinc fingers: DNA binding and protein–protein interactions. *Biol. Res.* 33, 21–30.
- Levine, R.B., Truman, J.W., 1985. Dendritic reorganization of abdominal motoneurons during metamorphosis of the moth, *Manduca sexta*. *J. Neurosci.* 5, 2424–2431.
- Lewis, E.B., 1978. A gene complex controlling segmentation in *Drosophila*. *Nature* 276, 565–570.
- Lisi, S., Mazzon, I., White, K., 2000. Diverse domains of THREAD/DIAP1 are required to inhibit apoptosis induced by REAPER and HID in *Drosophila*. *Genetics* 154, 669–678.
- Liu, Q.A., Hengartner, M.O., 1999. The molecular mechanism of programmed cell death in *C. elegans*. *Ann. New York Acad. Sci.* 887, 92–104.
- Liu, Z., Sun, C., Olejniczak, E.T., Meadows, R.P., Betz, S.F., et al., 2000. Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. *Nature* 408, 1004–1008.
- Lockshin, R., 1963. Programmed Cell Death in an Insect. Harvard University Press, Cambridge, MA.
- Lockshin, R.A., 1969. Programmed cell death: activation of lysis by a mechanism involving the synthesis of protein. *J. Insect Physiol.* 15, 1505–1516.
- Lockshin, R.A., Beaulaton, J., 1974. Programmed cell death: cytochemical evidence for lysosomes during the normal breakdown of the intersegmental muscles. *J. Ultrastruct. Res.* 46, 43–62.
- Lockshin, R.A., Beaulaton, J., 1979. Programmed cell death: electrophysiological and ultrastructural correlations in metamorphosing muscles of lepidopteran insects. *Tissue and Cell* 11, 803–819.
- Lockshin, R.A., Williams, C.A., 1964. Programmed cell death. 2. Endocrine potentiation of the breakdown of the intersegmental muscles of silkworms. *J. Insect Physiol.* 10, 643–649.
- Lockshin, R.A., Williams, C.A., 1965a. Programmed cell death. 1. Histology and cytology of the breakdown of the intersegmental muscles in saturniid moths. *J. Insect Physiol.* 11, 123–133.
- Lockshin, R.A., Williams, C.A., 1965b. Programmed cell death. 3. Neural control of the breakdown of the intersegmental muscles of silkworms. *J. Insect Physiol.* 11, 601–610.
- Lockshin, R.A., Williams, C.M., 1965c. Programmed cell death. 4. The influence of drugs on the breakdown of the intersegmental muscles of silkworms. *J. Insect Physiol.* 11, 803–809.
- Low, P., Bussell, K., Dawson, S.P., Billett, M.A., Mayer, R.J., et al., 1997. Expression of a 26S proteasome ATPase subunit, MS73, in muscles that undergo developmentally programmed cell death, and its control by ecdysteroid hormones in the insect *Manduca sexta*. *FEBS Lett.* 400, 345–349.
- Lundell, M.J., Lee, H.K., Perez, E., Chadwell, L., 2003. The regulation of apoptosis by Numb/Notch signaling in the serotonin lineage of *Drosophila*. *Development* 130, 4109–4121.
- MacDonald, R.L., Stoodley, M., 1998. Pathophysiology of cerebral ischemia. *Neurol. Med. Chir. Tokyo* 38, 1–11.
- McCall, K., Steller, H., 1997. Facing death in the fly: genetic analysis of apoptosis in *Drosophila*. *Trends Genet.* 13, 222–226.
- McNabb, S.L., Baker, J.D., Agapite, J., Steller, H., Riddiford, L.M., et al., 1997. Disruption of a behavioral sequence by targeted death of peptidergic neurons in *Drosophila*. *Neuron* 19, 813–823.
- Martins, L.M., 2002. The serine protease Omi/HtrA2: a second mammalian protein with a Reaper-like function. *Cell Death Differ.* 9, 699–701.
- Meier, P., Finch, A., Evan, G., 2000. Apoptosis in development. *Nature* 407, 796–801.
- Mesce, K.A., Fahrbach, S.E., 2002. Integration of endocrine signals that regulate insect ecdysis. *Front. Neuroendocrinol.* 23, 179–199.
- Metchnikoff, E., 1892. La phagocytose musculaire. 1. Atrophie des muscles pendant la transformation des batéaciens. *Ann. Inst. Pasteur* 6, 1–12.
- Miller, A., 1950. The internal anatomy and histology of the imago of *Drosophila melanogaster*. In: Demerec, M. (Ed.), *Biology of Drosophila*. John Wiley, New York, pp. 420–534.
- Miller, L.K., 1999. An exegesis of IAPs: salvation and surprises from BIR motifs. *Trends Cell Biol.* 9, 323–328.
- Milligan, C.M., Schwartz, L.M., 1997. Programmed cell death during animal development. *Br. Med. Bull.* 53, 570–590.
- Monsma, S.A., Booker, R., 1996a. Genesis of the adult retina and outer optic lobes of the moth, *Manduca sexta*. 1. Patterns of proliferation and cell death. *J. Comp. Neurol.* 367, 10–20.
- Monsma, S.A., Booker, R., 1996b. Genesis of the adult retina and outer optic lobes of the moth, *Manduca sexta*. 2. Effects of deafferentation and developmental hormone manipulation. *J. Comp. Neurol.* 367, 21–35.
- Montemayor, M.E., Fahrbach, S.E., Giometti, C.S., Roy, E.J., 1990. Characterization of a protein that appears in the nervous system of the moth *Manduca sexta* coincident with neuronal death. *FEBS Lett.* 276, 219–222.
- Muqit, M.M., Feany, M.B., 2002. Modelling neurodegenerative diseases in *Drosophila*: a fruitful approach? *Nature Rev. Neurosci.* 3, 237–243.
- Murphey, R.K., Godenschwege, T.A., 2002. New roles for ubiquitin in the assembly and function of neuronal circuits. *Neuron* 36, 5–8.
- Mutsuddi, M., Nambu, J.R., 1998. Neural disease: *Drosophila* degenerates for a good cause. *Curr. Biol.* 8, R809–R811.
- Myer, A., Schwartz, L.M., 1996. Allelic variation in the polyubiquitin gene in the tobacco hawkmoth *Manduca sexta* and its regulation by heat shock and programmed cell death. *Insect Biochem. Mol. Biol.* 26, 1037–1046.
- Nagy, L.M., Booker, R., Riddiford, L.M., 1991. Isolation and embryonic expression of an abdominal-A-like gene

- from the lepidopteran, *Manduca sexta*. *Development* 112, 119–129.
- Nijhout, H.F., 1994. *Insect Hormones*. Princeton University Press, Princeton, NJ.
- Nishikawa, A., Murata, E., Akita, M., Kaneko, K., Moriya, O., *et al.*, 1998. Role of macrophages in programmed cell death and remodeling of tail and body muscle of *Xenopus laevis* during metamorphosis. *Histochem. Cell Biol.* 109, 11–17.
- Nixon, R.A., Cataldo, A.M., 1993. The lysosomal system in neuronal cell death: a review. *Ann. New York Acad. Sci.* 679, 87–109.
- Olson, M.R., Holley, C.L., Gan, E.C., Colon-Ramos, D.A., Kaplan, B., *et al.*, 2003. A GH3-like domain in reaper required for mitochondrial localization and induction of IAP degradation. *J. Biol. Chem.* 278, 44758–44768.
- Orogozo, V., Schweisguth, F., Bellaiche, Y., 2002. Binary cell death decision regulated by unequal partitioning of Numb at mitosis. *Development* 129, 4677–4684.
- Park, J.H., Schroeder, A.J., Helfrich-Forster, C., Jackson, F.R., Ewer, J., 2003. Targeted ablation of CCAP neuropeptide-containing neurons of *Drosophila* causes specific defects in execution and timing of ecdysis behavior. *Development* 130, 2645–2656.
- Peterson, C., Carney, G.E., Taylor, B.J., White, K., 2002. reaper is required for neuroblast apoptosis during *Drosophila* development. *Development* 129, 1467–1476.
- Platt, N., da Silva, R.P., Gordon, S., 1998. Recognizing death: the phagocytosis of apoptotic cells. *Trends Cell Biol.* 8, 365–372.
- Raff, M.C., Barres, B.A., Burne, J.F., Coles, H.S., Ishizaki, Y., *et al.*, 1993. Programmed cell death and the control of cell survival: lessons from the nervous system. *Science* 262, 695–700.
- Rechsteiner, M., 1988. Ubiquitin. Plenum Press, New York.
- Renn, S.C.P., Park, J.H., Rosbash, M., Hall, J.C., Taghert, P.H., 1999. A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. *Cell* 99, 781–802.
- Rheuben, M.B., 1992. Degenerative changes in the muscle fibers of *Manduca sexta* during metamorphosis. *J. Exp. Biol.* 167, 91–117.
- Richardson, H., Kumar, S., 2002. Death to flies: *Drosophila* as a model system to study programmed cell death. *J. Immunol. Methods* 265, 21–38.
- Riddiford, L.M., 1993. Hormones and *Drosophila* development. In: Bate, M., Arias, A.M. (Eds.), *The Development of Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 899–939.
- Robinow, S., Draizen, T.A., Truman, J.W., 1997. Genes that induce apoptosis: transcriptional regulation in identified, doomed neurons of the *Drosophila* CNS. *Devel. Biol.* 190, 206–213.
- Robinow, S., Talbot, W.S., Hogness, D.S., Truman, J.W., 1993. Programmed cell death in the *Drosophila* CNS is ecdysone-regulated and coupled with a specific ecdysone receptor isoform. *Development* 119, 1251–1259.
- Robinson-Rechavi, M., Garcia, H., Laudet, V., 2003. The nuclear receptor superfamily. *J. Cell Sci.* 116, 585–586.
- Rodriguez, A., Oliver, H., Zou, H., Chen, P., Wang, X., *et al.*, 1999. Dark is a *Drosophila* homologue of Apaf-1/CED-4 and functions in an evolutionarily conserved death pathway. *Nature Cell Biol.* 1, 272–279.
- Rulifson, E.J., Kim, S.K., Nusse, R., 2002. Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. *Science* 296, 1118–1120.
- Runion, H.I., Pipa, R.L., 1970. Electrophysiological and endocrinological correlates during the metamorphic degeneration of a muscle fibre in *Galleria mellonella* (L.) (Lepidoptera). *J. Exp. Biol.* 53, 9–24.
- Sanchez-Herrero, E., Vernos, I., Marco, R., Morata, G., 1985. Genetic organization of *Drosophila* bithorax complex. *Nature* 313, 108–113.
- Sanson, B., 2001. Generating patterns from fields of cells: examples from *Drosophila* segmentation. *EMBO Rep.* 2, 1083–1088.
- Savill, J., Fadok, V., 2000. Corpse clearance defines the meaning of cell death. *Nature* 407, 784–788.
- Schmidt, H., Rickert, C., Bossing, T., Vef, O., Urban, J., *et al.*, 1997. The embryonic central nervous system lineages of *Drosophila melanogaster*. 2. Neuroblast lineages derived from the dorsal part of the neuroectoderm. *Devel. Biol.* 189, 186–204.
- Schneiderman, A.M., Matsumoto, S.G., Hildebrand, J.G., 1982. Trans-sexually grafted antennae influence development of sexually dimorphic neurons in moth brain. *Nature* 298, 844–846.
- Schnepf, B., Grumbling, G., Donaldson, T., Simcox, A., 1996. Vein is a novel component in the *Drosophila* epidermal growth factor receptor pathway with similarity to the neuregulins. *Genes Devel.* 10, 2302–2313.
- Schwartz, D.C., Hochstrasser, M., 2003. A superfamily of protein tags: ubiquitin, SUMO and related modifiers. *Trends Biochem. Sci.* 28, 321–328.
- Schwartz, L.M., 1992. Insect muscle as a model for programmed cell death. *J. Neurobiol.* 23, 1312–1326.
- Schwartz, L.M., Kosz, L., Kay, B.K., 1990a. Gene activation is required for developmentally programmed cell death. *Proc. Natl Acad. Sci. USA* 87, 6594–6598.
- Schwartz, L.M., Myer, A., Kosz, L., Engelstein, M., Maier, C., 1990b. Activation of polyubiquitin gene expression during developmentally programmed cell death. *Neuron* 5, 411–419.
- Schwartz, L.M., Ruff, R.L., 2002. Changes in contractile properties of skeletal muscle during developmentally programmed atrophy and death. *Am. J. Physiol. Cell Physiol.* 282, C1270–C1277.
- Schwartz, L.M., Smith, S.W., Jones, M.E.E., Osborne, B.A., 1993. Do all programmed cell deaths occur via apoptosis? *Proc. Natl Acad. Sci. USA* 90, 980–984.

- Schwartz, L.M., Truman, J.W., 1982. Peptide and steroid regulation of muscle degeneration in an insect. *Science* 215, 1420–1421.
- Schwartz, L.M., Truman, J.W., 1983. Hormonal control of rates of metamorphic development in the tobacco hornworm, *Manduca sexta*. *Devel. Biol.* 99, 103–114.
- Schwartz, L.M., Truman, J.W., 1984a. Cyclic GMP may serve as a second messenger in peptide-induced muscle degeneration in an insect. *Proc. Natl Acad. Sci. USA* 81, 6718–6722.
- Schwartz, L.M., Truman, J.W., 1984b. Hormonal control of muscle atrophy and degeneration in the moth *Antheraea polyphemus*. *J. Exp. Biol.* 111, 13–30.
- Sears, H.C., Kennedy, C.J., Garrity, P.A., 2003. Macrophage-mediated corpse engulfment is required for normal *Drosophila* CNS morphogenesis. *Development* 130, 3557–3565.
- Smith, C.A., Williams, G.T., Kingston, R., Jenkinson, E.J., Owen, J.J., 1989. Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. *Nature* 337, 181–184.
- Song, Z., McCall, K., Steller, H., 1997. DCP-1, a *Drosophila* cell death protease essential for development. *Science* 275, 536–540.
- Sonnenfeld, M.J., Jacobs, J.R., 1995a. Apoptosis of the midline glia during *Drosophila* embryogenesis: a correlation with axon contact. *Development* 121, 569–578.
- Sonnenfeld, M.J., Jacobs, J.R., 1995b. Macrophages and glia participate in the removal of apoptotic neurons from the *Drosophila* embryonic nervous system. *J. Comp. Neurol.* 359, 644–652.
- Spana, E.P., Doe, C.Q., 1996. Numb antagonizes Notch signaling to specify sibling neuron cell fates. *Neuron* 17, 21–26.
- Spana, E.P., Kopczynski, C., Goodman, C.S., Doe, C.Q., 1995. Asymmetric localization of numb autonomously determines sibling neuron identity in the *Drosophila* CNS. *Development* 121, 3489–3494.
- Srinivasula, S.M., Datta, P., Fan, X.J., Fernandes-Alnemri, T., Huang, Z., et al., 2000. Molecular determinants of the caspase-promoting activity of Smac/DIABLO and its role in the death receptor pathway. *J. Biol. Chem.* 275, 36152–36157.
- Srinivasula, S.M., Datta, P., Kobayashi, M., Wu, J.W., Fujioka, M., et al., 2002. *sickle*, a novel *Drosophila* death gene in the *reaper/hid/grim* region, encodes an IAP-inhibitory protein. *Curr. Biol.* 12, 125–130.
- Steller, H., 1995. Mechanisms and genes of cellular suicide. *Science* 267, 1445–1449.
- Steller, H., Grether, M.E., 1994. Programmed cell death in *Drosophila*. *Neuron* 13, 1269–1274.
- Stocker, R.F., Edwards, J.S., Truman, J.W., 1978. Fine structure of degenerating moth abdominal motorneurons after eclosion. *Cell Tissue Res.* 191, 317–331.
- Streichert, L.C., Pierce, J.T., Weeks, J.C., 1997. Steroid hormones act directly to trigger segment-specific programmed cell death of identified neurons *in vitro*. *Devel. Biol.* 183, 95–107.
- Sun, D., Ziegler, R., Milligan, C.E., Fahrback, S.E., Schwartz, L.M., 1995. Apoliphorin III is dramatically up-regulated during the programmed death of insect skeletal muscle and neurons. *J. Neurobiol.* 26, 119–129.
- Sutherland, E.W., 1972. Studies on the mechanism of hormone action. *Science* 177, 401–408.
- Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K., et al., 2001a. A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol. Cell* 8, 613–621.
- Suzuki, Y., Nakabayashi, Y., Takahashi, R., 2001b. Ubiquitin–protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 and enhances its anti-apoptotic effect in Fas-induced cell death. *Proc. Natl Acad. Sci. USA* 98, 8662–8667.
- Swindle, J., Ajioka, J., Eisen, H., Sanwal, B., Jacquemot, C., et al., 1988. The genomic organization of the ubiquitin genes of *Trypanosoma cruzi*. *EMBO J.* 7, 1121–1127.
- Takayanagi, K., Dawson, S., Reynolds, S.E., Mayer, R.J., 1996. Specific developmental changes in the regulatory subunits of the 26S proteasome in intersegmental muscles preceding eclosion in *Manduca sexta*. *Biochem. Biophys. Res. Commun.* 228, 517–523.
- Talbot, W.S., Swyryd, E.A., Hogness, D.S., 1993. *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. *Cell* 73, 1323–1337.
- Taylor, H.M., Truman, J.W., 1974. Metamorphosis of the abdominal ganglia of the tobacco hornworm, *Manduca sexta*: changes in population of identified motor neurons. *J. Comp. Physiol.* 90, 367–388.
- Tenev, T., Zachariou, A., Wilson, R., Paul, A., Meier, P., 2002. Jafrac2 is an IAP antagonist that promotes cell death by liberating Dronc from DIAP1. *EMBO J.* 21, 5118–5229.
- Tepass, U., Fessler, L.I., Aziz, A., Hartenstein, V., 1994. Embryonic origin of hemocytes and their relationship to cell death in *Drosophila*. *Development* 120, 1829–1837.
- Thorn, R.S., Truman, J.W., 1989. Sex-specific neuronal respecification during the metamorphosis of the genital segments of the tobacco hornworm moth *Manduca sexta*. *J. Comp. Neurol.* 284, 489–503.
- Thorn, R.S., Truman, J.W., 1994a. Sexual differentiation in the central nervous system of the moth *Manduca sexta*. 1. Sex and segment-specificity in production, differentiation, and survival of the imaginal midline neurons. *J. Neurobiol.* 25, 1039–1044.
- Thorn, R.S., Truman, J.W., 1994b. Sexual differentiation in the central nervous system of the moth, *Manduca sexta*. 2. Target dependence for the survival of the imaginal midline neurons. *J. Neurobiol.* 10, 1054–1066.
- Thress, K., Henzel, W., Shillinglaw, W., Kornbluth, S., 1998. Scythe: a novel reaper-binding apoptotic regulator. *EMBO J.* 17, 6135–6143.

- Tittel, J.N., Steller, H., 2000. A comparison of programmed cell death between species. *Genome Biol.* 1(3), REVIEWS0003.
- Thummel, C.S., 2001. Steroid-triggered death by autophagy. *BioEssays* 23, 677–682.
- Truman, J.W., 1983. Programmed cell death in the nervous system of an adult insect. *J. Comp. Neurol.* 216, 445–452.
- Truman, J.W., 1984. Ecdysteroids regulate the release and action of eclosion hormone in the moth *Manduca sexta*. In: Hoffman, J., Porchet, M. (Eds.), *Biosynthesis, Metabolism, and Mode of Action of Invertebrate Hormones*. Springer, New York, pp. 136–144.
- Truman, J.W., Bate, M., 1988. Spatial and temporal patterns of neurogenesis in the CNS of *Drosophila melanogaster*. *Devel. Biol.* 125, 146–157.
- Truman, J.W., Fahrbach, S.E., Kimura, K.-I., 1990. Hormones and programmed cell death: insights from invertebrate studies. In: Coleman, P., Higgins, G., Phelps, C. (Eds.), *Progress in Brain Research*. Elsevier, Amsterdam, pp. 25–35.
- Truman, J.W., Riddiford, L.M., 2002. Insect developmental hormones and their mechanism of action. In: Pfaff, D.W. (Ed.), *Hormones, Brain and Behavior*. Academic Press, New York, pp. 841–873.
- Truman, J.W., Schwartz, L.M., 1984. Steroid regulation of neuronal death in the moth nervous system. *J. Neurosci.* 4, 274–280.
- Truman, J.W., Talbot, W.S., Fahrbach, S.E., Hogness, D.S., 1994. Ecdysone receptor expression in the CNS correlates with stage-specific responses to ecdysteroids during *Drosophila* and *Manduca* development. *Development* 120, 219–234.
- Truman, J.W., Taylor, B.J., Awad, T.A., 1993. Formation of the adult nervous system. In: Bate, M., Arias, A.M. (Eds.), *The Development of Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1245–1275.
- Truman, J.W., Thorn, R.S., Robinow, S., 1992. Programmed neuronal death in insect development. *J. Neurobiol.* 23, 1295–1311.
- van Loo, G., van Gorp, M., Depuydt, B., Srinivasula, S.M., Rodriguez, I., et al., 2002. The serine protease Omi/HtrA2 is released from mitochondria during apoptosis: Omi interacts with caspase-inhibitor XIAP and induces enhanced caspase activity. *Cell Death Differ.* 9, 20–26.
- Vaux, D.L., Silke, J., 2003. Mammalian mitochondrial IAP binding proteins. *Biochem. Biophys. Res. Commun.* 304, 499–504.
- Verhagen, A.M., Coulson, E.J., Vaux, D.L., 2001. Inhibitor of apoptosis proteins and their relatives: IAPs and other BIRPs. *Genome Biol.* 2(7), REVIEWS3009.
- Verhagen, A.M., Ekert, P.G., Pakusch, M., Silke, J., Connolly, L.M., et al., 2000. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 102, 43–53.
- Verhagen, A.M., Silke, J., Ekert, P.G., Pakusch, M., Kaufmann, H., et al., 2002. HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins. *J. Biol. Chem.* 277, 445–454.
- Vernooy, S.Y., Copeland, J., Ghaboosi, N., Griffin, E.E., Yoo, S.J., et al., 2000. Cell death regulation in *Drosophila*: conservation of mechanism and unique insights. *J. Cell Biol.* 150, 69–76.
- Wang, S.L., Hawkins, C.J., Yoo, S.J., Muller, H.A., Hay, B.A., 1999. The *Drosophila* caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID. *Cell* 98, 453–463.
- Watanabe, K., Sasaki, F., 1974. Ultrastructural changes in regressing tail muscles of anuran tadpoles during metamorphosis. *Cell Tissue Res.* 155, 321–336.
- Weber, R., 1964. Ultrastructural changes in regressing tail muscles of *Xenopus* larvae at metamorphosis. *J. Cell Biol.* 68, 251–306.
- Weber, R., 1965. Inhibitory effect of actinomycin D on tail atrophy in *Xenopus* larvae at metamorphosis. *Experientia* 21, 665–666.
- Weeks, J.C., 1987. Time course of hormonal independence for developmental events in neurons and other cell types during insect metamorphosis. *Devel. Biol.* 124, 163–176.
- Weeks, J.C., 1999. Steroid hormones, dendritic remodeling and neuronal death: insights from insect metamorphosis. *Brain Behav. Evol.* 54, 51–60.
- Weeks, J.C., Davidson, S.K., 1994. Influence of interganglionic interactions on steroid-mediated dendritic reorganization and death of proleg motor neurons during metamorphosis in *Manduca sexta*. *J. Neurobiol.* 25, 535–554.
- Weeks, J.C., Davidson, S.K., Debu, B.H.G., 1993. Effects of a protein synthesis inhibitor on the hormonally mediated regression and death of motoneurons in the tobacco hornworm, *Manduca sexta*. *J. Neurobiol.* 24, 125–140.
- Weeks, J.C., Ernst-Utzschneider, K., 1989. Respecification of larval proleg motoneurons during metamorphosis of the tobacco hornworm, *Manduca sexta*: segmental dependence and hormonal regulation. *J. Neurobiol.* 20, 569–592.
- Weeks, J.C., Roberts, W.M., Trimble, D.L., 1992. Hormonal regulation and segmental specificity of motoneuron phenotype during metamorphosis of the tobacco hornworm, *Manduca sexta*. *Devel. Biol.* 149, 185–196.
- Weeks, J.C., Truman, J.W., 1985. Independent steroid control of the fates of motoneurons and their muscles during insect metamorphosis. *J. Neurosci.* 5, 2290–2300.
- White, K., Grether, M.E., Abrams, J.M., Young, L., Farrell, K., et al., 1994. Genetic control of programmed cell death in *Drosophila*. *Science* 264, 677–683.
- White, K., Tahaoglu, E., Steller, H., 1996. Cell killing by the *Drosophila* gene *reaper*. *Science* 271, 805–807.

- Wiborg, O., Pederson, M.S., Wind, A., Berglund, L.E., Marcker, K.A., *et al.*, 1985. The human ubiquitin multi-gene family: some genes contain multiple directly repeated ubiquitin coding sequences. *EMBO J.* 4, 755–759.
- Wilson, R., Goyal, L., Ditzel, M., Zachariou, A., Baker, D.A., *et al.*, 2002. The DIAP1 RING finger mediates ubiquitination of Dronc and is indispensable for regulating apoptosis. *Nature Cell Biol.* 4, 445–450.
- Wing, J.P., Karres, J.S., Ogdahl, J.L., Zhou, L., Schwartz, L.M., *et al.*, 2002. *Drosophila sickle* is a novel grim-reaper cell death activator. *Curr. Biol.* 12, 131–135.
- Wing, J.P., Nambu, J.R., 1998a. Apoptosis in *Drosophila*. In: Potten, C.S., Booth, C., Wilson, J.W. (Eds.), *Apoptosis Regulatory Genes*. Chapman and Hall, London, pp. 205–241.
- Wing, J.P., Schwartz, L.M., Nambu, J.R., 2001. The RHG motifs of *Drosophila* Reaper and Grim are important for their distinct cell death-inducing abilities. *Mech. Devel.* 102, 193–203.
- Wing, J.P., Zhou, L., Schwartz, L.M., Nambu, J.R., 1998b. Distinct cell killing properties of the *Drosophila reaper*, *head involution defective*, and *grim* genes. *Cell Death Differ.* 5, 930–939.
- Wu, J.W., Cocina, A.E., Chai, J., Hay, B.A., Shi, Y., 2001. Structural analysis of a functional DIAP1 fragment bound to Grim and Hid peptides. *Mol. Cell* 8, 95–104.
- Wyllie, A.H., Morris, R.G., Smith, A.L., Dunlop, D., 1984. Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. *J. Pathol.* 142, 67–77.
- Yaffe, D., Saxel, O., 1977. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature* 270, 725–727.
- Yang, Y., Fang, S., Jensen, J.P., Weissman, A.M., Ashwell, J.D., 2000. Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. *Science* 288, 874–877.
- Yoo, S.J., Huh, J.R., Muro, I., Yu, H., Wang, L., *et al.*, 2002. *hid*, *rpr* and *grim* negatively regulate DIAP1 levels through distinct mechanisms. *Nature Cell Biol.* 4, 416–424.
- Yoshizato, K., 1996. Cell death and histolysis in amphibian tail during metamorphosis. In: Gilbert, L.I., Tata, J.R., Atkinson, B.G. (Eds.), *Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells*. Academic Press, New York, pp. 647–671.
- Yuan, J., Shaham, S., Ledoux, S., Ellis, H.M., Horvitz, H.R., 1993. The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. *Cell* 75, 641–652.
- Zee, M.C., Weeks, J.C., 2001. Developmental change in the steroid hormone signal for cell-autonomous, segment-specific programmed cell death of a motoneuron. *Devel. Biol.* 235, 45–61.
- Zhang, H., Huang, Q., Ke, N., Matsuyama, S., Hammock, B., *et al.*, 2000. *Drosophila* pro-apoptotic Bcl-2/Bax homologue reveals evolutionary conservation of cell death mechanisms. *J. Biol. Chem.* 275, 27303–27306.
- Zhou, L., Hashimi, H., Schwartz, L.M., Nambu, J.R., 1995. Programmed cell death in the *Drosophila* central nervous system midline. *Curr. Biol.* 5, 784–790.
- Zhou, L., Schnitzler, A., Agapite, J., Schwartz, L.M., Steller, H., *et al.*, 1997. Cooperative functions of the *reaper* and *head involution defective* genes in the programmed cell death of *Drosophila* central nervous system midline cells. *Proc. Natl Acad. Sci. USA* 94, 5131–5136.
- Zhou, L., Song, Z., Tittel, J., Steller, H., 1999. HAC-1, a *Drosophila* homolog of APAF-1 and CED-4 functions in developmental and radiation-induced apoptosis. *Mol. Cell* 4, 745–755.
- Zuzarte-Luis, V., Hurlle, J.M., 2002. Programmed cell death in the developing limb. *Int. J. Devel. Biol.* 46, 871–876.

6 Prothoracicotropic Hormone

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6.1. General Introduction and Historical Background

The evolution of multicellular organisms possessing multiple cell types has required the coevolution of a variety of intercellular signals. These signals coordinate and control the multiplication and differentiation of a multitude of cell types from a single, initial zygotic cell. These signals also coordinate and control the interaction of these various cell types throughout the life of an organism. In vertebrates familiar to most people, i.e., reptiles, birds, and mammals, a newborn or hatched individual is generally not strikingly different from an adult and postembryonic development is relatively gradual; altricial birds and marsupials are exceptions in that individuals of these groups undergo embryonic-like development for a period of time after hatching or birth (see Truman and Riddiford, 2002). However, even in these latter vertebrate groups, developmental

changes are gradual and do not involve relatively sudden and major changes in morphology and physiology. In contrast, many invertebrates, including insects, undergo striking and periodic postembryonic changes in the rate of development and in morphology and physiology. The physical growth of insects is restricted by the extracellular cuticle, which is limited in its ability to accommodate expansion. Insects solve this problem by periodic molts in which the old cuticle is partly resorbed and partly shed and a new cuticle is laid down, allowing for a further period of growth. In addition, many insects undergo molts in which significant changes in structure occur, i.e., metamorphic molts (for more thorough discussions on the evolution of metamorphosis, see Sehna *et al.*, 1996; Truman and Riddiford, 2002). Controlling and coordinating these episodes of rapid developmental change is a suite of intercellular messengers (hormones). The

polyhydroxylated steroid hormone, 20-hydroxyecdysone (20E) is a major factor regulating molting and metamorphosis. 20E is produced from ecdysone (E), which itself has some hormonal activity (see Chapter 7). This chapter focuses on prothoracicotropic hormone (PTTH), a brain neuropeptide hormone, and its action in regulating the physiology of the prothoracic gland. For the actual pathway of ecdysteroid synthesis, starting with cholesterol or other sterols, the reader is directed to

It must be stated at the outset that the vast majority of data and discussion presented in this chapter come from studies of the Lepidoptera, especially the moth species *Manduca sexta* (tobacco hornworm) and *Bombyx mori* (domestic silkworm), simply because we have much more knowledge of PTTH or PTTH-like activity from this taxon than from any other group. The first section of this chapter reviews the field of PTTH research from the 1920s to the early 1980s; for further and more detailed pictures of this time period, see reviews by Ishizaki and Suzuki (1980), Gilbert *et al.* (1981); Granger and Bollenbacher (1981); Ishizaki and Suzuki (1984) and Bollenbacher and Granger (1985), among others.

In the second and third decades of the twentieth century, the Polish biologist Stefan Kopeć first provided evidence that the brain could control metamorphosis (e.g., Kopeć, 1922). Kopeć studied the larval–pupal molt of the gypsy moth, *Lymantria dispar*, employing simple techniques like the ligation of larvae into anterior and posterior sections, and brain extirpation. His data demonstrated that the larval–pupal molt of the gypsy moth was dependent upon the brain during a critical period in the last instar larval stage. After this stage, head ligation failed to prevent pupation. Kopeć (1922) found also that this control was not dependent on intact nervous connections with regions posterior to the brain. The studies of Kopeć represent the beginning of the field of neuroendocrinology but their importance was not recognized at the time. However, later workers, notably Wigglesworth, took similar approaches with other species of insects and demonstrated again that the brain was important for larval molting at certain critical periods and that the active principle appeared to be produced in a part of the brain containing large neurosecretory cells (e.g., Wigglesworth, 1934, 1940).

In the same time period, other studies, again using simple techniques like transplantation and transection, found that the brain-derived factor was not sufficient to stimulate molting and metamorphosis. Rather, a second component produced in the thorax was necessary (e.g., Burtt, 1938; Fukuda, 1941;

Williams 1947). This material originated in the prothoracic gland or ring gland and was later shown to be an ecdysteroid precursor to 20E, i.e., 3dE in Lepidoptera (Kiriishi *et al.*, 1990), or E and makisterone A in *Drosophila* (Pak and Gilbert, 1987).

The chemical nature of PTTH was addressed in studies beginning in 1958, when Kobayashi and Kimura (1958) found that high dose of an ether extract of *B. mori* pupal brains was able to induce adult development in a *Bombyx* dauer pupa assay. This result suggested that PTTH was a lipoidal moiety, such as a sterol. In contrast, Ichikawa and Ishizaki (1961) found that a simple aqueous extract of *Bombyx* brains could elicit development in a *Samia* pupal assay, an observation not supporting a lipoidal nature for PTTH, and in a later study, provided direct evidence that *Bombyx* PTTH was a protein (Ichikawa and Ishizaki, 1963). Schneiderman and Gilbert (1964), using the *Samia* assay, explicitly tested the possibility that PTTH was cholesterol or another sterol and concluded that a sterol PTTH was highly unlikely. During the 1960s and 1970s, data accumulated from a number of studies indicated that *Bombyx* PTTH was proteinaceous in nature, but attempts to ascertain the molecular weight yielded at least two peaks of activity, one ≤ 5 kDa and a second ≥ 20 kDa (e.g., Kobayashi and Yamazaki, 1966).

In the 1970s and 1980s, a second lepidopteran, the tobacco hornworm *M. sexta*, gained favor as a model organism for the elucidation of the nature of PTTH. Initial experiments with *Manduca* utilized a pupal assay (Gibbs and Riddiford, 1977) but a system measuring ecdysteroid synthesis by prothoracic glands *in vitro* soon gained prominence (Bollenbacher *et al.*, 1979). Using the pupal assay, Kingan (1981) estimated the molecular weight of *Manduca* PTTH to be ≈ 25 kDa. A similar estimate (MW ≈ 29 kDa) resulted from assays employing the *in vitro* technique but this approach also provided evidence for a second, smaller PTTH (MW = 6–7 kDa) (Bollenbacher *et al.*, 1984).

The site of PTTH synthesis within the brain was first investigated by Wigglesworth (1940) who, by implanting various brain subregions into host animals, identified a dorsal region of the *Rhodnius* brain containing large neurosecretory cells as the source of PTTH activity. Further studies, in species such as *Hyalophora cecropia*, *Samia cynthia* and *M. sexta*, also provided evidence that PTTH was produced within regions of the dorsolateral or medial-lateral brain that contained large neurosecretory cells. This methodology was refined and ultimately culminated in the identification of the

source of PTTH as two pairs of neurosecretory cells (one pair per side) in the brain of *Manduca* (Agui *et al.*, 1979).

The corpus cardiacum (CC) of the retrocerebral complex was originally suggested to be the site of PTTH release, based on structural characteristics of the organ and the observation that axons of some cerebral neurosecretory cells terminate in the CC. However, physiological studies involving organ transplants and extracts implicated the corpus allatum (CA), the other component of the retrocerebral complex, as the site of PTTH release. *In vitro* studies utilizing isolated CCs and CAs from *Manduca* revealed that the CAs contained considerably more PTTH than CCs (Agui *et al.*, 1979, 1980). This result was also obtained when high levels of potassium were used to stimulate PTTH release from isolated CCs and CAs; potassium caused a several-fold increase in PTTH release from both organs (Carrow *et al.*, 1981) but the increase from CCs barely matched the spontaneous release from CAs. PTTH activity in the CC could be explained by the fact that axons that terminate in the CA traverse the CA and isolation of the CC would carry along any PTTH caught in transit through this organ. Additional evidence that the CAs are the neurohemal organ for the PTTH-producing neurosecretory cells was the observation that CA PTTH content correlated positively with brain PTTH content (Agui *et al.*, 1980).

The episodic nature of insect growth and morphological development reflects, *in sensu lato*, the periodic peaks of circulating molting hormone, i.e., 20E. The existence of these ecdysteroid peaks implies that peaks in PTTH synthesis and release also exist. A variety of studies tried to determine when PTTH peaks occur and what factors influence the release of PTTH into the hemolymph. Wigglesworth (1933) performed pioneering studies on the control of *Rhodnius* PTTH release. He found that distension of the intestinal tract was the most important determinant of blood meal-dependent molting. Multiple small meals were not effective but artificially increasing distension by blocking the anus with paraffin notably decreased the size of a blood meal required. A meal of saline can also trigger (but not sustain) a molt, indicating that for *Rhodnius* at least, nutritional signals are not critical (Beckel and Friend, 1964). Meal-related distension appears to be sensed by abdominal stretch or pressure receptors in insects such as *Rhodnius* (Wigglesworth, 1934). This type of regulation is not surprising in blood-sucking insects that rapidly change the fill state of their gut but, somewhat surprisingly, it appears also to occur in some insects that are continuous rather

than episodic feeders, like the milkweed bug, *Onco-peltus fasciatus* (Nijhout, 1979). In this species, sub-critical weight nymphs inflated with air or saline will commence molting.

In many other insects, the physical and physiological conditions that permit and control PTTH release are subtle and partially known. In *Manduca*, it appears that a critical weight must be achieved before a molt is triggered (Nijhout, 1981); however, this weight is not a fixed quantity. Rather, the critical weight is a function of the weight and/or size at the time of the last molt. Animals that were very small or very large at the fourth to fifth larval instar molt will exhibit critical weights smaller or larger than the typical individual. Further complications revolve around the element of time. Animals that remain below critical size for a long period may eventually molt to the next stage (*Manduca*: Nijhout, 1981), or undergo stationary larval molts without growth (*Galleria mellonella*: Allegret, 1964). PTTH release may also be a "gated" phenomenon. That is, PTTH release occurs only during a specific time of the day and only in animals that meet critical criteria, like size, weight or allometric relationships between structures or physiological variables. Individuals that reach critical size within a diel cycle but after that day's gate period will not release PTTH until the next gate on the following day.

The concept of a critical size or similar threshold parameter that must be met to initiate PTTH release implies that there will be a single PTTH release per larval instar. This appears to be true through the penultimate larval instar but careful assessment of PTTH activity in *Manduca* hemolymph revealed two periods of significant levels of circulating PTTH during the last larval instar (Bollenbacher and Gilbert, 1981). In this species, three small PTTH peaks were seen about 4 days after the molt to the fifth instar while a single larger peak occurred about 2 days later. The first period of PTTH release is gated but the second appears to be neither gated nor affected by photoperiod (Bollenbacher, unpublished data cited in Bollenbacher and Granger, 1985). These two periods of higher circulating PTTH correspond to the two head critical periods (HCPs) that are found in the last larval instar of *Manduca*. HCPs represent periods of development before which the brain is necessary for progression to the next molt, as determined by ligation studies. In the fifth (last) larval instar of *Manduca*, the two periods of higher hemolymph titers of PTTH activity immediately precede and partially overlap two peaks in circulating ecdysteroids (Bollenbacher and Gilbert, 1981). The first small ecdysteroid peak

determines that the next molt will be a metamorphic one (the commitment peak: Riddiford, 1976) while the second large peak actually elicits the molt.

The existence of daily temporal gates for PTTH release suggested that a circadian clock plays a role in molting and metamorphosis (see Chapter 9). In fact, such a clock does appear to function in *Manduca* because shifting the photoperiod shifted the time of the gate (e.g., Truman, 1972). Furthermore, the entrainment of this circadian cycling was not dependent on retinal stimulation because larvae with cauterized ocelli still reacted to photoperiodic cues (Truman, 1972). A more overarching effect of photoperiod can be seen in the production of diapause. For instance, in *Antheraea* and *Manduca*, which exhibit pupal–diapause, short days experienced during the last larval instar result in the inhibition of PTTH release during early pupal–adult development (e.g., Williams, 1967; Bowen *et al.*, 1984a); the brain PTTH content of these diapausing pupae and that of nondiapausing pupae are similar, at least at the beginning of the pupal stage. Temperature often plays an important role in breaking diapause and an elevation of temperature has been interpreted to cause the release of PTTH (Bollenbacher and Granger, 1985), with a resultant rise in circulating ecdysteroids that triggers the resumption of development. The interposition of hormonal signals between light-cycle sensing and the inhibition of PTTH release and/or synthesis can involve the juvenile hormones (JHs). In species exhibiting larval diapause, like *Diatraea grandiosella*, the persistence of relatively high JH levels controls diapause (e.g., Yin and Chippendale, 1973). Ligation experiments indicated that the head was involved in JH-related larval diapause, resulting in the suggestion that JH inhibited the release of an ecdysiotropin, i.e., PTTH (Chippendale and Yin, 1976).

In summary, by the time of the publication of *Comprehensive Insect Physiology, Biochemistry and Pharmacology* in 1985, PTTH had progressed from being an undefined brain factor controlling molting (Kopeć, 1922) to being identified as one of two brain neuropeptides that specifically regulated ecdysteroid synthesis in the prothoracic gland (e.g., Bollenbacher *et al.*, 1984), and that was synthesized in a very limited set of brain neurosecretory cells (Agui *et al.*, 1979). In the remainder of this chapter, the characterization and purification of PTTH will be detailed, as will the intracellular signaling pathways by which PTTH activates the prothoracic gland. On the whole, this phase of PTTH research has involved more complicated biochemical and cell biological experiments than earlier work. However,

even though the methodology described has become more complicated and specialized, it is hoped that the discussions and speculations presented in this phase will remain as broad and as “biological” as those generated during that earlier, “simpler” period of PTTH research.

6.2. Prothoracicotropic Hormone (PTTH): Characterization, Purification, and Cloning

6.2.1. Purification and Cloning of the PTTHs of *Bombyx* and other Lepidoptera

Two species of Lepidoptera, *B. mori* and *M. sexta*, have emerged as the premier model species for studying the endocrinology of insect molting and metamorphosis. As described above, evidence suggested that, for these species, PTTH activity resided in proteins exhibiting two distinct molecular weights, one of 4–7 kDa and the second of 20–30 kDa. Early work with *Bombyx* brain extracts utilized a cross-species assay to determine PTTH activity. This assay, using debrained *Samia* pupae, suggested that the most effective *Bombyx* PTTH (4K-PTTH) was the smaller protein. However, as efforts to purify and further characterize *Bombyx* PTTH progressed, a *Bombyx* pupal assay was developed (Ishizaki *et al.*, 1983a). This assay revealed that when *Bombyx* PTTH preparations were tested with debrained *Bombyx* pupae, *Bombyx* PTTH appeared to be the larger molecule while the small PTTH, identified with the *Samia* assay, was not effective at physiologically meaningful doses (Ishizaki *et al.*, 1983a, 1983b). This result was confirmed when the small and large PTTHs were tested for their ecdysteroidogenic effect *in vitro* on *Bombyx* prothoracic glands (Kiriishi *et al.*, 1992).

The effort to isolate and sequence *Bombyx* PTTH shifted rapidly to the “large” PTTH form following the 1983 results; the smaller molecule, subsequently named bombyxin, is discussed briefly below (see Section 6.2.5). Within a few years, the sequence of the amino terminus (13 amino acids) of *Bombyx* PTTH (called 22K-PTTH in the publications of the day) was obtained after a purification effort that involved processing 500 000 adult male heads (Kataoka *et al.*, 1987). The final steps of this purification involved HPLC separations of proteins and this technique suggested that PTTH might exhibit heterogeneity in sequence and/or structure. However, the nature of that heterogeneity was unknown, since only one of four potential PTTH peaks was sequenced. In addition to this limited amount of amino acid sequence, this study is significant since

further pursued. Evidence suggesting that the *Manduca* brain might express a small PTTH in addition to the larger peptide is discussed in Section 6.2.5. Additional molecular studies, again using probes derived from the *Bombyx* PTTH sequence, have yielded lepidopteran PTTH sequences from three additional species: *Samia cynthia ricini* (Ishizaki and Suzuki, 1994); *Antheraea pernyi* (Sauman and Reppert, 1996a); and *H. cecropia* (Sehnal *et al.*, 2002) (see the following section). Recombinant *Antheraea* PTTH has been expressed and shown to have bioactivity, being capable of inducing adult development in debrained pupae (Sauman and Reppert, 1996a). The bioactivity of the proteins coded for by the *Samia* and *Hyalophora* sequences has not yet been tested.

Preliminary determinations of the molecular weight of PTTH have been made recently for a few additional lepidopteran species, such as the gypsy moth *L. dispar* (Kelly *et al.*, 1992; Fescemyer *et al.*, 1995), primarily using *in vitro* assays of ecdysteroidogenesis and variously treated brain extracts. These studies revealed two molecular weight ranges for PTTH, i.e., small and large. However, the molecular weight obtained for the large *Lymantria* PTTH (11 000–12 000) varied notably from those determined for the purified and cloned forms such as *Bombyx* PTTH (native weight \approx 25 000–30 000). This difference is probably a function of the sample treatment (acidic organic extraction versus aqueous extraction) and molecular weight analysis methods (HPLC versus SDS-PAGE), since when essentially the same extraction and HPLC methodology were applied to *Manduca* brain extracts, a similarly low molecular weight determination for “big” PTTH was obtained (11.5 kDa; Kelly *et al.*, 1996).

6.2.2. Characterization of the PTTH of Non-Lepidoptera

Despite the importance of the hemipteran *Rhodnius* in early studies demonstrating a brain-derived prothoracicotropic factor (e.g., Wigglesworth, 1934, 1940), relatively little work has been done on molecularly characterizing PTTH activity from species other than lepidopterans. In *Rhodnius*, Vafopoulou *et al.* (1996) found that brain extracts contained a protease-sensitive PTTH activity (see **Chapter 4**). Later work suggested that this activity was greater than 10 kDa in molecular weight when immunoblots of *Rhodnius* brain proteins or medium from *in vitro* incubations of brains were probed with an antibody against *Bombyx* PTTH (Vafopoulou and Steel, 2002). If nonreducing gel conditions were used, the strongest immunoreactive band was \approx 68 kDa, but reducing conditions yielded only a

single band at \approx 17 kDa. This latter molecular weight is the same as that calculated for *Bombyx* PTTH under reducing electrophoresis conditions (Mizoguchi *et al.*, 1990). It is also very similar to the molecular weights determined for *Manduca* PTTH, again employing the *Bombyx* antibody after reducing SDS-PAGE (\approx 16 kDa; Rybczynski *et al.*, 1996), and for the *Antheraea* PTTH, using an *Antheraea* PTTH antibody and strong reducing conditions (\approx 15 kDa; Sauman and Reppert, 1996a). Also, as demonstrated earlier for *Manduca* brain extract (Rybczynski *et al.*, 1996), an anti-*Bombyx* PTTH antibody removed PTTH activity from a *Rhodnius* brain extract (Vafopoulou and Steel, 2002).

The growing use of *Drosophila melanogaster* as a model organism for the study of development, neuronal function, and many other fields has not been accompanied by a concomitant increase in our knowledge of *Drosophila* PTTH. The small size and rapid development of *Drosophila* have made it difficult to perform classic endocrinology on this species, e.g., measuring ecdysteroid titers, accumulating large quantities of brain proteins, or isolating the ring gland for *in vitro* studies of ecdysteroid synthesis. In addition, the composite nature of the ring gland, which contains multiple cell types in addition to the prothoracic gland cells, complicates the interpretation of experiments in a way that is not seen with the moth glands. Nevertheless, an *in vitro* assay for ring gland ecdysteroid synthesis has been developed (Redfern, 1983) and used to search for PTTH activity. The results of these studies have not yielded a clear or consistent picture of *Drosophila* PTTH, nor, in fact, conclusive evidence that *Drosophila* possesses a PTTH that is much like the lepidopteran molecules.

In a series of studies, Henrich and collaborators characterized PTTH activity from the central nervous system of *Drosophila* (Henrich *et al.*, 1987a, 1987b; Henrich, 1995). They found that PTTH activity was present in both the brain and in the ventral ganglion; the latter location is in contradistinction to the Lepidoptera where PTTH activity is found only in the brain and in the corpus allatum, the neurohemal organ for PTTH release. Note that in *Drosophila* and other higher flies, the ring gland, which contains the prothoracic gland, also includes the corpus allatum. Also relevant is the finding that an antibody against *Bombyx* PTTH binds to specific cells both in the brain and in ventral ganglionic regions of *Drosophila* (Žitňan *et al.*, 1993), thus supporting the conclusion of Henrich (1995) that *Drosophila* PTTH activity was not restricted to the brain. An attempt to estimate the molecular weight

of this putative PTTH yielded equivocal results. Ultrafiltration through a 10-kDa filter resulted in PTTH activity in both the filtrate and retentate (Henrich, 1995). This might indicate the presence of two PTTH-like molecules, i.e., a big and a small PTTH, or simply the presence of a single PTTH of a molecular weight and shape that results in incompletely partitioning under the ultrafiltration protocol employed.

Two other studies yielded very different estimates of *Drosophila* PTTH molecular weight. Pak *et al.* (1992), using column chromatography, found two peaks of PTTH activity, one of ≈ 4 kDa and the other larger, at ≈ 16 kDa. In contrast, Kim *et al.* (1997), using a more complex chromatographic purification, concluded that *Drosophila* PTTH was a much larger, heavily glycosylated molecule of ≈ 66 kDa, with a core protein of ≈ 45 kDa. This group also subjected two reduced fragments of their purified PTTH to amino acid sequence analysis and found no homology with other protein sequences known at that time. However, a search of the current *Drosophila* molecular databases revealed that the sequence of these two fragments match portions of the deduced amino acid sequence encoded by *Drosophila* gene BG:DS000180.7 (unpublished analysis). This protein is cysteine-rich, with a MW of ≈ 45 kDa, and contains sequence motifs that place it in the EGF protein family, thus suggesting that it is a secreted protein. Based on its sequence, the DS000180.7 gene product has been hypothesized to be involved in cell–cell adhesion (Hynes and Zhao, 2000). As a member of the EGF family of proteins, the DS000180.7 gene product cannot be ruled out as an intercellular signaling molecule, i.e., as a prothoracicotropic hormone, without a functional test. What is clear currently, is that this protein shows no detectable amino acid similarity to the demonstrated PTTHs of the Lepidoptera (unpublished analysis), when compared with those sequences using a standard comparison program like Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990).

Further clouding the interpretation of the *Drosophila* PTTH studies was the requirement for relatively large amounts of putative PTTH material that was also, relative to lepidopteran material, comparatively poor in eliciting increased ecdysteroidogenesis. Thus, in the *Drosophila* studies, Henrich and coworkers (Henrich *et al.*, 1987a, 1987b; Henrich, 1995) and Pak *et al.* (1992) needed to use approximately eight brain-ventral ganglion equivalents of extract to stimulate ecdysteroid synthesis to two to three times basal, while Kim *et al.* (1997) employed 500 ng of purified protein to achieve a similar

activation. In contrast, lepidopteran PTTHs are effective at much lower doses; for instance, in *Manduca*, 0.25 brain equivalents or 0.25–0.5 ng of pure PTTH consistently results in a four to six times increase in ecdysteroidogenesis (Gilbert *et al.*, 2000) and activations of steroidogenesis of eight to ten times basal are not rare (personal observation). Further differences between putative *Drosophila* PTTH(s) and the lepidopteran PTTHs are discussed below (see Section 6.3.4). In addition to the work just discussed, the *Drosophila* genome project has offered a second approach to identify a PTTH candidate for this species. A search of this database with lepidopteran PTTH amino acid sequences revealed two candidate *Drosophila* sequences with intriguing similarities to the moth PTTHs and these are discussed in the next section.

Mosquitoes, such as the African malaria mosquito *Anopheles gambiae* and the yellow fever mosquito *Aedes aegypti*, are important disease vectors and have been the subject of many studies incorporating basic and applied approaches to their biology and control. In adult females of these species, a blood meal stimulates both the release of brain-derived gonadotropins, like ovary ecdysteroidogenic hormone, and of insulin-like molecules that stimulate ovarian ecdysteroid synthesis. Preadult development in mosquitoes appears to depend on ecdysteroid hormones, as in other insects; however, the morphological source of ecdysteroid production is not clear. Jenkins *et al.* (1992) provided evidence that the prothoracic gland of *A. aegypti*, which is part of a composite, ring gland-like organ, is inactive and that unknown cell types in the thorax and abdomen produce ecdysteroids. At the time of writing (2003), a genome-sequencing project is also under way for *A. aegypti* but the limited database currently available does not contain any putative PTTH homologs. However, in this context it is intriguing that the genome of *A. gambiae* might code for a PTTH-like protein. Searches of the *A. gambiae* genome database with lepidopteran and putative *Drosophila* PTTH amino acid sequences reveal two candidate gene products, which are discussed in the next section.

Antibodies against PTTH afford an additional tool to search for PTTH-like proteins and have been used in a number of studies, in addition to the immunoprecipitations of *Manduca*, *Drosophila*, and *Rhodnius* studies discussed above (Žitňan *et al.*, 1993; Dai *et al.*, 1994; Rybczynski *et al.*, 1996; Vafopoulou and Steel, 2002). Závodská *et al.* (2003) used polyclonal anti-*Antheraea* PTTH antibodies in an immunohistochemical survey of the central nervous systems of 12 species of insects in

10 orders: Archaeognatha, Ephemera, Odonata, Orthoptera, Plecoptera, Hemiptera, Coleoptera, Hymenoptera, Tricoptera, and Diptera. Positive signals were seen in all species surveyed except *Locusta migratoria* (Orthoptera), with immunoreactive cells found mainly in the protocerebrum and less commonly in the subesophageal ganglion (the mayfly *Siphonurus armatus* (Ephemera) and the damselfly *Ischnura elegans* (Odonata)). The number of putative PTTH-containing cells ranged from two or three pairs (Archaeognatha, Hemiptera, and Hymenoptera) to five or more pairs (Plecoptera, Coleoptera, Tricoptera, and Diptera) and only in the stonefly *Perla burmeisteriana* (Plecoptera) a signal was detected in a potential neurohemal organ, the corpus cardiacum. These results are intriguing but must be interpreted with caution, pending further information such as determination of the size of the recognized protein or the immunoprecipitation of PTTH activity by the antibody. In this context, results obtained with an anti-*Manduca* PTTH antibody must serve as a cautionary tale. This monoclonal antibody recognized only a small number of cells in the CNS, including but not limited to the demonstrated prothoracicotropes (Westbrook *et al.*, 1993), and apparently also bound to active PTTH (Muehleisen *et al.*, 1993). Yet, the protein isolated by an immunoaffinity column constructed with this antibody proved to be an intracellular retinoid-binding protein (Mansfield *et al.*, 1998) and not a secreted neurohormone.

6.2.3. Sequence Analyses and Comparisons of PTTHs

The amino acid sequence and structure of *Bombyx* PTTH have been characterized in a number of studies, beginning with Kataoka *et al.* (1987). **Figure 1** summarizes the major features of this protein. *Bombyx* PTTH appears to be synthesized as a 224-amino acid prohormone that is cleaved to yield an active peptide of 109 amino acids (Kawakami *et al.*, 1990). The first 29 residues comprise a relatively hydrophobic region that is terminated by a trio of basic amino acids and fulfill the criteria defining a signal peptide. A second trio of basic residues immediately precedes the beginning of the mature PTTH hormone. Both these basic areas were believed to define peptide cleavage sites. Between these two sites lies a pair of basic amino acids that has been hypothesized to be an additional cleavage site (Kawakami *et al.*, 1990). The presence of these three potential peptide processing sites has led to speculation that the region between the signal peptide and the mature PTTH sequence might be cleaved into two smaller peptides and that these small molecules

serve a physiological function, such as modulating juvenile hormone synthesis in the corpus allatum (Kawakami *et al.*, 1990; Ishizaki and Suzuki, 1992). However, there were no experimental data to support this speculation and this is still the case today.

A dimeric structure for *Bombyx* PTTH was long suspected and this was confirmed as part of the massive PTTH purification study that resulted in the determination of almost the entire amino acid sequence (Kataoka *et al.*, 1991). This study also indicated that PTTH was a homodimer joined by one or more disulfide bonds and that asparagine-linked glycosylation (see **Figure 1**) was a likely cause for the disparity between the observed monomer MW of ≈ 17 kDa and the predicted MW of $\approx 12\,700$ (Kawakami *et al.*, 1990). Further elucidation of the structure of *Bombyx* PTTH was provided by the incisive work of Ishibashi *et al.* (1994). This group used enzyme digestions of partially reduced recombinant PTTH to determine the intra- and interdimeric disulfide bonds (see **Figure 2**). This recombinant PTTH, expressed in bacteria (*Escherichia coli*) without glycosylation, had about 50% of the biological activity per nanogram of native PTTH. This result indicated that glycosylation is not necessary for biological activity although it may be required for maximum activity. *Bombyx* and other lepidopteran PTTHs (see below) do not have any significant homologs among vertebrate proteins, based on amino acid sequence (Kawakami *et al.*, 1990; R. Rybczynski, unpublished BLAST analysis of vertebrate protein data bases.) However, Noguti *et al.* (1995) have shown that *Bombyx* PTTH exhibits an interesting arrangement of its intramonomeric disulfide bonds that is very much like that seen in some members of the vertebrate growth factor superfamily (β NGF, TGF- β 2, and PDGF-BB). These workers suggest that PTTH is, in fact, a member of this superfamily and that PTTH and vertebrate growth factors share a common ancestor. It must be pointed out, however, that some insects, i.e., *Drosophila*, also possess genes belonging to the growth factor superfamily, such as *tolloid* and *60A*, with a significant amino acid identity to the vertebrate proteins in addition to the cysteine/disulfide bond conservation (Sampath *et al.*, 1993). Furthermore, the intracellular signaling events activated by vertebrate growth factors like TGF- β , NGF, and PDGF are considerably different than those elicited by PTTH (see Section 6.3.4), involving receptors that are also kinases and that trigger quite different ligand-dependent intracellular processes (see Massagué, 1998; Haluska and Adjei, 2001; Huang and Reichardt,

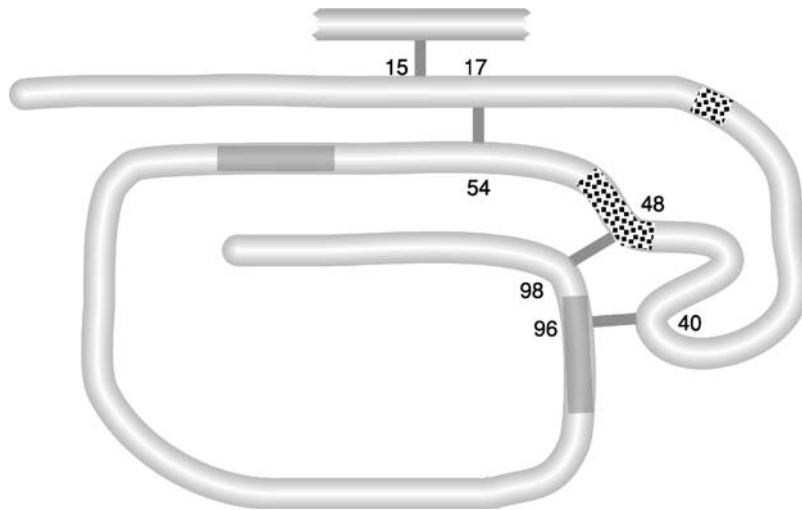


Figure 2 The structure of mature PTTHs, based on the *Bombyx* structure elucidated by Ishibashi *et al.* (1994). Dark shading indicates regions predicted to be hydrophobic in all five lepidopteran sequences while stippling indicates regions predicted to be hydrophobic in three lepidopteran sequences (Rybczynski, unpublished data). Numbers indicate the positions of the cysteines involved in intra- and intermonomeric bonds, in the mature *Bombyx* PTTH sequence.

2003). Thus, if PTTHs are members of the vertebrate growth factor superfamily, they have diverged extensively in amino acid sequence from other insect members of that superfamily. Furthermore, the co-evolved events that PTTH initiate in their target cells would have to have diverged just as drastically from their vertebrate and insect counterparts. Perhaps resolution of this interesting puzzle will come from genomic studies of invertebrates more primitive than insects. In this regard, it is salient that the genome of the nematode *Caenorhabditis elegans* contains identifiable homologs to TGF proteins, e.g., *daf-7* (Inoue and Thomas, 2001), but appears to contain no gene coding for a PTTH-like protein (R. Rybczynski, unpublished data).

The amino acid sequences of the five lepidopteran PTTHs discussed above and of two dipteran candidate PTTHs are shown in Figure 3. The two dipteran sequences were found by searching the *D. melanogaster* and *A. gambiae* genomes with the complete *Bombyx* PTTH amino acid sequence. At present, no experimental data are available to indicate the function(s) of either of these two dipteran proteins and they are included here not only because of their general, although low, sequence identity and similarity with the lepidopteran PTTHs but, more particularly, because of the conservation of cysteine residues, i.e., it seems likely that these proteins are structurally similar to the demonstrated PTTHs. Note that due to the dynamic state of the *Anopheles* genome sequencing project at the time of writing (2003), the *Anopheles* sequence included here is undoubtedly incomplete and will be subject to

revision. This may also be true of the candidate *Drosophila* sequence, although to a lesser extent.

All five lepidopteran PTTHs appear to be synthesized as prohormones that begin with a hydrophobic signal peptide sequence. The *Drosophila* protein is shown with a possible signal peptide sequence (Figure 3) but this region does not unequivocally meet the criteria for a cleaved signal peptide as defined by Nielsen *et al.* (1997). Following the signal peptide, these proteins exhibit only moderate sequence identity until an area preceding the mature PTTH sequence by about 35 amino acids. This cluster of greater amino acid conservation among the lepidopteran PTTHs might indicate that this region of the prohormone indeed has a function, as proposed by Ishizaki and Suzuki (1992). Note that the position of basic amino acids between the signal peptide and the mature hormone is not identical among the lepidopteran PTTHs and thus the 2 Da and 6 kDa peptide regions hypothesized for *Bombyx* (Kawakami *et al.*, 1990) do not have equivalents in *Samia*, *Antheraea*, and *Hyalophora*. A variety of proprotein convertases exist that recognize a variety of monobasic, dibasic, and tribasic amino acid patterns as well as specific sites lacking basic amino acids (see Loh *et al.*, 1984; Mains *et al.*, 1990; Seidah and Prat, 2002). Without experimental evidence, it is premature to predict where the preproPTTHs might be cleaved, in addition to the known site yielding the mature monomeric unit. The cellular location for the processing of PTTH from the longer, initial translation product to the shorter mature peptide form is not definitively

confirmed by immunogold electron microscopy in both *Manduca* and *Bombyx*, using the same anti-*Bombyx* PTTH antibody (Dai *et al.*, 1994, 1995).

The mature lepidopteran PTTHs begin with glycine-asparagine (GN) or glycine-aspartic acid (GD) in the lepidopteran PTTHs and the sequence from this glycine to the carboxy end has been confirmed to possess ecdysteroidogenic activity directly (*in vitro* prothoracic gland assays) in *Bombyx* (Kawakami *et al.*, 1990) and *Manduca* (Gilbert *et al.*, 2000), and indirectly (development of de-brained pupae) in *Antheraea* (Sauman and Reppert, 1996a). Proteins beginning with glycines can be modified by N-terminal myristylation, yielding a hydrophobic moiety that often serves as a membrane anchor. No evidence for this or any other N-terminal or C-terminal posttranslational modifications were found during the sequencing of *Bombyx* PTTH (Kataoka *et al.*, 1991) and the derived sequences of the PTTHs do not begin or end with the amino acids most likely to be so modified, e.g., N-terminal alanines (methylation site) or serines (acetylation site) or C-terminal glycines (amidation substrate). The mature PTTH peptides all contain seven cysteines, except for the putative *Anopheles* sequence, which is preliminary and probably incomplete (Figure 3). Given the close spacing similarity among the sequences, it is likely that all PTTHs are linked by disulfide bonds and folded in the same manner as demonstrated for *Bombyx* PTTH (Ishibashi *et al.*, 1994; see Figure 2). The lepidopteran PTTHs also all contain consensus sites for N-linked glycosylation (Figure 3). Given a consistent disparity of 4000–5000 between molecular weights predicted by amino acid sequence versus larger molecular weights observed after SDS-PAGE, it seems likely that all five of these proteins are indeed glycosylated (for *Bombyx* compare Kawakami *et al.*, 1990 and Kataoka *et al.*, 1991; for *Manduca* compare Rybczynski *et al.*, 1996 and Shionoya *et al.*, 2003; for *Antheraea* see Sauman and Reppert, 1996a). The candidate

Drosophila PTTH amino acid sequence does not contain a consensus site for N-linked glycosylation but does possess several for O-linked glycosylation (see Figure 3). Note that sites for O-linked glycosylation are difficult to predict and that only one of the potential sites in the *Drosophila* sequence falls within the putative mature peptide region. The processed (mature) PTTH sequences are, on the whole, hydrophilic proteins, with three or four short hydrophobic regions, depending on the species. Figure 2 shows a diagrammatic representation of the location of these hydrophobic regions in the lepidopteran sequences. Whether or not these hydrophobic regions play significant roles in determining monomeric or dimeric structures, or participate in receptor interactions are unanswered questions.

Table 1 summarizes the amino acid identities and similarities for pair-wise comparisons between six of the seven mature PTTH sequences presented in Figure 3; the candidate *Anopheles* sequence is omitted from the analysis due to the preliminary nature of the data. The similarities of these PTTH sequences are summarized graphically in Figure 4, using a phylogenetic tree neighbor-joining program (Saitou and Nei, 1987) applied to the mature PTTH amino acid sequences. In this analysis, the three Saturnid moths (*Antheraea*, *Hyalophora*, and *Samia*) form a clear-cut group, as do the two dipterans; *Bombyx* and *Manduca* form an outlier group in which the relationship of the two PTTH sequences do not closely match the conventional view of their taxonomic relationship. If the same analysis is performed with the prohormone rather than mature PTTH sequences, the results are essentially the same, reflecting the very similar, albeit modest, degrees of amino acid identity and similarity in the mature and prohormone sequences (see Table 1). The *Hyalophora* and *Samia* sequences are intriguingly similar and it would be very interesting to test if the PTTHs of these two species would significantly cross-activate prothoracic gland ecdysteroid synthesis. Such a comparison might

Table 1 Comparison of the amino acid identities and similarities for mature (bioactive) PTTHs

	Bombyx	Antheraea	Samia	Manduca	Hyalophora	Drosophila
<i>Bombyx</i> (224) ^a	X	52% ^b (75%) ^c	55% (73%)	59% (76%)	54% (73%)	22% (34%)
<i>Antheraea</i> (221)		X	68% (84%)	50% (68%)	71% (85%)	25% (41%)
<i>Samia</i> (236)			X	48% (73%)	94% (98%)	23% (36%)
<i>Manduca</i> (217)				X	48% (72%)	20% (39%)
<i>Hyalophora</i> (251)					X	24% (39%)
<i>Drosophila</i> (212)						X

^aNumber of amino acids in sequence.

^bAmino acid identity.

^cAmino acid similarity.

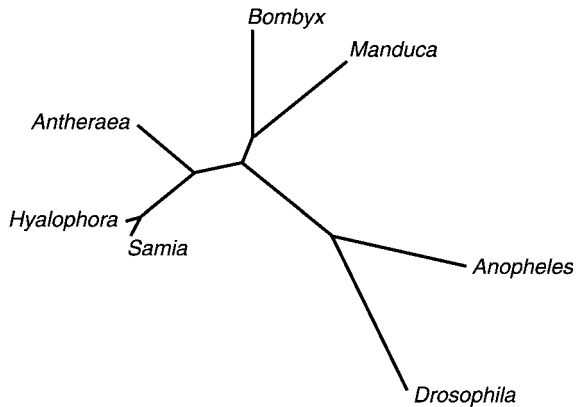


Figure 4 Graphical representation of mature PTTH amino acid sequence relationships, using a phylogenetic tree neighbor-joining program (Saitou and Nei, 1987).

enable some informed speculation as to the location of the receptor-binding regions of PTTHs.

Two additional lepidopteran PTTHs have been cloned recently from the closely related species *Heliothis virescens* (Xu and Denlinger, 2003) and *Heliocoverpa zea* (Xu *et al.*, 2003). The deduced amino acid sequences of these two PTTHs revealed that the major features of lepidopteran PTTHs, as discussed above, are conserved in these two new sequences, e.g., the proteolytic site yielding the mature peptide and the number and position of the seven conserved cysteines. Comparison of the deduced amino acid sequences of the mature peptide indicated that the *Heliothis* and *Heliocoverpa* are very similar (94% identity) but the identity with other lepidopteran PTTHs is much lower (48–59%) (Xu *et al.*, 2003).

6.2.4. Are PTTHs Species Specific?

With amino acid identities and similarities averaging around 50% and 80%, respectively, it is difficult to predict *a priori* whether PTTHs should act across species at biologically meaningful concentrations. Actual tests for cross-species bioactivity have been equivocal on this question. Agui *et al.* (1983) tested three lepidopteran pupal brain extracts with prothoracic glands from the three species *M. sexta*, *M. brassicae*, and *B. mori*. Their data suggested that conspecific PTTHs were generally most efficacious at eliciting ecdysteroid synthesis but that heterospecific PTTHs were also effective at doses (brain equivalents) from one half to about four times that of the conspecific molecule. This study was not able to take into account possible interspecific differences in endogenous PTTH levels and the brain extracts employed had not been size-selected to remove small PTTH or bombyxin (see Section

6.2.5). Several studies have re-examined the issue of potential *Manduca–Bombyx* PTTH cross-species activation of ecdysteroidogenesis. Gray *et al.* (1994) and Rybczynski, Mizoguchi and Gilbert (unpublished data) did not find activation of *Manduca* ecdysteroidogenesis by crude, native (size-selected) or by recombinant (pure) *Bombyx* PTTH, respectively. Additionally, *Manduca* crude PTTH was not able to activate *Bombyx* prothoracic glands in an *in vivo* dauer assay (Ishizaki quoted in Gray *et al.*, 1994). Similarly, *Bombyx* crude PTTH did not stimulate development by debrained *Samia* pupae, when separated from the much smaller bombyxin molecule originally thought to be PTTH (Ishizaki *et al.*, 1983b; see also Kiriishi *et al.*, 1992) and *Antheraea* recombinant PTTH did not activate *Manduca* prothoracic glands *in vitro* (Rybczynski and Gilbert, unpublished data).

Yokoyama *et al.* (1996) tested brain extracts prepared from pupal day 0 brains of four species of swallowtail butterflies (*Papilio xuthus*, *P. machaon*, *P. bianor*, and *P. helenus*) and found cross-specific activation of prothoracic gland ecdysteroid synthesis *in vitro*, with similar doses for half-maximal and maximal activation being relatively independent of the species of origin. These results are somewhat in contrast to the moth cross-species studies discussed above and this difference may be a function of the evolutionary closeness of the *Papilio* species versus the evolutionary distance of the moth species.

The possibility of cross-species PTTH activity has also been examined using dipteran ring glands and brain extracts. Roberts and Gilbert (1986) found that ecdysteroid synthesis by ring glands from post-feeding larval *Sarcophaga bullata* was readily activated by extracts from *Sarcophaga* prepupal brains and that these ring glands were not activated by *Manduca* brain extract. In contrast, *Manduca* prothoracic glands showed a stage-specific response to *Sarcophaga* brain extract, with larval but not pupal glands responding positively at relatively low doses of extract, i.e., less than one brain equivalent. Henrich (1995) addressed the cross-species question with studies on the *Drosophila* ring gland. As discussed above, the activation of *Drosophila* ring gland ecdysteroidogenesis by *Drosophila* brain or ganglionic extracts is not robust. Nevertheless, in a small study, Henrich (1995) found that both small and large *Manduca* PTTH preparations were able to elicit increased steroidogenesis by the ring gland with the former possibly being more efficacious. In contrast, a preliminary study with pure recombinant *Manduca* PTTH was not able to detect stimulation of *Drosophila* ecdysteroid synthesis (Rybczynski, unpublished data).

Both *Rhodnius* and *Bombyx* have served as important experimental organisms in research aimed at understanding PTTH. Vafopoulou and Steel (1997) have explored the effect of *Bombyx* PTTH on ecdysteroid synthesis by *Rhodnius* prothoracic glands under *in vitro* conditions. They found that recombinant *Bombyx* PTTH elicited a statistically significant increase in ecdysteroidogenesis relative to controls, with effective concentrations similar to that observed in *Bombyx*–*Bombyx* experiments. This is an intriguing and surprising result, given the evolutionary distance between *Rhodnius* (Hemiptera) and *Bombyx* (Lepidoptera). A puzzling feature of the results is the very sharp optimum in concentration seen with the *Bombyx* PTTH (8 ng ml^{-1}), with decreasing response seen at 10 ng ml^{-1} and above, although a similar, albeit broader, optimum is also seen with *Rhodnius* brain extracts (Vafopoulou *et al.*, 1996). Also of note is the approximately two-fold increase in ecdysteroid synthesis achieved with *Bombyx* PTTH-stimulated *Rhodnius* glands under the same conditions with which *Rhodnius* brain extract elicited nearly a five-fold increase (*cf.* Vafopoulou *et al.*, 1996; Vafopoulou and Steel, 1997). Thus, care must be exercised in interpreting these data, especially given observations in *Manduca* that small increases in ecdysteroid synthesis can be obtained with nonspecific stimuli (Bollenbacher *et al.*, 1983).

In summary, the question of PTTH species specificity remains open. The cloning, sequencing and expression of recombinant pure PTTHs will offer a more rigorous tool to investigate this topic and perhaps by doing so, gain insight into the portion of PTTH that productively interacts with PTTH receptors. If the region that contacts the receptor appears to be relatively small, then chimeric recombinant PTTHs can be used to investigate ligand-receptor interactions. At present, it seems premature to speculate on which portion(s) of the PTTH protein are necessary and sufficient for binding to the PTTH receptor and whether the area(s) that confer species-specificity are congruent with the former sites. It does seem likely that the most N-terminal region of the mature PTTH molecule is not involved in receptor binding since an antibody to this region failed to block PTTH-stimulated ecdysteroid synthesis by *Manduca* prothoracic glands (Rybczynski *et al.*, 1996). If sequence divergence is any guide to areas conferring species-specificity and receptor binding, then one region of interest can be pointed out (considering only the lepidopteran species), i.e., an area between a conserved triplet of basic amino acids and a conserved serine that is located slightly past the middle of the

mature peptide (see fourth of the five sets of rows in Figure 3). This area is also a hydrophilic region but as might be expected for a peptide hormone released into an aqueous environment, most of the mature PTTH peptide is predicted to be hydrophilic. Note that this region lies outside the common fold area that sequence-based modeling indicated that PTTH might share with members of the vertebrate growth factor superfamily (Noguti *et al.*, 1995). Note also that this PTTH region is not positionally equivalent to the regions of the platelet-derived growth factor (PDGF) or brain-derived neurotrophic factor (BDNF) molecules, for example, that are believed to be crucial for receptor interaction (LaRoche *et al.*, 1992; O'Leary and Hughes, 2003); however, like the regions in these vertebrate molecules, this portion of the PTTH molecule lies in an area predicted to be within a hydrophilic area, extending out from the common fold area (Noguti *et al.*, 1995).

6.2.5. Bombyxin and Small PTTH

The small neuropeptide bombyxin was initially characterized as *Bombyx* PTTH, using a *Samia* assay, until it was found that purified bombyxin was ineffective at stimulating ecdysteroid synthesis in *Bombyx* itself (see Section 6.2.1). The purification and amino acid sequencing of bombyxin, which began when it was considered to be *Bombyx* PTTH, revealed that there was actually a family of bombyxin proteins with sequence homology to the A and B chains of human insulin (see Ishizaki and Suzuki, 1994). Jhoti *et al.* (1987) modeled the three-dimensional structure of a bombyxin and found that it could assume an insulin-like tertiary structure. Despite these sequence and structural similarities, the functions of bombyxins, with nearly 40 genes in *Bombyx* (Kondo *et al.*, 1996), remain practically unknown. Specific receptors in lepidopteran ovarian cells have been identified, suggesting a role in reproduction (Fullbright *et al.*, 1997). Roles in ovarian and embryonic development have also been suggested (Orikasa *et al.*, 1993; Tanaka *et al.*, 1995). Whether or not ovarian ecdysteroid synthesis might be affected is an unaddressed question. Evidence also exists that a bombyxin may function in carbohydrate metabolism, in an insulin-like manner (Satake *et al.*, 1997; Masumura *et al.*, 2000). Recent work by Nijhout and Gunnert (2002) has demonstrated another role for a bombyxin in the butterfly *Precis coenia*. In this species, bombyxin II acts as a growth factor in concert with 20-hydroxyecdysone, stimulating cell division in cultured wing imaginal discs.

Finally, in regard to bombyxin, the ability of *Bombyx* bombyxin, effective at 30 pM, to stimulate adult development in debrained *Samia* pupae is still not understood (see Ishizaki and Suzuki, 1994). Nagata *et al.* (1999) used the deduced amino acid sequence of two *Samia* bombyxin homologs to synthesize the corresponding peptides. These peptides were then used in the *Samia* debrained pupae, where they proved to be effective at stimulating adult development at estimated hemolymph concentrations of 1–5 nM, a concentration at least 30 times higher than the *Bombyx* bombyxin. Given the plethora of bombyxin genes in *Bombyx*, and presumably other Lepidoptera, the possibility that one of these genes might code for a true prothoracicotropic protein cannot be ruled out. An additional factor must be kept in mind when considering bombyxins, i.e., the ability of bombyxins to activate adult development does not prove that bombyxins directly stimulate prothoracic gland ecdysteroidogenesis. In addition to the study by Kiriishi *et al.* (1992), demonstrating the relative ineffectiveness of bombyxin in stimulating ecdysteroid synthesis by isolated prothoracic glands (see Section 6.2.1), Vafopoulou and Steel (1997) found that *Bombyx* bombyxin was much less effective than *Bombyx* PTTH at stimulating ecdysteroid synthesis by *Rhodnius* glands *in vitro*. Other tests *in vitro* of small ecdysteroidogenic molecules have used large amounts of size-fractionated brain extracts, e.g., 50 brain equivalents per prothoracic gland, making it difficult to determine the physiological relevance of the stimulatory molecules, presumed to be bombyxins (Endo *et al.*, 1990; Fujimoto *et al.*, 1991).

What role insulin-like factors play in the acute regulation of steroidogenesis in taxa other than the Lepidoptera is also an open question. The *Drosophila* genome does not appear to contain any definite bombyxin homologs (R. Rybczynski, unpublished BLAST analysis), but it does contain seven genes that, like the bombyxins, code for proteins containing characteristic insulin motifs (Brogiolo *et al.*, 2001). These seven genes (*Drosophila* insulin-like proteins: DILP 1–7) do not show significant homology to bombyxins outside of the insulin motifs and, unlike the bombyxins, are not limited in expression to neuronal cells (Brogiolo *et al.*, 2001). It is not known if any of the DILP gene products participate in regulating ecdysteroidogenesis.

Despite the demonstration that the so-called small PTTH of *Bombyx*, now known as bombyxin, is very unlikely to be a physiologically important PTTH in this species, the question persists – is there more than one brain-derived PTTH? As discussed above (Section 6.2.2), size-fractionated brain extracts

frequently exhibit two peaks of ecdysteroidogenic activity, with one peak at 20 000–30 000 MW and a second, smaller peak below 10 000 MW. In most cases, there are simply no data beyond approximate molecular weight and dose (brain equivalents) that can address this question. The observations indicate a much larger dose requirement for the so-called small PTTHs. Additionally, a smaller maximum response (e.g., Endo *et al.*, 1990, 1997; Yokoyama *et al.*, 1996) suggests, but does not prove, that these so-called small PTTHs may be bombyxin homologs; as noted for *Bombyx* prothoracic glands tested *in vitro* (Kiriishi *et al.*, 1992), PTTH stimulated ecdysteroidogenesis much more effectively than did bombyxin. However, there are some data from *Manduca* that suggest that small PTTH in this species may be a molecule other than a bombyxin. Bollenbacher *et al.* (1984) described a small PTTH (MW = 6–7 kDa) that has subsequently been partially characterized in regard to its developmental expression and the signal transduction events it elicits in prothoracic glands. *Manduca* small PTTH appears to be much more abundant in pupal than in larval brains, while “big” PTTH shows only a slight increase in the pupal versus larval brain (O’Brien *et al.*, 1986). Bollenbacher *et al.* (1984) reported that small PTTH was notably less effective at stimulating ecdysteroid synthesis in pupal prothoracic glands than in larval glands; however, a study by O’Brien *et al.* (1986) showed no such difference. Like big PTTH (see Section 6.3.4), *Manduca* small PTTH appears to stimulate ecdysteroid synthesis via Ca²⁺ influx and Ca²⁺-dependent cAMP accumulation (Watson *et al.*, 1993; Hayes *et al.*, 1995). These observations concerning intracellular signaling suggest that *Manduca* small PTTH might not be a bombyxin homolog because, as members of the insulin peptide family, bombyxin would not be expected to stimulate either Ca²⁺ influx or Ca²⁺-dependent cAMP (see Combettes-Souverain and Issad, 1998). However, this is an inference as authentic bombyxin-stimulated intracellular events do not appear to have been studied. It should also be pointed out that for small PTTH to have the same effects as PTTH on Ca²⁺ influx and Ca²⁺-dependent cAMP generation is surprising and confusing, especially if small PTTH does not differentially activate larval glands (*cf.* conflicting data in Bollenbacher *et al.*, 1984 and O’Brien *et al.*, 1986). In fact, it has been hypothesized that small PTTH might be an active proteolytic fragment of PTTH (Gilbert *et al.*, 2002); if so, it is unclear whether small PTTH is generated as an artifact during PTTH extractions, or is a naturally occurring processed form.

Besides bombyxin and *Manduca* small PTH, other, noncerebral, ecdysiotropic activities have been described, e.g., factors extracted from the proctodea of *Manduca*, the European corn borer, *Ostrinia nubilalis*, and the gypsy moth, *L. dispar* (Gelman *et al.*, 1991; Gelman and Beckage, 1995). These data are not completely surprising, given the precedent of the vertebrate gut as an endocrine organ. However, a definitive role for such factors *in vivo* has not been established and further discussion of these candidate ecdysiotropes is outside the scope of this review.

6.3. Prothoracicotropic Hormone: Synthesis and Release

6.3.1. Sites of Synthesis and Release

Agui *et al.* (1979), using micro-dissections, demonstrated functionally that *Manduca* PTH was present only in two pairs of dorsolateral brain neurosecretory cells. Subsequently, a number of lepidopteran PTHs have been purified and/or cloned and antibodies raised against the derived sequences (see Section 6.2.1). The use of cDNA probes for *in situ* localization of these PTH mRNAs supported the findings of Agui *et al.* (1979) in all species tested (*Bombyx*: Kawakami *et al.*, 1990; *Antheraea*: Sauman and Reppert, 1996a; and *Manduca*: Shionoya *et al.*, 2003). Similarly, the use of antibodies in immunohistochemical studies also supported the early *Manduca* work (*Bombyx*: Mizoguchi *et al.*, 1990; *Antheraea*: Sauman and Reppert, 1996a; and *Manduca*: Gilbert *et al.*, 2000) (see Figure 5). Of particular note was the co-localization of PTH mRNA and protein to the same cells in *Antheraea* brain (Sauman and Reppert, 1996a). This co-localization has been assumed to be true but has only been explicitly demonstrated in *Antheraea*.

The antibody studies discussed above were conspecific studies, e.g., an anti-*Bombyx* PTH antibody was used to probe *Bombyx* tissues. However, as discussed in Sections 6.2.1 and 6.2.2, anti-PTH antibodies have been used also in heterospecific immunohistochemical studies. Anti-*Bombyx* PTH antibodies were used to probe *Manduca* (Dai *et al.*, 1994), *Samia* (Yagi *et al.*, 1995), *D. melanogaster* (Žitňan *et al.*, 1993), and *Locusta* (Goltzene *et al.*, 1992). In *Manduca* and *Samia* brains, the antibody decorated two pairs of lateral neurosecretory cells. These cells appeared to be the same as seen in *Bombyx* brain, in the conspecific probing summarized above. In *Drosophila*, Žitňan *et al.* (1993) found that the anti-*Bombyx* PTH bound to two superior mediolateral cells that might be equivalent

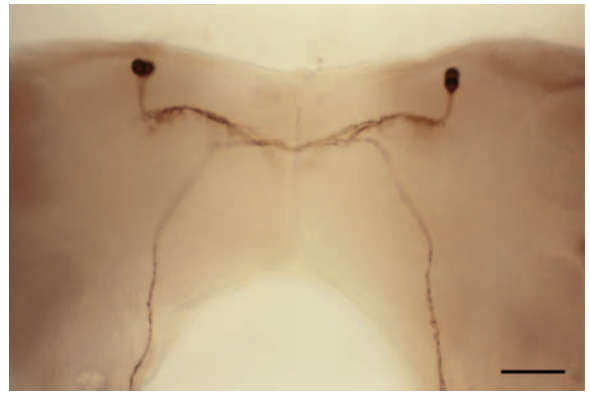


Figure 5 Immunocytochemical localization of PTH in the pupal brain of *Manduca sexta* showing that PTH is detectable only in the cell bodies and axons of two pairs of lateral neurosecretory cells. Scale bar = 100 μ m. (Data from Gilbert, L.I., Rybczynski, R., Song, Q., Mizoguchi, A., Morreale, R., *et al.*, 2000. Dynamic regulation of prothoracic gland ecdysteroidogenesis: *Manduca sexta* recombinant prothoracicotropic hormone and brain extracts have identical effects. *Insect Biochem. Mol. Biol.* 30, 1079–1089.)

to the lepidopteran PTH-positive cells. Also positive were axons and axon terminals in the neurohemal region of the ring gland, as to be expected based on the site of PTH release determined in Lepidoptera. However, a number of additional cells in the subesophageal thoracic and abdominal ganglia were also positive, particularly in the adult. It is unknown if this last result indicates multiple sites of PTH synthesis or a cross-reactivity to a protein besides PTH.

PTH immunoreactivity in *Locusta* is dependent on the antibody employed. Goltzene *et al.* (1992) found that a number of brain neurosecretory-type cells reacted positively with an anti-*Bombyx* PTH antibody while the use of an anti-*Antheraea* PTH antibody revealed no positive reactions (Závodská *et al.*, 2003). This same anti-*Antheraea* PTH antiserum was used to survey a wide variety of insects (Závodská *et al.*, 2003; see Section 6.2.2); until we know more about the nature of PTH in these species, it is not possible to interpret the smorgasbord of immunohistochemical patterns seen among the surveyed taxa.

Functional evidence that the corpus allatum and not the corpus cardiacum was the neurohemal organ for lepidopteran PTH was provided by Agui *et al.* (1979, 1980) and Carrow *et al.* (1981) and immunohistochemical studies have supported this conclusion (Mizoguchi *et al.*, 1990; Dai *et al.*, 1994). If the additional immunohistochemically positive cells seen in some studies (e.g., Žitňan *et al.*, 1993, Závodská *et al.*, 2003) actually do contain PTH,

it is presumably synthesized and released there. Further discussion of these “outlier” cells awaits confirmation that the regions of interest contain bioactive PTHH.

6.3.2. Control of Release: Neurotransmitters

PTHH release is apparently ultimately controlled by a number of factors, including photoperiod, size and nutritional state of the animal (see Section 6.1). Proximally, a number of studies have pointed out the likelihood that PTHH release is directed by cholinergic neurons synapsing with the prothoracicotropes. It must be emphasized that the data discussed in this section in the following studies do not definitively identify PTHH as the active molecule released from the brain, although this is most likely the case.

Carrow *et al.* (1981) showed that PTHH could be released by depolarizing *Manduca* (brain) neurons with a high potassium concentration in the presence of Ca^{2+} ; a similar result was obtained when *Bombyx* brains were treated with high K^+ in the presence or absence of external Ca^{2+} . Lester and Gilbert (1986, 1987) found that acetylcholine accumulation and α -bungarotoxin binding sites exhibited temporal peaks in the brain of fifth instar *Manduca* larvae and that these peaks coincided with periods of PTHH release and increased ecdysteroid synthesis. Of course, it is not likely that all the changes in the cholinergic systems of the brain that occur at these stages directly feed into the PTHH-producing neurons but these data are supportive for a role of cholinergic neurons in PTHH biology. Direct evidence that cholinergic stimulation can result in PTHH release comes from several *in vitro* studies of *Bombyx* and *Mamestra*. Agui (1989) showed that acetylcholine and cholinergic agonists caused cultured *Mamestra* brains to release PTHH into the medium while dopaminergic agents have little or no effect. Shirai *et al.* (1994) obtained similar results, using the acetylcholine agonist, carbachol, and further refined the analysis by using both muscarinic and nicotinic agents. They found that treatment with muscarine caused PTHH release from *Bombyx* brains *in vitro*, and that carbachol-induced release was blocked by the muscarinic antagonist atropine. Nicotine had no effect on PTHH release, with the composite data indicating the involvement of a muscarinic acetylcholine receptor. Later immunohistochemical studies revealed that the PTHH-producing cells of *Bombyx* do indeed express a muscarinic acetylcholine receptor (Aizono *et al.*, 1997). Shirai *et al.* (1994) further determined the effects of a calmodulin inhibitor, a phospholipase C inhibitor (PLC) and a protein

kinase C (PKC) inhibitor on carbachol-induced PTHH release. All three drugs inhibited the effects of carbachol, suggesting roles for Ca^{2+} -calmodulin, PKC, and PLC in this system. A role for Ca^{2+} was more directly demonstrated, when it was found that Ca^{2+} -ionophore also caused PTHH release (Shirai *et al.*, 1995). Finally in regard to the cholinergic neurotransmission, Shirai *et al.* (1998) investigated the possible role for small G-proteins in carbachol-stimulated PTHH release. The poorly hydrolyzable GTP analog $\text{GTP}\gamma\text{S}$ was found to stimulate PTHH release in the presence of a PLC inhibitor; the use of the PLC inhibitor removed the positive effect that the $\text{GTP}\gamma\text{S}$ would have on the larger heterotrimeric G-protein directly associated with muscarinic receptors (see Hamilton *et al.*, 1995).

The experiments of Agui (1989) indicated that dopamine might have a small effect on PTHH release *in vitro*. This possibility was tested with *Bombyx* brains and it was found that dopamine and serotonin increased PTHH release, while norepinephrine had no effect (Shirai *et al.*, 1995). The effects of dopamine and serotonin were notably slower than that of carbachol, suggesting that the first two compounds might be acting at some distance from the PTHH-producing cells. It must be emphasized, though, that there are no data that demonstrate that cholinergic agonists directly act upon the prothoracicotropes.

In contrast to observations in *Bombyx* (Shirai *et al.*, 1995), serotonin had an inhibitory effect on PTHH release, as measured by prothoracic gland ecdysteroid synthesis, during short-term (3 h) co-cultures of cockroach (*Periplaneta americana*) brains and prothoracic glands (Richter *et al.*, 2000). No effect of serotonin on isolated prothoracic gland ecdysteroid synthesis was found. Serotonin is the precursor to melatonin, the latter playing an important role as neuromodulator associated with darkness in diel light/dark cycles. In contrast to the results with serotonin, Richter *et al.* (2000) found that melatonin increased PTHH release in brains and prothoracic gland co-cultures during both short-term (photophase) and long-term (12 h: scotophase) incubations. A melatonin receptor antagonist, luzindole, blocked the melatonin effect. Again, neither melatonin nor luzindole had an effect on prothoracic gland ecdysteroid synthesis in the absence of the brain. These results raise the possibility that melatonin may play a role in modulating temporal patterns of PTHH release. However, as Richter *et al.* (2000) pointed out, there was a lack of daily rhythmicity in the molting of cockroaches in their colony, suggesting that there was no rhythmicity to PTHH release either, thus indicating

that an *in vivo* role for melatonin is problematic (see Section 6.3.4 and Chapter 9) for further information on cycles of PTTH release).

6.3.3. Control of Synthesis and Release: Juvenile Hormone

Insect molts, be they simple or metamorphic, are under the general control of 20-hydroxyecdysone (20E). Of course, 20E does not act alone and there are additional hormones, like ecdysis-triggering hormone and eclosion hormone, that act downstream from 20E, playing very proximate roles in controlling ecdysis behavior (see Mesce and Fahrback, 2002). However, juvenile hormones (JHs), which also participate in regulating molting and metamorphosis (see Chapter 8), modulate the action of 20E in a way that makes them partners with 20E rather than dependent factors. Juvenile hormones are epoxidated sesquiterpenoids produced in, and released from, the corpus allatum, which is also the site of PTTH release. Depending on the taxon and the developmental stage, a corpus allatum may synthesize one or two methylated JHs as well as JH acids (see Gilbert *et al.*, 1996; Henrich *et al.*, 1999; and Chapter 8).

JH is an important hormone in some aspects of insect reproduction (see Nijhout, 1994) but the relevant role of JHs, in regard to 20E and hence PTTH, is the ability of JHs to direct a molt either into a simple or into a metamorphic pathway. Basically, the presence of circulating JH during an ecdysteroid peak results in the next molt being a simple one, i.e., larva to larva, but if JH levels are very low during an ecdysteroid peak, then the ensuing molt will be a metamorphic molt, e.g., larva to pupa. In the last larval instar, of many or most insects, a small release of PTTH appears to trigger a small ecdysteroid release from the prothoracic gland during a period of very low JH titer. This small increase in ecdysteroid titer (Bollenbacher *et al.*, 1981) is termed the commitment peak because it commits the animal to a metamorphic molt (Riddiford, 1976).

How JHs modulate molt quality is not well understood. A considerable number of studies have demonstrated that JH or JH analogs (JHAs) (see Chapter 8) have effects on ecdysteroid titers when applied topically, injected, or fed to preadult insects. In these studies involving intact larvae, pupae, debrained animals, or isolated abdomens, JHs appeared to modulate PTTH synthesis and/or release, and perhaps affected the PTTH signaling pathway in the prothoracic gland or the gland's capacity for ecdysteroidogenesis. Two periods of JH influence have been identified in such studies during the last larval instar; the influence of JH

during the penultimate instar is different and is treated separately below.

In Lepidoptera, JH hemolymph titers decline dramatically in the first few days of the last larval instar (for *Manduca*, see Baker *et al.*, 1987; for *Bombyx*, see Nimi and Sakurai, 1997). The JH decline is followed by a release of PTTH and the resultant small peak in the ecdysteroid titer termed the commitment peak (see Section 6.3.1). Application of juvenoids (JHs or JH analogs: JHAs) before the commitment peak results in a delay of development, e.g., deferral of the cessation of feeding and wandering (see Riddiford, 1994, 1996). In *Manduca*, Rountree and Bollenbacher (1986) found that prothoracic glands from early fifth instar larvae treated with JH-1 or ZR512 (a JHA) for 10 h prior to extirpation still responded to PTTH with increased ecdysteroid synthesis, although the normal developmental increase in basal ecdysteroidogenesis was inhibited. However, when the brain-retrocerebral complexes from such animals were implanted into head-ligated larvae, they exhibited a delay in activating development, relative to control complexes. This result suggested that JHs controlled the time of PTTH release at this time, although the data do not rule out an effect on PTTH content/synthesis in the complexes, as well. In a later study of *Manduca* glands, Watson and Bollenbacher (1988) did find a reduced response to PTTH *in vitro*, but this study involved animals treated with a JHA for 2 days, starting at an earlier time point. Thus, the conclusions reached may reflect the experimental paradigms as well as the animal's intrinsic biology.

In *Bombyx*, similar results have been obtained. Sakurai (1984), using the technique of brain removal, concluded that PTTH release occurred early in the fifth instar shortly after a precipitous decline in JH titer, as found for *Manduca*. Furthermore, allatectomy late in the fourth instar shortened the phagoperiod in the fifth instar, and brain removal performed on these larvae at 48 h into the fifth instar did not block metamorphosis. Sakurai also found that JH application early in the fifth instar prolonged the larval period. The combined data suggested again that JH in the early fifth instar blocked PTTH release. Sakurai *et al.* (1989) provided evidence that JH inhibits prothoracic gland activity in *Bombyx* by showing that allatectomy of early fifth instar larvae accelerated the period of PTTH sensitivity in glands, but that this acceleration was blocked by JH treatment.

The above studies utilized either JHs, or JH analogs with JH-like chemical structures, i.e., terpenoids. However, the nonterpenoid chemical fenoxycarb, a carbamate, has also been shown to exhibit JH-like

effects when applied to larvae. Leonardi *et al.* (1996) found that single, topically applied doses of fenoxycarb, as low as 10 femtograms, were sufficient to disrupt larval–pupal ecdysis in *Bombyx*. Also studying *Bombyx*, Monconduit and Mauchamp (1998) showed that very low doses of fenoxycarb, 1 ng and below, were sufficient to induce permanent larvae when administered in the food during the phagoperiod of the last larval instar. This treatment also considerably depressed the ecdysteroid synthesis of prothoracic glands when measured *in vitro*. The glands still exhibited a qualitative response to a (crude) PTTH preparation, but the quantitative response was greatly curtailed. Dedos and Fugo (1996) found that fenoxycarb significantly and directly inhibited prothoracic gland steroidogenesis under *in vitro* conditions; however, the effective doses required in these *in vitro* experiments were much higher (0.5–1 µg/gland) than employed by Leonardi *et al.* (1996) and Monconduit and Mauchamp (1998). Thus, the possibility that fenoxycarb at these doses had effects not attributable to JH-like activities cannot be ruled out, as suggested by Mulye and Gordon (1993) and Leonardi *et al.* (1996), among others.

When exogenous JH or JH analogs are administered after the commitment peak period of last larval instars, quite different results are obtained, i.e., JHs accelerate development and shorten the period to pupation (see Sakurai and Gilbert, 1990; Smith, 1995). Brain removal or ligation after the commitment peak period does not affect this accelerative ability of JH, indicating that the brain and hence PTTH release are not the targets of JH at this time (Safranek *et al.*, 1980; Gruetzmacher *et al.*, 1984a). Intriguing in this regard are the observations of Sakurai and Williams (1989). They implanted prothoracic glands extirpated from feeding (day 2) fifth instar *Manduca* larvae into pupae lacking a cephalic complex. Treatment of these pupae with either 20E or the JH analog hydroprene resulted in an inhibition of steroidogenesis when the prothoracic glands were studied subsequently *in vitro*. However, if both 20E and hydroprene were administered, an increase in ecdysteroid synthesis was seen. These conditions simulate the postcommitment peak period in larvae, when JH (particularly JH acids) and 20E are both high. Thus, as Smith (1995) suggested, the prepupal ecdysteroid peak may be initiated by PTTH and maintained by JH, with the eventual decline of JH titer resulting in the decline of ecdysteroid levels that occurs shortly before larval–pupal ecdysis.

The relationship between JH and the brain–prothoracic gland axis observed in the last larval

instar is not characteristic of every developmental stage. Stages prior to the last larval instar lack the commitment peak of ecdysteroids, along with the PTTH release that triggers it. In *Manduca*, the fourth (penultimate) larval instar is characterized by high to moderate JH levels throughout (see Riddiford, 1996; and Chapter 8). The fourth instar JH titer minimum is reached shortly before ecdysis to the fifth instar and ecdysteroid levels in the hemolymph begin rising while JH levels are relatively high, albeit falling. In *Bombyx*, the case is somewhat more complicated. Peaks in the JH titer occur during the early and in the middle to late portions of the penultimate instar, with the minimum between the two peaks being higher in concentration and shorter in duration than the minimum measured during the last (fifth) instar (Nimi and Sakurai, 1997).

Fain and Riddiford (1976) showed that head ligation of fourth instar *Manduca* larvae before a presumed head critical period resulted in precocious pupation; this operation isolated the sources of both PTTH and JH from the prothoracic glands. Injection of 20E immediately after ligation resulted instead in a larval molt. Delaying the 20E treatment resulted in a progressive loss of the larval response but JH injection maintained the 20E-induced larval molt. Lonard *et al.* (1996) found that application of the JH analog methoprene to fourth instar *Manduca* larvae affected neither the ecdysteroid titer *in vivo* nor the ecdysteroidogenic activity of prothoracic glands measured *in vitro*; however, allatectomy abolished the ecdysteroid peak, which could be restored by the application of methoprene. Further methoprene experiments with debrained and neck-ligated animals indicated that the restorative effect of methoprene involved the brain and was interpreted to indicate the existence of JH-dependent PTTH release.

Sakurai (1983) observed results in *Bombyx* similar to those obtained by Fain and Riddiford (1976), i.e., head ligation early in the fourth instar resulted in precocious pupation and application of methoprene shifted the postligation molts back to larval or larval–pupal intermediates. Unlike the case of JH in *Manduca*, methoprene was effective at reversing precocious pupations only when administered about 24 h after ligation, with larvae treated directly after ligation simply showing no further development. This difference between the two species may reflect a fundamental difference in the ecdysteroidogenic competence of the prothoracic gland in the time immediately following larval ecdysis (see Section 6.3.4). Sakurai also found that application of 20E to debrained *Bombyx*, still possessing corpora allata, resulted in larval rather than pupal molts.

These data from *Manduca* and *Bombyx* indicate that high levels of 20E in the relative absence of JHs will result in a pupal molt, even prior to the last larval instar. However, under ordinary circumstances, PTTH release and ecdysteroid synthesis take place against a significant JH titer background in stages other than the last larval instar.

The relationship between JHs and the PTTH-prothoracic gland axis during pupal–adult development has not been studied extensively, perhaps because there is no JH in early lepidopteran pharate adults, and therefore the experiments are not physiological. In general, application of exogenous juvenoids to pupae shortens the time until adult ecdysis, and the juvenoid must be administered about the time of pupal ecdysis or termination of pupal diapause (see Sehna, 1983). Using *Bombyx*, Dedos and Fugo (1999a) injected the potent juvenoid fenoxycarb at pupal ecdysis and found that the ecdysteroids were considerably elevated above control during subsequent pupal–adult development. *In vitro* analysis of the prothoracic glands from treated animals showed elevated ecdysteroid secretion relative to controls; additionally, brain-retrocerebral complexes from fenoxycarb-treated animals secreted more PTTH than controls did, after 3 h of *in vitro* incubation; however, there was no difference from controls if the incubation was only 1 h. These data were interpreted to indicate that fenoxycarb stimulated ecdysteroid synthesis by promoting PTTH release. However, the observed delay in the fenoxycarb-facilitated increase in PTTH release *in vitro* could also be interpreted as a release from *in vivo* inhibition, once the brain-retrocerebral complexes were removed from the larvae.

Although all the studies cited above, and many others not discussed here, clearly point out the existence of cross-talk between the brain-prothoracic gland axis and juvenile hormones, considerable care must be exercised in interpreting these experiments. Ligations, extirpations, and tissue transplants have provided valuable insights into insect endocrinology over the years but these methodologies are rarely clean. For instance, allatectomy removes not only the site of juvenile hormone synthesis but also the neurohemal organ for normal PTTH release. Similarly, brain removal eliminates a source of many neuropeptides as well as a biochemical clock involved in regulating many diel cycles.

Experiments involving hormone additions to intact or nonintact animals also have to be interpreted carefully. Hormones are powerful drugs when given in large doses or at unusual times. For example, fenoxycarb-treated pupae that had elevated

ecdysteroid titers also exhibited malformed rectums (Dedos and Fugo, 1999a) and whether or not this result tells us something informative about ecdysteroid titer regulation during normal adult development is open to debate. JH has often been suggested to have a negative effect on PTTH secretion in the last larval instar (see above) based on experiments in which larvae were treated with JH or JH analogs early in the instar. However, recently Mizoguchi (2001) measured the PTTH titer in JH-treated fourth and fifth instar *Bombyx* larvae, using time-resolved fluorimmunoassay. His data showed that, in fact, JH treatment had just the opposite effect, i.e., JH treatment raised the PTTH hemolymph titer! Thus, experiments in which PTTH secretion was measured *in vitro* following JH treatment of the donor larvae appear to be misleading in an attempt to understand the relationship between JH and PTTH.

Differences between species in the basic intercellular or intracellular “wiring” of their endocrinological systems may also confound experimental manipulations. An obvious example is the variation among species in prothoracic gland innervation. *Manduca* and *Bombyx* glands appear not to be innervated while prothoracic glands in other species clearly are (see Section 6.5.4). Brain extirpation in the latter species could have consequences on the prothoracic gland beyond the obvious removal of the source of PTTH. Less obvious but potentially important are developmental differences between species that complicate the ability to make generalizations. For instance, during the earliest portion of the last larval instar, *Bombyx* prothoracic glands are refractory to PTTH stimulation while *Manduca* glands are responsive (Sakurai, 1983). This refractoriness stems from a developmentally specific deficit in PTTH receptors or some other very early-acting component of the PTTH signal transduction cascade (Gu *et al.*, 1996; see also Section 6.5.1).

It must also be emphasized that direct effects of JHs on prothoracic glands have been seen rarely, indicating the probable participation of one or more additional cell types and/or hormones and/or neuronal connections in JH effects. In the rare cases where a direct effect has been demonstrated *in vitro*, the concentrations of JHs or JHAs employed are notably above physiological limits. In addition to the effect of 1 μg ($\approx 65 \mu\text{M}$) fenoxycarb on *Bombyx* prothoracic glands discussed above (Dedos and Fugo, 1996), Richard and Gilbert (1991) reported that JHB3 and JHIII both inhibited the secretion of ecdysteroids from *Drosophila* brain–ring gland complexes; however, millimolar concentrations were needed to elicit the effect.

6.3.4. PTTH Hemolymph Titer

The periodic increases in ecdysteroid synthesis and hemolymph titer that control molting and molt quality have long been interpreted to indicate that PTTH was itself released periodically into the hemolymph. However, testing this hypothesis has not been simple. Unlike ecdysone and 20E, a sensitive radioimmunoassay for PTTH has not been available until very recently. Instead, the measurement of PTTH hemolymph titers has been done primarily via *in vitro* analysis of prothoracic gland ecdysteroidogenesis using hemolymph extracts.

Bollenbacher and Gilbert (1981) determined the brain and hemolymph titers of PTTH in *Manduca* during the fourth and fifth larval instars, using an *in vitro* bioassay system and discovered little concordance between measured brain- and hemolymph-derived PTTH activities. Brain titers of PTTH rose fairly steadily during this period, punctuated by minor decreases or plateaus. A small dip in brain PTTH was seen immediately after the molt to the fifth larval instar and a larger decrease seen about 1 day prior to the pupal molt. In contrast, hemolymph PTTH activity was low throughout this period with only a few very sharp peaks. In the fourth instar, a single PTTH peak occurred late on day one, also reported later by Bollenbacher *et al.* (1987), with a second small peak spanning the molt to the fifth larval instar. During the fifth larval instar proper, four peaks were noted. Three medium-sized peaks clustered on and about the fourth day, preceding and overlapping the commitment peak of ecdysteroid synthesis. A much larger PTTH hemolymph titer was measured on day six, at the rising edge of the major larval ecdysteroid titer that precedes the metamorphic molt to pupal-adult development and closely anticipating apolysis. The PTTH titer appeared to be rising again, just before pupal ecdysis, but titers were not determined beyond this point.

Later studies of *Manduca* brain PTTH content indicated that PTTH levels in the brain increased considerably during pupal-adult development (O'Brien *et al.*, 1986) but hemolymph titers have yet to be determined at this stage for *Manduca*. These studies suggested that PTTH titers in the brain are not closely correlated with circulating levels, unlike the levels in the corpus allatum (Agui *et al.*, 1980). The PTTH titers described in the Bollenbacher and Gilbert (1981) study, while intriguing, must be considered preliminary for three reasons. First, the bioassay may miss small peaks that are not high enough to activate prothoracic glands *in vitro* but which might act *in vivo* i.e.,

very low levels of PTTH *in vitro* might be able to stimulate prothoracic gland steroidogenesis with a slower time course than that utilized in the *in vitro* assays. Second, very low levels of PTTH might have biologically significant effects on the prothoracic gland besides stimulating ecdysteroidogenesis. Third, if the so-called small PTTH (see Section 6.2.5) is secreted into the hemolymph, then an unknown fraction of the PTTH activity detected in the hemolymph might reflect this incompletely characterized protein rather than “big” PTTH.

Shirai *et al.* (1993) first addressed the topic of PTTH hemolymph titers in *B. mori*. Again, an assay was used in which fifth larval instar hemolymph extracts were tested for PTTH ecdysteroidogenic activity with prothoracic glands *in vitro*. In addition, the secretion of PTTH activity by brain-endocrine complexes was measured with the *in vitro* gland assay. Hemolymph ecdysteroid levels were also determined via radioimmunoassay. The results of this study suggested that five peaks of PTTH activity were present in the hemolymph of *Bombyx* fifth instar larvae. Two of these PTTH peaks occurred in the first half of the fifth instar, clearly before a small ecdysteroid peak on day seven or day eight to nine, depending on the particular *Bombyx* strain-cross utilized (*Shunrei* x *Shougetsu* and *Kinshu* x *Shouwa*, respectively). This small ecdysteroid peak was presumed to be the commitment peak, as seen in *Manduca*. A third PTTH peak occurred the day before the putative commitment peak while the fourth peak encompassed the time span of the commitment peak and just overlapped the beginning of the large premolt ecdysteroid peak. The fifth hemolymph peak detected by Shirai *et al.* (1993) began almost immediately after the finish of the fourth peak and overlapped with approximately the first third of the large ecdysteroid peak. These workers also found four to five peaks of PTTH activity when brain-endocrine complexes were assayed *in vitro*. These peaks anticipated the hemolymph peaks by about one day. These results generally supported the hemolymph peak data and also suggest that, at least in *Bombyx*, isolated brain-endocrine complexes might be released prematurely from negative controls that function *in vivo*. These hemolymph and *in vitro* secretion results are intriguing because they suggest that two to three releases of PTTH may not be ecdysteroidogenic, but might serve additional functions, such as “priming” the prothoracic gland to produce higher levels of ecdysteroids at a later time. This “priming” could represent a trophic effect of PTTH, in which transcription and translation of components of the ecdysteroidogenic synthetic pathway occur.

As was the case for the *Manduca* hemolymph PTTT data (Bollenbacher and Gilbert, 1981), the data obtained by Shirai *et al.* (1993) must be interpreted carefully for several reasons. First, the hemolymph and incubation medium assays measured total PTTT activity, but did not determine if the activity stems from PTTT and/or from other molecules such as small PTTT or bombyxin. Second, the peaks of PTTT activity obtained from both the hemolymph extracts and the brain-endocrine incubations were surprisingly similar in height, i.e., PTTT activity/ml, during the fifth instar, in contrast to the data obtained by Bollenbacher and Gilbert (1981). Third, because the *Bombyx* prothoracic gland is not responsive to PTTT at the beginning of the fifth instar (see Sections 6.3.3 and 6.5.1), a release of PTTT in the first day or two of the fifth larval instar of *Bombyx* would not be expected to result in a corresponding ecdysteroid peak. However, the lack of an ecdysteroid peak *in vivo* in response to the PTTT activity peak measured around days four to five is surprising, and calls into question the extraction and incubation results.

The development of a highly specific antibody against *Bombyx* PTTT (Mizoguchi *et al.*, 1990) afforded an opportunity to develop a more facile and sensitive method to determine hemolymph PTTT titers. Dai *et al.* (1995) and Mizoguchi *et al.* (2001, 2002) used this antibody in a time-resolved fluoroimmunoassay to study *Bombyx* PTTT hemolymph titers during larval-pupal and pupal-adult development. In addition, Dai *et al.* (1995) utilized this anti-PTTT antibody to assess the PTTT content of brain-retrocerebral complexes, using immunogold electron microscopy.

In the first of these three studies (Dai *et al.*, 1995), the hemolymph PTTT titer was moderately high at the beginning of the fourth instar and reached a minimum about 36 h later (scotophase of day 1, fourth instar (IV₁)). A larger peak ensued, with a slight dip in the photophase of IV₂, with a minimum again just before the molt to the fifth instar. The fifth instar PTTT titer exhibited a small but distinct peak during the first photophase on V₀ with minima 12 h later (V₁) and just at the larval-pupal molt (V₉-P₀). In between the two minima, hemolymph levels climbed fairly steadily to a plateau from V₆ through V₈, at which point a rapid decline to the molt minimum began. The parallel determinations of hemolymph ecdysteroid titer and PTTT antibody reactivity in the corpus allatum (the site of PTTT release; see Section 6.3.1) indicated that there was no obvious correlation between PTTT and ecdysteroid hemolymph titers, except during the fourth instar, when both of the PTTT peaks were associated

with ecdysteroid peaks. The commitment and large premolt ecdysteroid increases of the fifth larval instar both occurred during the plateau period of high PTTT titer. In contrast, PTTT immunostaining in the corpus allatum was low during the early fifth instar peak (V₁) and during most of the later hemolymph plateau phase of PTTT titer; however, a striking transitory increase in PTTT immunostaining was seen on V₇, just before the rapid decline in hemolymph PTTT titer commenced.

Subsequent to these intriguing observations by Dai *et al.* (1995), it was discovered that the method used to extract the hemolymph for the PTTT fluoroimmunoassay included a hemolymph factor that contributed an unknown amount to the measured PTTT titer (Mizoguchi *et al.*, 2001). Consequently, Mizoguchi *et al.* (2001) repeated the determination of *Bombyx* hemolymph and ecdysteroid titers, using a revised hemolymph treatment protocol. This study also extended through the period of pupal-adult development and included a parallel ecdysteroid titer determination, as well as an analysis of brain PTTT content, using the time-resolved fluoroimmunoassay. Mizoguchi *et al.* (2002) provided a third determination of fifth instar and pupal-adult *Bombyx* hemolymph PTTT and ecdysteroid titers, using the revised fluoroimmunoassay, in conjunction with a study directed at discovering potential rhythmicity in PTTT secretion and the influence of diel light cycles. These two more recent studies contrast in several ways with the data obtained by Dai *et al.* (1995) and Shirai *et al.* (1993). Like Dai *et al.* (1995), Mizoguchi *et al.* (2001) found two hemolymph PTTT peaks in the fourth instar, but only the second one was accompanied by a clear-cut rise in hemolymph ecdysteroids. During the fifth instar, Mizoguchi and colleagues detected three rises and fall of hemolymph PTTT, with the instar ending in a period of rapidly increasing PTTT levels that carried on into pupal-adult development. These studies involved three different crosses of *Bombyx* races and four separate determinations (in the following section, the name of the female race in a cross is given first, and the male second). The earliest of these increases took place on V₄ and rose just slightly above background PTTT levels. Of the two crosses for which complimentary ecdysteroid titers are available, the *Kinshu* x *Showa* (Mizoguchi *et al.*, 2001) exhibited ecdysteroid peaks corresponding to all three PTTT rises (albeit the first ecdysteroid peak is very small). In the *J106* x *Daizo* hybrid (Mizoguchi *et al.*, 2002), only the second and third PTTT peaks are correlated with measurable ecdysteroid peaks, i.e., the small, presumptive commitment and the much larger premolt peaks. In

contradistinction to the associated ecdysteroid peaks, the relative heights of the second and third PTTH increases vary with the hybrid, i.e., the V_5 peak is distinctly higher than the V_7 peak in the *Kinshu* x *Showa* hybrid (Mizoguchi *et al.*, 2001) while in the *J106* x *Daizo* hybrid, the later peak (V_6) is much larger (Mizoguchi *et al.*, 2002). The immunoassay survey of fifth instar brain PTTH by Mizoguchi *et al.* (2001) revealed a period of gradual increase, starting from the first day of the instar and peaking on the fifth and sixth days, followed by a rapid decline to a minimum on the ninth day. PTTH levels then rose slightly on the ninth day and remained static through pupation on the following day.

Pupal–adult PTTH hemolymph titers were analyzed four times by Mizoguchi and colleagues and indicated one or two high peaks of concentration extending over the first half of the stage followed by a period of much lower levels and ending with a variety of levels, low, intermediate, or high, depending on the cross (Mizoguchi *et al.*, 2001, 2002). The two parallel ecdysteroid titers exhibited much less variation in temporal patterning. A large, fairly symmetric peak was centered about 48 h after pupal ecdysis and ecdysteroid levels declined rapidly and evenly to very low concentrations by the time of adult ecdysis. The major difference between the two pupal–adult ecdysteroid titers was that of concentration. The *Kinshu* x *Showa* racial hybrids reached levels of more than 7 μg equivalents per ml (Mizoguchi *et al.*, 2001) while the *J106* x *Daizo* hybrids peaked at only about 1.5 μg equivalents per ml (Mizoguchi *et al.*, 2002).

The five immunologic determinations of *Bombyx* PTTH hemolymph titers, and the three of ecdysteroids, made by Dai *et al.*, 1995 and Mizoguchi *et al.* (2001, 2002) paint a fairly similar picture of circulating PTTH levels in *Bombyx*, yet there are several important discrepancies among the results that need to be resolved. First, the small yet distinct early ecdysteroid peak seen in the fourth instar by Dai *et al.* (1995), occurring in consort with the first of the two PTTH rises, was not consistently found in other assays of fourth instar ecdysteroid titers, e.g., possibly seen by Kiguchi and Agui (1981), but not by Gu and Chow (1996) or Mizoguchi *et al.* (2001). Verifying, or disproving, the existence of this ecdysteroid increase is necessary to understand the role(s) of PTTH at this time. Second, the status of hemolymph PTTH levels at the beginning of pupal–adult life is not clear. In two of four assays, a broad peak of hemolymph PTTH was found in early pupal–adult life centered on day 2 (*Kinshu* x *Showa*: Mizoguchi *et al.*, 2001; *Showa* x *Kinshu*

raised at 25 °C: Mizoguchi *et al.*, 2002). In a third profile (*Showa* x *Kinshu* raised at 25 °C: Mizoguchi *et al.*, 2002), a peak on the second day after pupal ecdysis was followed by an approximately 2-day plateau of intermediate values, before finishing the last half of pupal–adult life with lower levels but with considerable variation, especially in the 36 h before adult ecdysis. In the fourth instance (*J106* x *Daizo*: Mizoguchi *et al.*, 2002), a high, narrow peak of hemolymph PTTH occurred on the first day after ecdysis, followed by a second, broader, peak about 36 h later. A third topic of concern is the level of PTTH circulating at the end of pupal–adult development. Mizoguchi *et al.* (2001) reported an adult ecdysis peak (≈ 250 pg rPTTH equivalents) that was as high as that seen at the beginning of pupal–adult development, when ecdysteroid levels are also very high. In contrast, PTTH hemolymph titers in the other three pupal–adult studies were only 25% to 50% of the earlier pupal–adult peaks (Mizoguchi *et al.*, 2002).

It is not possible currently to assess the reasons for the observed large temporal and concentration variations in PTTH titer. Multiple racial hybrids of *Bombyx* were used and some data came from animals raised on mulberry leaves (Shirai *et al.*, 1993), rather than on an artificial diet. The length of the fifth instar ranged from 8 to 12 days while pupal–adult development varied from 8 to 11 days. The possibility that further stage-specific or hybrid-specific factors are influencing the PTTH titer data cannot be ruled out. However, since hybrid-specific differences in ecdysteroid titers were also found, especially in the peak concentrations, and ecdysteroid assays are well standardized, it seems likely that the PTTH titer variations are mainly biologically based. Given this assumption, it is possible to present an average or summary picture of PTTH and ecdysteroid hemolymph titers in *Bombyx* (Figure 6). Several conclusions can be drawn from these data. First, it is clear that PTTH increases do not always result in a detectable increase in hemolymph ecdysteroid concentrations and these results suggest that the gland was nearly or absolutely refractory to PTTH stimulation *in vivo* at times when a response was easily detected *in vitro*, e.g., V_6 and V_9 (Okuda *et al.*, 1985). This disparity suggests that the prothoracic gland may be subject to negative controls at some stages and that this inhibition of activity is lost when the glands are placed *in vitro*. Another obvious inference stemming from these observations is that PTTH might stimulate nonsteroidogenic events at these times, perhaps in tissues other than, or in addition to, the prothoracic gland.

A second conclusion that originates from the examination of the hemolymph titers of PTTH and

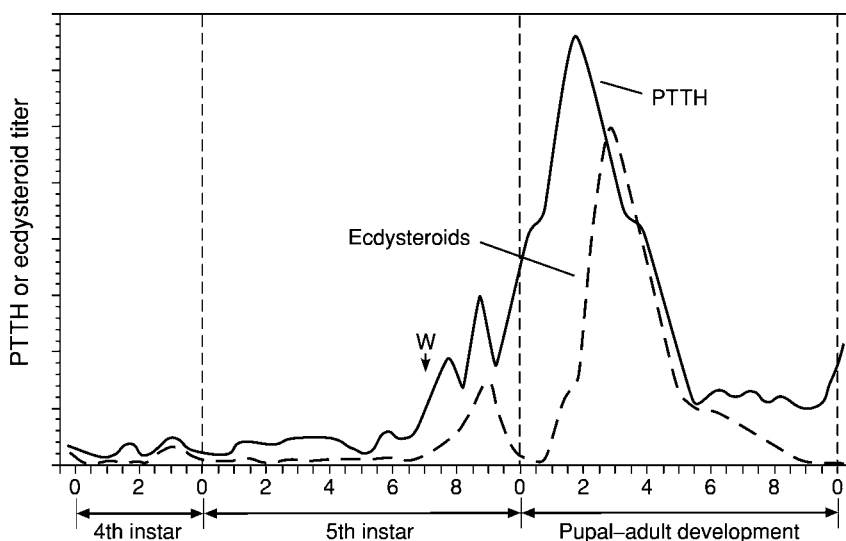


Figure 6 Typical PTTH and ecdysteroid titers in *Bombyx mori* hemolymph. Values are a composite of determinations from Dai *et al.* (1995) and Mizoguchi *et al.* (2001, 2002) and any individual, original profile may vary notably from the constructed profile shown here. W indicates wandering stage.

ecdysteroids is that there might be a negative correlation between peak concentrations of PTTH and peak concentrations of ecdysteroids during pupal-adult development. Thus, during this stage the *Kinshu* x *Showa* hybrid experienced a maximum titer of PTTH ≈ 270 pg rPTTH equivalents/ml while the associated ecdysteroid peak was ≈ 7000 ng 20E equivalents/ml (Mizoguchi *et al.*, 2001); in contrast, in the *J106* x *Daizo* hybrids, the temporally comparable peaks were ≈ 670 pg rPTTH/ml equivalents and ≈ 1500 ng 20E equivalents/ml, respectively (Mizoguchi *et al.*, 2002).

Third, while peak concentrations of PTTH are not good predictors of pupa-adult ecdysteroid levels, a suggestive two point (!) concordance does occur in the fifth instar. The maximum fifth instar PTTH titer in the *Kinshu* x *Showa* hybrid (≈ 90 pg rPTTH equivalents/ml) was about half that seen in the *J106* x *Daizo* hybrid, but the peak ecdysteroid level in the *Kinshu* x *Showa* hybrid was more than twice that of the *J106* x *Daizo* cross (≈ 1600 ng and ≈ 600 ng 20E equivalents/ml, respectively) (Mizoguchi *et al.*, 2001, 2002).

Fourth, in the *J106* x *Daizo* hybrids, a large temporally narrow peak of PTTH (≈ 880 pg rPTTH equivalents/ml) occurred immediately after larval-pupal ecdysis, which was not associated with an ecdysteroid peak and which was not seen in the other PTTH titer profiles (Mizoguchi *et al.*, 2001, 2002). The failure to see a corresponding ecdysteroid peak may not be surprising, given evidence from *in vitro* studies indicating that the prothoracic gland might be refractory to PTTH stimulation during the period of larval-pupal ecdysis (Okuda *et al.*, 1985).

What this peak does suggest is that our understanding of factors involved in PTTH release and metabolism is rudimentary. One missing factor in this equation may well be juvenile hormone, the values of which were not addressed in these studies, but other hormones and factors are also likely involved.

Finally, the variation found in PTTH and ecdysteroid titers and in developmental rates, originating from methodologically similar studies (Dai *et al.*, 1995; Mizoguchi *et al.*, 2001, 2002), clearly indicate that more data are desirable from *Bombyx* and from other insects such as *Manduca*. Only then will we obtain a reliable picture of how this important piece of the insect endocrinological system functions. The open questions are many. For instance, how much does the magnitude of a PTTH peak matter? Do very high levels have unique biological effects on the prothoracic glands, or do they simply delay processes that depend on low PTTH levels for their expression or regulation? What are the targets and consequences of the orphan peaks, i.e., PTTH increases that do not elicit ecdysteroidogenesis? What is the role of PTTH in late pupal-adult development and during adult life, when prothoracic glands are generally absent due to programmed cell death?

6.3.5. PTTH: Rhythms and Cycles

The above discussion of PTTH hemolymph titers has concentrated on the issues of the timing and magnitude of stage-specific peaks. However, a number of studies have yielded data that showed that some PTTH peaks are "gated," that is, occur only within a narrow window within a 24 h light-dark

cycle. Some evidence also suggests that a circadian variation in PTTH titer is overlaid upon the broader sweeps of changing PTTH levels. Steel and Vafapoulou (see Chapter 9) cover the subject of circadian rhythms in detail (see also Giebultowicz, 2000; Steel and Vafapoulou, 2002); only a brief treatment is given here.

Head critical period (HCP) is a term used to refer to a developmental period before which the presence of the head (brain) is necessary for later events to transpire. In the case of ecdysteroid-triggered events, the HCP has generally been used as a marker for PTTH release. Studies of the HCP for molting provided the first data indicating that an endocrine-dependent event in insect development involved circadian cycles (Truman, 1972). This work revealed that larval molting and the HCP for molt initiation in *Manduca* and *A. pernyi* were temporally gated. Since this HCP and ecdysis are ultimately controlled by PTTH, via ecdysteroid levels, these observations implicated the involvement of a circadian clock in PTTH release. Further work in *Manduca* (Truman and Riddiford, 1974) demonstrated that the PTTH release responsible for inducing wandering in the last larval instar, as revealed through HCP timing, was also temporally gated, again indicating the participation of a circadian clock. In *Samia cynthia ricini*, the HCP for gut purging was also gated (Fujishita and Ishizaki, 1982) but not every HCP is necessarily under the control of a photoperiodically controlled circadian clock. For instance in *Samia*, in contrast to the HCP for gut purging, the HCP for pupal ecdysis, which follows wandering by 2 days, was unaffected by shifts in the photoperiod conditions and appeared to be a constant (≈ 96 h) (Fujishita and Ishizaki, 1982). Light-entrained circadian rhythms in endocrine-dependent events are not confined to the Lepidoptera, of course, and have been studied in a number of other taxa, such as the Diptera. For instance, in *S. bullata* (Roberts, 1984) and *S. argyrostoma* (Richard *et al.*, 1987) larval wandering is under strong photoperiodic control. A similar result was seen with *D. melanogaster*, if they were raised under long day (16:8) and low-density conditions (Roberts *et al.*, 1987).

More recent work on *Rhodnius prolixus* and the recent surveys of PTTH hemolymph titers have suggested that there might be diel variations in titer, and presumably PTTH release, which are superimposed upon the larger peaks and plateaus of circulating PTTH levels. In *Bombyx*, data from the fifth larval instar and from pupal–adult development (Sakurai *et al.*, 1998; Mizoguchi *et al.*, 2001) suggested that there may be such a daily cycling of PTTH hemolymph levels; a similar suggestion was seen in the

in vitro secretory activity of prothoracic glands from the last 4 days of the fifth instar (Sakurai *et al.*, 1998). To examine this possibility more directly, Mizoguchi *et al.* (2001) phase shifted two groups of initially synchronous *Bombyx* larvae on the first day of the fifth instar, such that their light–dark periods were displaced by 12 h from one another. After 5 days, both groups exhibited a peak in PTTH hemolymph titer that began at the scotophase–photophase transition and peaked during the photophase, supporting the hypothesis that light–dark cycles influence some periods of PTTH release. However, the careful temporal analysis of PTTH titers afforded by this study did not indicate that every apparent PTTH peak was so influenced. In both phase-shifted populations, a second PTTH peak was observed that varied between the two groups in time of appearance (30 h versus 48 h), with one of the following peaks occurring during the scotophase and the other during the photophase. Thus, this study did not support the notion of small daily PTTH peaks.

The strongest and most direct evidence for circadian rhythms in PTTH levels and consequent ecdysteroid hemolymph levels comes from studies of the bug *R. prolixus* and the cockroach *P. americana*. In *Rhodnius*, during the last two-thirds of the final larval instar, PTTH synthesis and release appears to be controlled by a circadian clock, with peaks occurring during the scotophase (Vafopoulou and Steel, 1996; see Chapter 9). The implications of this rhythmicity for ecdysteroid synthesis are not straightforward, as studies *in vitro* revealed that the *Rhodnius* prothoracic gland exhibited circadian cycling of ecdysteroid synthesis in the absence of PTTH, although with a phase shift to peaks during the photophase (Pelc and Steel, 1997). Steroid synthesis in animals that had been either decapitated or paralyzed with tetrodotoxin, to ablate PTTH release, exhibited a rhythmicity similar to that seen *in vitro*, and the composite results indicate that PTTH is an important entraining factor for the circadian clock regulating ecdysteroid synthesis. The endogenous clock of the prothoracic gland is light entrained, specifically by a “lights-off” cue (Vafopoulou and Steel 1998, 2001). A “lights-off” cue also functions in controlling PTTH release, as shown in experiments where PTTH release was abolished under continuous light but promptly restored after transfer to dark conditions (Vafopoulou and Steel 1998, 2001). Further complexity in the control of ecdysteroidogenesis by PTTH in *Rhodnius* was indicated by the discovery that prothoracic glands also express a daily rhythm in PTTH responsiveness, i.e., PTTH receptivity is gated (Vafopoulou and Steel, 1999).

In the cockroach *P. americana*, isolated prothoracic glands did not exhibit a circadian cycling of ecdysteroid synthesis *in vitro* when they had been extirpated from stages that exhibit daily cycles of ecdysteroids in the hemolymph, i.e., the last several days of the last larval instar (Richter, 2001). However, if the *Periplaneta* glands were co-cultured with brains taken from such late instar animals, then a diel rhythm in ecdysteroidogenesis resulted, with a peak in the scotophase matching the *in vivo* hemolymph pattern (Richter, 2001). As discussed earlier (Section 6.3.2), melatonin dramatically increased the secretion of PTTH from brains *in vitro* during a scotophase, with lesser increases seen during a photophase (Richter, 2001). These data suggested that PTTH release might be, at least partially and at only some stages, regulated by melatoninergic neurons. This PTTH cycling would result in a circadian variation in ecdysteroid synthesis and hemolymph titer. Nonetheless, further support for this hypothesis has yet to be marshaled. In particular, a demonstration of PTTH cycling *in vivo*, preferably in the hemolymph titer, would be supportive because factors other than PTTH might be driving prothoracic gland cycles, e.g., input from nerves that synapse with the prothoracic gland in *Periplaneta* (Richter, 1985).

The existence of light-dependent cyclic or rhythmic behavior by an organism or a tissue or single cell indicates the presence of a light-sensing system as well as an actual molecular-based clock. In cases where isolated brains or prothoracic glands cyclically release hormones under *in vitro* conditions, the sensing apparatus and clock(s) must reside within some or all of the cells under study. A light-sensing system implies the presence of a photoreceptive protein or a photopigment coupled to a transduction system. In the instance of PTTH and ecdysteroid cycling observed *in vitro* in *Rhodnius* tissues, such a sensing system must be extraretinal. In *Drosophila*, one such protein is known, cryptochrome, which absorbs in the blue, and functions as an extraocular photoreceptor in the lateral neurons of the brain (see Stanewsky, 2002, 2003). In a series of clever experiments involving gland transplantation, selective illumination of larval regions and phase-shifting of gut purge, Mizoguchi and Ishizaki (1982) showed that the prothoracic gland of *Samia cynthia* possesses both a photoreceptor and an endogenous clock; it seems likely that a cryptochrome is the photoreceptor in this system.

The actual cellular molecular clock has been studied extensively in *Drosophila* where the central oscillator is formed by a complex feedback loop involving the transcription, translation, and

phosphorylation of at least seven genes: *period*, *timeless*, *clock*, *cycle*, *doubletime*, *vriille*, and *shaggy* (Stanewsky, 2002, 2003), and possibly *cryptochrome*, which appears to function as part of the “central oscillator” in some peripheral tissues (Krishnan *et al.*, 2001). The presence of cryptochrome in the prothoracic glands has not been determined but the *period* and *timeless* proteins have been immunologically detected in the *Rhodnius* gland (Terry and Steel, cited in Steel and Vafapoulou, 2002) and in the prothoracic gland portion of the *Drosophila* ring gland (Myers *et al.*, 2003). At least some of the proteins involved in the *Drosophila* central oscillator shuttle between the nucleus and the cytoplasm, and cycles of abundance of both protein and mRNA are characteristic. For a more thorough understanding of these processes, (Saunders (2002)).

The expression of the *period* protein in insect brains has been the subject of several immunohistochemical studies in insects other than *Drosophila*. In the *Rhodnius* brain, a small group of neurons adjacent to the prothoracicotropes (one per hemisphere) were immunopositive for the *period* and *timeless* proteins (Terry and Steel, cited in Steel and Vafapoulou, 2002). Sauman and Reppert (1996a) found that two pairs of dorsolateral neurons, per hemisphere, were immunopositive for *period* in the brain of the moth *A. pernyi*, and that the cell bodies were in contact with the two pairs of dorsolateral neurosecretory cells that were immunopositive for PTTH. Interestingly, the *period* positive cells sent their axons to the corpus cardiacum whereas PTTH-producing neurons course contralaterally to terminate in the corpus allatum. Thus, if the PTTH- and *period*-producing cells communicate with one another it is likely to be at the level of the cell bodies, perhaps via tight junctions, or in the corpus cardiacum, where the “*period* cells” could transmit information to boutons on the axons of the “PTTH neurons.” Interestingly, only cytoplasmic and axonal *period* staining was observed in the *Antheraea* brain (Sauman and Reppert, 1996b); the expected nuclear presence was not seen, although temporal cycling of protein and mRNA was observed. Závodská *et al.* (2003) surveyed the cephalic nervous systems of 14 species of insects, representing six orders, using immunohistochemical methods and antibodies against *period*, PTTH, and pigment-dispersing hormone (PDH, also known as pigment-dispersing factor), as well as eclosion hormone. PDH-expressing neurons are also important in circadian processes, although their role appears to be information transfer between cells rather than direct participation in the intracellular molecular

clock oscillator (Stengl and Homberg, 1994; Helfrich-Förster *et al.*, 2000). A detailed recapitulation of the data obtained in this study is beyond the purposes of this review but a few results are worth noting. First, *period* was widely distributed, with most species showing antibody reactivity in cells of the optic lobe, pars intercerebralis, and the dorsolateral protocerebrum. Second, *period* staining was limited to the cytoplasm. Third, PDH exhibited the most consistent immunoreactivity, being found in the optic lobes of all 14 species. Finally, none of the cells immunopositive for any one of the four antigens was also immunopositive for any of the other three antigens. Thus, to the extent that these four proteins play a role in circadian cycles, the data suggest that such cycles are controlled, at least within the brain, by a nexus of cells rather than a discrete nucleus.

Obviously, we are currently still in the early stages of understanding the molecular and physiological mechanisms that control cyclical PTTH release. The studies reviewed here, as well as others, suggest that there are several ways in which the PTTH-prothoracic gland axis may entrain rhythms in steroid levels. It is not yet possible to say whether these varied circadian systems sort out along taxonomic, or ecological, or other grounds. Furthermore, we know essentially nothing about the removal or degradation of PTTH from the insect circulation. Changes in the PTTH hemolymph titer must be regulated not only by synthesis and release, but also by processes of sequestration and/or proteolysis. Whether or not there are PTTH-specific uptake, elimination, or proteolytic pathways is totally unknown, but it seems unlikely that the prothoracic gland will be the only tissue found to be involved in these processes. More work on these problems is clearly needed, involving not only “modern” molecular genetics, but “old-fashioned” biochemistry and physiology as well.

6.3.6. PTTH and Diapause

All organisms face variations in their environment, and even in the most equable habitats, confront periods during which they must alter their behavior and/or their physiology to cope with stressful conditions. When such nonfavorable circumstances are neither too severe nor prolonged, behavioral changes or simple homeostatic mechanisms are generally evoked. When these changes are harsher, such as nonseasonal drought or the protracted cold of temperate zone winters, then more sweeping actions are taken. At the physiological level, insects and many other arthropods deal with unpredictable

environmental challenges by becoming quiescent. Simple quiescence involves rapid and temporary cessation of activity and the animal quickly resumes its activity when the hostile conditions cease. When the adverse conditions are relatively predictable, e.g., winter or seasonal drought, then the solution of most insects is to enter diapause. Diapause is a type of dormancy that involves perception of one or more predictable environmental cues that anticipate and predict regularly occurring, unfavorable conditions. Animals in diapause express physiological mechanisms that greatly increase their ability to resist environmental challenges that range from desiccation to freeze-damage resistance. The cues that trigger diapause are not necessarily the conditions that the behavior has evolved to escape or moderate, e.g., organisms that enter a winter diapause state may be stimulated to do so by shorter day length rather than by falling temperatures. Diapause involves a genetically determined response that is controlled by the neuroendocrine system. Diapause is obligate or facultative, and undergone by embryos, larvae, pupae, or adults, depending on the species. For broader views of diapause than presented here, see Tauber *et al.* (1986); Danks (1987).

The PTTH-ecdysteroid axis has been implicated in the control of both larval and pupal diapause. *Manduca* larvae molt into diapausing pupae after being raised under a short-day (e.g., 12 h) photoperiod. Bowen *et al.* (1984a) found that such animals exhibit a much reduced hemolymph ecdysteroid titer relative to developing pupae. Low ecdysteroid titers have been documented in other Lepidoptera and other taxa that undergo a pupal diapause, e.g., the moths *H. cecropia* (McDaniel, 1979) and *Heliothis virescens* (Loeb, 1982), and the flesh flies *Sarcophaga crassipalpis* (Walker and Denlinger, 1980) and *S. argyrostoma* (Richard *et al.*, 1987). Bowen *et al.* (1984a) showed that two factors resulted in the low levels of circulating ecdysteroids during diapause. First, diapause prothoracic glands were found to be refractory to PTTH stimulation when tested *in vitro*. Smith *et al.* (1986a) found that the refractory state in diapausing pupae could be simulated in nondiapausing pupae by removal of the brain, indicating that the presence of a brain-derived factor is necessary to retain prothoracic gland responsiveness. PTTH is, of course, a prime candidate to function in such a trophic context (see Section 6.3.6) but a role for other hormones cannot be ruled out. Smith *et al.* (1986a) also determined that the refractory “blockade” in the *Manduca* prothoracic gland is downstream from the PTTH receptor, Ca^{2+} -influx, and cAMP generation (see Section 6.3.4 for a discussion of the PTTH transduction

cascade). The use of a calcium ionophore and a cell-permeable cAMP analog both failed to stimulate ecdysteroid synthesis in the diapause glands, although these agents are as effective as PTH when applied to nondiapause glands. However, PTH stimulated cAMP accumulation within diapause glands, in fact to levels higher than seen under nondiapause conditions. This observation indicates that there is not also a block upstream from cAMP generation, such as a downregulation in the numbers of PTH receptors or adenylate cyclase molecules. Similar data, indicating a signal transduction lesion downstream from cAMP generation, were obtained by Richard and Saunders (1987), studying diapause in the flesh fly *Calliphora vicina*. Basal protein kinase A activity in *Manduca* day 10 diapause glands was $\approx 20\%$ lower than that seen in nondiapause day 0 pupal glands, but both pupal and diapause protein kinase activity approximately doubled in response to dibutyl cAMP (Smith *et al.*, 1987a). Thus, lowered protein kinase A activity is likely contributory to the refractory nature of diapause prothoracic glands but other factors are undoubtedly involved.

Bowen *et al.* (1984a) suggest that the second reason for low ecdysteroid levels in diapause pupae is the absence of PTH. These workers found that, in *Manduca*, during the first 20 days of diapause, the content of PTH in the brain or in the brain-retrocerebral complex did not differ significantly from that in nondiapausing, developing pupae. Bowen and colleagues construed these data to indicate that PTH release was greatly curtailed during diapause but the interpretation of this result is, unfortunately, not straightforward since it is possible that PTH catabolism in the hemolymph was increased; note that the hemolymph PTH titer was not measured in this case. In fact, Bowen *et al.* (1986) studied the release of PTH *in vitro* from *Manduca* brain-retrocerebral complexes and found no difference between complexes from nondiapause and diapause pupae. PTH release *in vitro* from diapause complexes could have been an artifact stemming from dissection or incubation conditions, or might indicate a release from an *in vivo* inhibitory factor that is lost or inhibited itself under the *in vitro* regimen. If PTH release is indeed inhibited *in vivo* in diapausing pupae, then data from nematodes may provide a clue to one step in its control, i.e., Tissenbaum *et al.* (2000) found that the diapause-like dauer state of *C. elegans* and *Ancylostoma caninum* was broken rapidly by muscarinic agents. Furthermore, dauer recovery was inhibited by atropine, a muscarinic antagonist. This finding is of note because both Agui (1989) and Shirai *et al.* (1994)

showed that cholinergic agents caused the release *in vitro* of PTH from lepidopteran larval complexes. A dopaminergic pathway may also play a role in diapause, but in contrast to the muscarinic system, dopamine may be involved in inducing or maintaining diapause. Some insects that undergo pupal diapause may exhibit a more complex control of the PTH-prothoracic gland axis. Williams (1967) was not able to detect PTH activity in brains from diapausing *H. cecropia* or *Samia cynthia*, in contrast to *A. pernyi*. These data suggest that the stronger diapause in *Hyalophora* and *Samia* is characterized by a period of very low PTH levels in the brain; whether or not the prothoracic glands are refractory to stimulation at these times was not investigated.

Recently, Xu and Denlinger (2003) studied the levels of PTH mRNA in brains of *H. virescens* nondiapause and prediapause larvae, and nondiapause and diapause pupae. They found that mRNA levels in nondiapause animals remained relatively constant through the fifth instar until about day 10 of pupal-adult development. However, in prediapause larvae, PTH mRNA levels dropped at the onset of wandering and, in diapause pupae, the mRNA levels remained low through at least the first 10 days of diapause. These results may reveal the mechanism behind the low PTH levels seen in diapausing *H. cecropia* or *S. cynthia* pupae, as reported by Williams (1967).

Data from *Manduca* indicate that the PTH-producing neurosecretory cells undergo morphological and electrophysiological changes during diapause. Hartfelder *et al.* (1994) found no significant differences in cellular ultrastructure between developing pupae and early diapause pupae. However, as diapause progressed, a number of cellular structures exhibited changes, e.g., neurosecretory granules were concentrated into large clusters that were separated by well-organized rough endoplasmic reticulum, notably fewer Golgi complexes were seen, and interdigitations with glial cells were decreased. When diapause was terminated by exposure to warm conditions (26 °C versus 4 °C), the PTH cells reverted to prediapause morphology within 3 days. Progressive differences in electrophysiological properties between diapausing and developing pupae were found by Tomioka *et al.* (1995), who noted that the diapausing PTH cells gradually became less excitable. This relative refractoriness was caused by a rise in the threshold value for action potential generation and a decrease in the input resistance.

The endocrine control of larval diapause appears to be more varied than pupal diapause. In

the European corn borer, *O. nubilalis*, larval diapause is similar to *Manduca*. Hemolymph ecdysteroid titers are significantly lower in prediapause larvae than in developing animals (Bean and Beck, 1983) and brain PTTH levels were the same in diapause and nondiapause animals, for at least the first 4 weeks of diapause (Gelman *et al.*, 1992). Gelman and colleagues also found that diapause prothoracic glands were highly refractory to PTTH stimulation, as seen in *Manduca*. It must be pointed out that although these results are very reminiscent of the *Manduca* findings, the only PTTH activity detected in diapause brains of *Ostrinia* had an apparent MW of ≤ 5 kDa (Gelman *et al.*, 1992). In contrast, the south-western corn borer, *Diatraea grandiosella*, which undergoes up to three stationary molts during diapause, appears to maintain an active PTTH-prothoracic gland axis during larval diapause; its diapause is controlled by high JH levels (Chippendale and Yin, 1973; Yin and Chippendale, 1973; Chippendale, 1984). High JH levels are also responsible for provoking and sustaining larval diapause in the rice-stem borer, *Chilo suppressalis* (Yagi and Fukaya, 1974); the exact state of the PTTH-prothoracic gland axis during larval life in this species appears to be unknown.

In larval and pupal diapause that are characterized by low ecdysteroid levels, the available evidence suggests that the relative quiescence of the prothoracic glands reflects decreased PTTH release and stimulation. This in turn suggests that the photoperiodic clock involved in the control of PTTH in short-day versus long-day developmental programs might reside in the brain, where PTTH is synthesized. This hypothesis was elegantly addressed with experiments that involved *in vitro* reprogramming of larval brains with altered light cycles (Bowen *et al.*, 1984b). In brief, brains from short-day larvae were exposed to a long-day light treatment for 3 days *in vitro* and then implanted into short-day larvae destined to enter diapause. Approximately half of these host larvae did not enter diapause, i.e., the implanted brains the diapause program. If the transplanted brains received a short-day light treatment *in vitro* prior to implantation, then very few host larvae failed to enter diapause. The composite data showed that the three main regulators of pupal diapause in *Manduca* reside in the brain: photoreceptor, clock, and hormonal effector. The main hormonal actor in this developmental program is clearly PTTH, but other factors might well be involved (see Chapter 9). For instance, brains in animals destined to diapause might secrete an ecdysiostatic factor to downregu-

late prothoracic gland activity in addition to curtailing PTTH release after pupal ecdysis (see Section 6.5.2).

The literature on diapause in insects is very large and only a very small fraction of it has been reviewed here. Given the long evolutionary history of insects, it is likely that there are additional variations in the ways in which environmental cues, receptors, PTTH, and the prothoracic glands interact to induce or modulate diapause.

6.4. PTTH: Signal Transduction in the Prothoracic Gland

Hormones are intercellular messengers that alter the behavior of the target cells. PTTH has only one known target tissue, the prothoracic gland (the possibility that PTTH affects other tissues is discussed in Section 6.3.6), and the ultimate response of the gland to PTTH is to increase ecdysteroid production. This section considers the transduction changes in the prothoracic gland elicited by PTTH, and how these changes connect to increased ecdysteroidogenesis. The reader is referred to for a discussion of the actual synthetic pathway leading from sterols to ecdysteroids. The present discussion has been organized to present the biochemical events of PTTH signal transduction in a sequential or temporal order, so far as we currently understand them. Of course, as events proceed from the initial contact of PTTH with the prothoracic gland and the PTTH receptor, it becomes increasingly likely that multiple PTTH-dependent phenomena occur simultaneously; indeed, it would be a mistake to conceive of any signal transduction cascade as a linear, vectorial process, rather than a complex nexus of interconnected pathways. By using an “outside-in” cellular approach to this topic, a broad chronological view will not be presented, but within each of the subtopics, some history of the field will be afforded. An overview of the PTTH signal transduction pathway, as discussed below, is provided in Figure 7.

6.4.1. The PTTH Receptor and Associated Proteins

A PTTH receptor has not yet been purified or cloned, so the present section is speculation based on data primarily from *Manduca* and *Bombyx*, and using information gleaned from other characterized peptide receptor systems. PTTH is a circulating, hydrophilic protein and can be expected to bind to a receptor that exposes an extracellular binding site and traverses the cell membrane. PTTH binding to

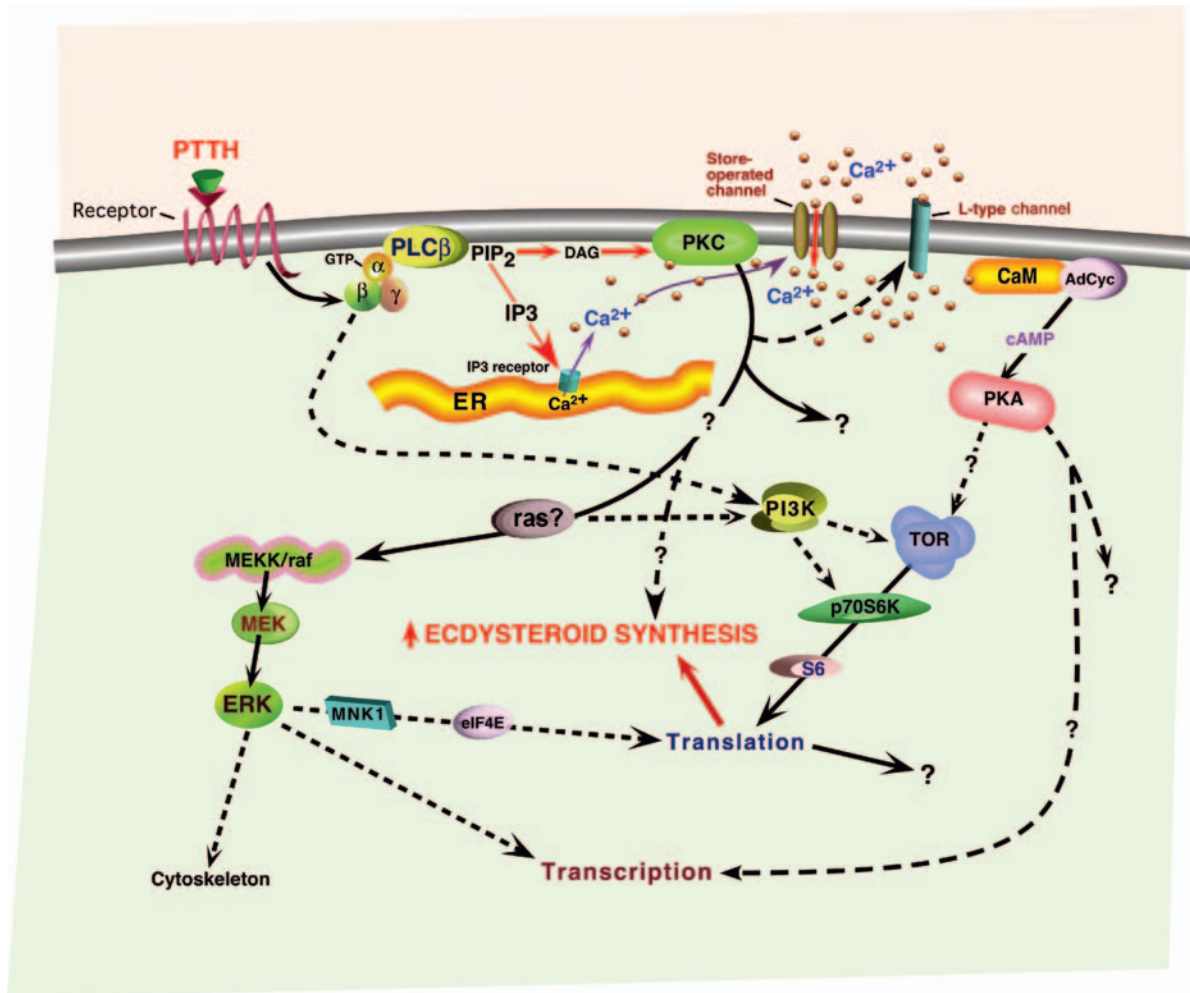


Figure 7 The PTTH signal transduction cascade in prothoracic gland cells. Solid lines indicate demonstrated or highly likely interactions while dashed lines indicate hypothetical relationships. Abbreviations: PTTH, prothoracicotropic hormone; PLC β , phospholipase C β ; PIP $_2$, phosphatidylinositol-4,5-bisphosphate; DAG, diacylglycerol; IP $_3$, inositol triphosphate; GDP, guanosine diphosphate; GTP, guanosine triphosphate; cAMP, cyclic adenosine monophosphate; CaM, calmodulin; AdCyc, adenylate cyclase; ER, endoplasmic reticulum; PKA, protein kinase A; PI3K, phosphoinositide 3OH-dependent kinase; TOR, target of rapamycin; MEKK, MEK kinase; MEK, MAPK/ERK kinase; ERK, extracellular signal-regulated kinase; S6, ribosomal protein S6; p70S6K, 70-kDa S6 kinase; MNK 1, MAP kinase-interacting kinase 1; ras, a small GTP binding protein; raf, raf serine-threonine kinase; and eIF-4E, eukaryotic translation initiation factor 4E.

the receptor is likely a high affinity interaction, with a K_D in the very low nanomolar or high picomolar range. Such an affinity is typical for peptide receptors and is also suggested by the findings that *Manduca* recombinant PTTH at ≈ 0.300 nM was sufficient to maximally stimulate ecdysteroidogenesis *in vitro* (Gilbert *et al.*, 2000). More limited data from *Bombyx* also indicated that a similarly low concentration (≈ 1.7 nM) of *Bombyx* recombinant PTTH was sufficient to elicit maximal ecdysteroidogenesis *in vitro* (Dedos *et al.*, 1999). Eukaryotic membrane receptors fall into several broad classes and it seem most likely that the PTTH receptor belongs to the G-protein coupled receptor (GPCR)

superfamily (for an insect-oriented review, see Chapter 14). This is a very large group of proteins that bind a diverse array of ligands. GPCRs span the membrane seven times, with connecting intracellular and extracellular loops. The ligand-binding site is located in an extracellular region. Intracellular areas of the GPCRs interact with the heterotrimeric G-proteins, which are the first-level effectors of an activated ligand-bound GPCR. The direct and indirect targets of activated G-proteins, released from a GPCR by ligand-occupancy, are many and include adenyl cyclase (see Pieroni *et al.*, 1993), which generates the second messenger cAMP from ATP. PTTH evokes a rapid generation of cAMP in

prothoracic gland cells (see Section 6.4.2) and, currently, it appears that the generation of cAMP in response to an extracellular ligand almost always involves a GPCR. GPCRs range from <400 to \approx 1000 amino acids in size, are glycosylated, and occur as hetero- or homooligomers (see Gazi *et al.*, 2002).

The heterotrimeric G-proteins (α , β , and γ) that characterize GPCRs are a genetically diverse group. In both mammals and insects, a considerable number of $G\alpha$, $G\beta$, and $G\gamma$ proteins are expressed and the specific subunits that associate with a given GPCR determine to a large degree the particular signaling pathways activated by that GPCR. For example, $G\alpha_s$ stimulates adenylate cyclase activity and $G\alpha_q$ activates phospholipase C (see Offermanns, 2003) and some $G\beta\gamma$ subunits can also activate phospholipase C (see Boyer *et al.*, 1994; Morris and Scarlata, 1997). The presence of G proteins in *Manduca* prothoracic glands was investigated by Meller and Gilbert (1990) using Western blotting and cross-linking techniques with antibodies against mammalian $G\alpha$ and $G\beta$ proteins. Their results suggested that several $G\alpha$ and $G\beta$ proteins are expressed in the glands and that they likely occur, as expected, in multimeric complexes. Meller and Gilbert also used an antibody against $G\alpha_s$ in an immunoprecipitation experiment and found that $G\alpha_s$ in the prothoracic gland was associated with adenylate cyclase. This observation is congruent with the proposal that the PTTH receptor is a GPCR but, obviously, the association of the G protein–cyclase complex with another receptor cannot be ruled out. Girgenrath and Smith (1996) studied the effects of pertussis and cholera toxins on PTTH-stimulated ecdysteroid synthesis in *Manduca* prothoracic glands; these toxins inhibit and stimulate, respectively, the activity of some G proteins. Pertussis toxin had no effect on PTTH-stimulated ecdysteroid synthesis, but cholera toxin stimulated ecdysteroid synthesis. Cholera toxin also stimulated ecdysteroid synthesis by *Bombyx* prothoracic glands (Dedos and Fugo, 1999c). These observations suggested that PTTH acts through a $G\alpha_s$ protein. Pertussis toxin also had no effect on mastoparan- or PTTH-stimulated Ca^{2+} influx in *Manduca* prothoracic glands (Birkenbeil, 2000).

Several additional pharmacological agents that are known to have effects on GPCR–G protein interactions have been used to investigate further the role of G proteins in PTTH signaling. Mastoparan, a wasp venom that activates G proteins among its other effects caused a dose-dependent increase in intracellular Ca^{2+} (Birkenbeil, 1999), but a dose-dependent decrease in ecdysteroid production in

Manduca prothoracic gland cells (Birkenbeil, 2000). Injection of GDP- β -S, an inhibitor of G protein activation, failed to block PTTH-stimulated Ca^{2+} influx; PTTH-stimulated ecdysteroid synthesis was not measured in this context (Birkenbeil, 2000). In contrast, the compound suramin, which uncouples G proteins from GPCRs among other effects, blocked PTTH-stimulated Ca^{2+} influx in *Bombyx* (Birkenbeil, 2000) while in *Manduca*, suramin inhibited PTTH-stimulated ecdysteroid synthesis (Rybczynski and Gilbert, unpublished data). The collective data from these laboratories suggest that the PTTH is coupled to a G protein and that the prothoracic gland expresses a number of $G\alpha$ proteins, at least one of which is not coupled to the PTTH receptor. The supposition that prothoracic glands express multiple $G\alpha$ proteins is in accord with the immunoblotting results of Meller and Gilbert (1990) discussed above and it is possible that PTTH receptors couple to more than one type of G protein.

If the PTTH receptor is a GPCR, then a number of proteins, besides the G proteins, likely associate with the receptor, at least at some times. These include proteins that are involved in receptor desensitization, such as a PTTH receptor-specific kinase, second messenger kinases (like cAMP-dependent protein kinase A), regulators of G protein signaling (RGS proteins) and arrestins (which bind phosphorylated receptors). If the PTTH receptor undergoes agonist-induced internalization, then proteins comprising the relevant endocytic pathway, e.g., clathrin, and subsequent degradative or recycling pathways would be periodically associated with the receptor. Desensitization and receptor cycling are complex topics. Another fascinating group of GPCR-interacting proteins is the RAMP (receptor activity modifying proteins), a family of proteins identified in vertebrates (see Morfis *et al.*, 2003). RAMPs contain a single transmembrane domain, heterodimerize with GPCRs, and the specific RAMP in the heterodimer can determine the ligand recognized by the receptor complex. Developmentally specific RAMP associations with a PTTH receptor could explain the puzzling decrease in the ability of small PTTH to stimulate *Manduca* pupal glands (see Section 6.2.5). Naturally, such an interaction is purely hypothetical at this point, given that a search of the *Drosophila* genome database did not reveal any obvious RAMP homologs (unpublished data).

Further speculation on the PTTH receptor and associated proteins is premature at this time and the field will only progress once an authentic receptor has been isolated and characterized. The

field of membrane receptors is a vast one and for detailed discussions on GPCRs, the interested reader should see reviews by Böhm *et al.* (1997); Vázquez-Prado *et al.* (2003), and Chapter 14, among many others.

6.4.2. Second Messengers: Ca²⁺, cAMP, and Inositols

The activation of GPCRs results in a transient increase of second messenger molecules within the cell. These small molecules carry and expand the intracellular “reach” of the receptor ligand, the first messenger, by binding to numerous target molecules. Ca²⁺ is perhaps the most common of second messengers. The concentration of free Ca²⁺ in the cytoplasm is well buffered and transient increases alter the behavior of Ca²⁺-binding proteins. Increases in Ca²⁺ can originate from the extracellular environment as well as from internal stores, such as those in the endoplasmic reticulum (see Berridge *et al.*, 2000). Smith *et al.* (1985) found that extracellular Ca²⁺ was crucial for PTTH-stimulated ecdysteroid synthesis in *Manduca* prothoracic glands and that Ca²⁺ influx was a very early event in the PTTH transduction pathway, i.e., cAMP analogs that stimulate ecdysteroid synthesis are still effective in the absence of extracellular Ca²⁺, indicating that Ca²⁺ influx precedes cAMP synthesis (see below). Smith *et al.* (1985) reported also that basal ecdysteroidogenesis was insensitive to the presence or absence of external Ca²⁺, as also later noted by Gu *et al.* (1998) for *Bombyx* prothoracic glands, and Henrich (1995) for *Drosophila* ring glands. In contrast, Meller *et al.* (1990) found that basal ecdysteroidogenesis in *Manduca* glands was decreased, although not absent, in Ca²⁺-free medium, and varied with stage of development; Dedos and Fugo (1999b) uncovered a similar, developmentally specific Ca²⁺-dependency for basal ecdysteroid synthesis in *Bombyx* glands. Thus, whether or not basal ecdysteroidogenesis is responsive to short-term changes in external Ca²⁺ is not certain; differences in experimental technique might well determine a gland's sensitivity in this regard.

External Ca²⁺ has been shown to be important for PTTH-stimulated ecdysteroidogenesis in at least two other insect species. PTTH-stimulated ecdysteroid synthesis by *Bombyx* prothoracic glands also required the presence of Ca²⁺ in the external medium (Gu *et al.*, 1998) and PTTH-containing neural extracts failed to stimulate ecdysteroid synthesis by *Drosophila* ring glands in the absence of external Ca²⁺ (Henrich, 1995). The importance of Ca²⁺ influx to PTTH-stimulated ecdysteroid synthesis was further demonstrated by the ability of the Ca²⁺

ionophore A23187 to stimulate ecdysteroidogenesis in *Manduca* (Smith and Gilbert, 1986) and *Bombyx* (Gu *et al.*, 1998) prothoracic glands.

Several studies using microfluorometric Ca²⁺ measurements explicitly verified the assumption that PTTH causes increases in prothoracic gland cytoplasmic free Ca²⁺ ([Ca²⁺]_i) and that the external environment was the primary source of this Ca²⁺. Birkenbeil (1996) showed that a PTTH preparation caused a rise in [Ca²⁺]_i in prothoracic glands of the wax moth, *G. mellonella*, while Birkenbeil and Dedos (2002) confirmed the interpretation that PTTH caused Ca²⁺ influx in *Bombyx* glands. In *Manduca*, Birkenbeil (1999, 2000) showed that PTTH preparations stimulated Ca²⁺ influx, and this work has been extended with pure *Manduca* recombinant PTTH (Fellner, Rybczynski, and Gilbert, unpublished data).

The modes by which free Ca²⁺ enters the cytoplasm of prothoracic glands in response to PTTH stimulation has been addressed chiefly in *Manduca*, *Galleria*, and *Bombyx*. In *Manduca* and *Bombyx*, cationic lanthanum inhibited PTTH-stimulated ecdysteroid synthesis (Smith *et al.*, 1985; Birkenbeil and Dedos, 2002); lanthanum is a nonspecific blocker of many plasma membrane ion channels. Identification of the precise types of Ca²⁺ channels that function in PTTH signaling has proven difficult. The Ca²⁺ channel agonist Bay K8644, which activates voltage-gated Ca²⁺ channels, stimulated ecdysteroid synthesis in both *Manduca* and *Bombyx* prothoracic glands (Smith *et al.*, 1987b; Dedos and Fugo, 1999b) but it is not known how this compound interacts with PTTH-stimulated Ca²⁺ influx, i.e., is it additive, synergistic, or antagonistic? In *Manduca*, the L-type channel blocker nitrendipine partially inhibited PTTH-stimulated ecdysteroid synthesis by prothoracic glands (Smith *et al.*, 1987; Girgenrath and Smith, 1996). However, nitrendipine was not found to inhibit PTTH-stimulated Ca²⁺ influx by Birkenbeil (1996, 1999). To confuse the issue more, an inhibitory effect of nifedipine, another L-type channel blocker, was demonstrated by Fellner *et al.* (unpublished data) while Birkenbeil (1999) did not observe any effect of nifedipine. Birkenbeil did note an inhibitory effect of amiloride, a T-type Ca²⁺ channel blocker, on PTTH-stimulated Ca²⁺ influx, but, at the concentration used, amiloride affects other proteins in addition to the T-type Ca²⁺ channels. In *Bombyx*, similarly conflicting data have been obtained. The L-type channel inhibitors verapamil (Gu *et al.*, 1998) and nitrendipine (Dedos and Fugo, 2001) both inhibited PTTH-stimulated ecdysteroidogenesis in *Bombyx* prothoracic glands but nitrendipine had no apparent effect

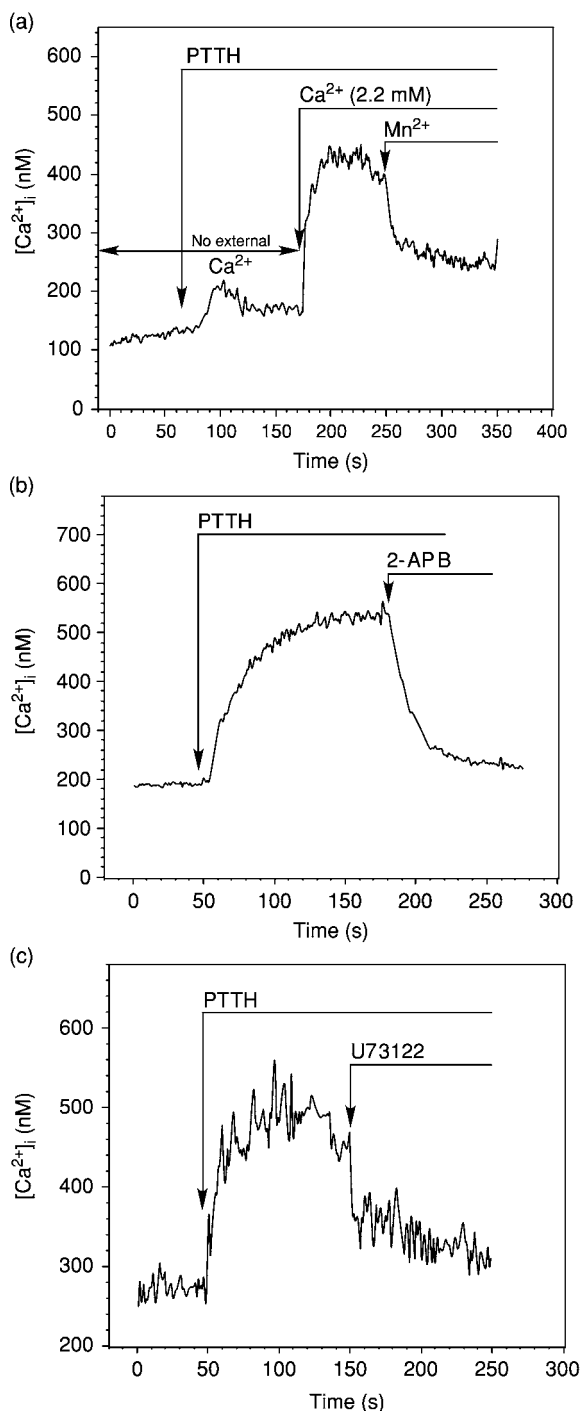


Figure 8 Free Ca^{2+} levels in prothoracic gland cells of *Manduca sexta* larvae, measured by fura-2 fluorescence. Data shown are representative plots from single cells. (a) The effect of PTTH on free cellular Ca^{2+} levels in the absence of external Ca^{2+} , and following the addition of Ca^{2+} to the incubation medium. The drop in free cellular Ca^{2+} levels following the addition of Mn^{2+} to the external medium is due to the entry of Mn^{2+} through store-operated channels. Mn^{2+} -dependent quenching of the Ca^{2+} fura-2 fluorescence indicates that the observed fluorescence results from Ca^{2+} binding to the fura, and not the simple accumulation of the dye within the cell. (b) 2-APB, an inhibitor of Ca^{2+} release from IP_3 gated stores on free cellular Ca^{2+} levels,

on PTTH-stimulated cytoplasmic free Ca^{2+} (Birkenbeil and Dedos, 2002). The contribution of T-type Ca^{2+} channels to *Bombyx* PTTH signaling is not clear; two inhibitors of this channel class, bepridil and mibefradil, had no effect on PTTH-stimulated $[Ca^{2+}]_i$ changes, while the less specific amiloride inhibited $[Ca^{2+}]_i$ increases. These perplexing results may reflect methodological differences among the studies. It also seems likely that the identification of an insect channel type based purely on pharmacological reagents characterized in vertebrates may be risky (Girgenrath and Smith, 1996; Rybczynski and Gilbert, 2003).

Fellner *et al.* (unpublished data) investigated the kinetics of PTTH-stimulated increases in $[Ca^{2+}]_i$ in *Manduca* prothoracic glands and found that PTTH caused a rapid increase in $[Ca^{2+}]_i$ with levels plateauing within ≈ 30 s to 1 min. Furthermore, when prothoracic glands were incubated in the absence of external Ca^{2+} a small, transient $[Ca^{2+}]_i$ peak was seen followed by a large peak if Ca^{2+} is added to the external medium (see Figure 8a). These results suggested that PTTH first causes a release of Ca^{2+} from internal stores followed by a larger influx of external Ca^{2+} , with the large influx of Ca^{2+} being due substantially to the opening of store-operated channels. Store-operated channels are Ca^{2+} channels that respond to increases in internal Ca^{2+} (see Clementi and Meldolesi, 1996). The compound 2-aminoethoxydiphenyl borate (APB), an inhibitor of Ca^{2+} release from IP_3 -sensitive stores in the endoplasmic reticulum, inhibited the PTTH-stimulated increase of $[Ca^{2+}]_i$, indicating that PTTH acts through the IP_3 receptor (Figure 8b) TMB-8, a second inhibitor of the release of Ca^{2+} from internal stores, also greatly diminished the PTTH-stimulated increase of $[Ca^{2+}]_i$, thus confirming the early participation of internal Ca^{2+} stores in the PTTH signal transduction pathway. It must be noted that Birkenbeil (1996, 1999) did not observe TMB-8 inhibition of PTTH-stimulated Ca^{2+} influx in *Galleria* or *Manduca* prothoracic glands. The reason for this is unknown. The composite data indicate that PTTH-stimulated increases in $[Ca^{2+}]_i$ originated from two Ca^{2+} pools: (1) an internal, IP_3 -controlled endoplasmic reticulum store and (2) the extracellular milieu. The participation of an IP_3 -controlled endoplasmic reticulum store in the early events of PTTH-stimulated Ca^{2+} mobilization was further

truncates the PTTH-stimulated rise in free cellular Ca^{2+} . (c) U73122, an inhibitor of $PLC\beta$, ablates the PTTH-stimulated rise in the concentration of free cellular Ca^{2+} . (Data from Fellner, Rybczynski, and Gilbert, unpublished data.)

confirmed by the ability of a phospholipase C inhibitor (U73122) to block or interrupt $[Ca^{2+}]_i$ increases (Figure 8c); phospholipase C activity is required to generate IP_3 from its PIP_2 precursor.

Entry from the extracellular pool proceeds through at least two types of channels. One is a store-operated channel and the second is a voltage-gated channel, probably of the L-type. The opening of the voltage-gated channel may result from changes in transmembrane potential caused by operation of the store-operated channels, but other factors cannot be ruled out. For example, *Drosophila* dihydropyridine-sensitive Ca^{2+} channel activity can be modulated by either PLC or protein kinase A signaling pathways (Gu and Singh, 1997).

These results with *Manduca*, including sensitivity to TMB-8, were largely congruent with those reached in a similar study utilizing *Bombyx* prothoracic glands (Birkenbeil and Dedos, 2002), although at least two differences existed. First, no small increase in $[Ca^{2+}]_i$ was actually noted when *Bombyx* glands were stimulated with PTTH under Ca^{2+} -free conditions; however, the time resolution of the *Bombyx* study was too coarse to detect a transient peak such as found in *Manduca*. Second, the rapidity with which PTTH-stimulated $[Ca^{2+}]_i$ reached maximal levels was considerably slower and more variable in *Bombyx* (from ≈ 2 to >10 min) than in *Manduca* (≈ 20 s to 1 min). The drawn-out nature of the *Bombyx* $[Ca^{2+}]_i$ response is surprising; when $[Ca^{2+}]_i$ increases are a primary response to receptor activation, the kinetics of $[Ca^{2+}]_i$ increases are generally rapid and temporally stereotyped. At present it is unclear whether the differences in $[Ca^{2+}]_i$ kinetics are consequences of variant research protocols or represent biological differences between the species, e.g., the ability of a reagent to pass through the basal lamina surrounding the prothoracic gland might vary between *Manduca* and *Bombyx*. Regardless of the differences, the *Bombyx* data are consistent with the interpretation that PTTH causes release of Ca^{2+} from internal pools, followed by the opening of store-operated channels and voltage-gated channels.

The existence of a PTTH-stimulated, small $[Ca^{2+}]_i$ peak in *Manduca*, and presumably in *Bombyx*, sensitive to APB and TMB-8 inhibition, suggested that inositol metabolism played a role in PTTH-stimulated signal transduction, i.e., the generation of IP_3 by phospholipase C (PLC) and the subsequent release of IP_3 -sensitive endoplasmic reticulum Ca^{2+} stores. Support for this hypothesis came from both *Bombyx* and *Manduca*. In the silkworm, the phospholipase C inhibitor, 2-nitro-4-carboxyphenyl-N, N-diphenylcarbamate, blocked PTTH-stimulated

ecdysteroidogenesis but had no effect on basal synthesis. In *Manduca*, the phospholipase C inhibitor U73122, likewise blocked PTTH-stimulated ecdysteroidogenesis and was without effect on basal production (Rybczynski *et al.*, unpublished data). Smith and Gilbert (1989) postulated the participation of phospholipase C in PTTH-stimulated ecdysteroidogenesis but attempts to detect PTTH-stimulated increases in PIP_2 metabolites like IP_3 have not been successful (Girgenrath and Smith, 1996; Dedos and Fugo, 2001). The failure to detect IP_3 might reflect a difficult-to-detect, small and transient IP_3 peak, if the APB-sensitive change in $[Ca^{2+}]_i$ is any guide to IP_3 behavior in the prothoracic gland. Clearly, in light of the results obtained with phospholipase C inhibitors, the question of PTTH-stimulated IP_3 generation should be re-addressed. Indirect evidence supporting the participation of the IP_3 pathway in ecdysteroidogenesis have also resulted from the study of *Drosophila* carrying mutations in the IP_3 receptor gene (Venkatesh and Hasan, 1997; Venkatesh *et al.*, 2001). Mutations in this gene caused molting delays and larval lethality that could be partially by feeding larvae food that contained 20-hydroxyecdysone.

PTTH may stimulate changes in $[Ca^{2+}]_i$ and in Ca^{2+} -dependent events indirectly, as well as directly through receptor interactions. Electrophysiological studies using *Manduca* prothoracic glands *in vitro* revealed that Ca^{2+} -dependent action potentials could spread from cell to cell (Eusebio and Moody, 1986). PTTH and signals from nerves (see Section 6.5.2) are candidate triggers for such action potentials, but whether or not this occurs *in vivo* is unknown. Gap junctions and intercellular bridges have been found in *Manduca* prothoracic glands and these structures may allow the spread of signaling molecules, like cAMP, among cells (Dai *et al.*, 1994). Thus, Ca^{2+} -dependent action potentials, gap junctions, and intercellular bridges all could serve to synchronize the behavior of the prothoracic gland cells in response to PTTH or other hormones. Measurements of resting $[Ca^{2+}]_i$ in *Manduca* cells indicated that cells of the prothoracic gland are indeed heterogeneous (Fellner, Rybczynski, and Gilbert, unpublished data), but, as yet, the significance of cell-to-cell differences is unknown. If cells are heterogeneous *in vivo* in their sensitivity to PTTH or other signals, signal-spreading among cells could serve to make the whole prothoracic gland respond at signal levels set by the most responsive cells. Note that a synchronization of cellular responses could downregulate the prothoracic gland's response to PTTH just as readily as facilitate it.

Both Ca^{2+} ions and cAMP are second messengers, although there are many cases in which the generation of cAMP is dependent upon prior intracellular changes in $[\text{Ca}^{2+}]_i$. This is the case in the prothoracic gland, where a role for cAMP in PTTH-stimulated ecdysteroid synthesis was first suggested by measurements of adenylate cyclase activity and intracellular cAMP in glands synthesizing large versus small amounts of ecdysteroids (Vedeckis and Gilbert, 1973; Vedeckis *et al.*, 1974, 1976). An explicit demonstration of the role of cAMP in ecdysteroid synthesis was obtained a decade later when *Manduca* PTTH preparations were shown to stimulate cAMP synthesis in a Ca^{2+} -dependent manner, i.e., extracellular Ca^{2+} was required for PTTH-stimulated cAMP synthesis (Smith *et al.*, 1984, 1985, 1986b). Further data supporting the upstream position of $[\text{Ca}^{2+}]_i$ relative to cAMP in the PTTH transduction cascade derive from observations that calcium ionophore mimics PTTH in eliciting both steroid and cAMP synthesis (Smith *et al.*, 1985, 1986b). In addition, the cell-permeable cAMP analog dibutyryl cAMP, as well as agents that activate adenylate cyclase and inhibit cAMP phosphodiesterase activity, all stimulated ecdysteroid synthesis in prothoracic glands incubated in Ca^{2+} -free medium (Smith *et al.*, 1985, 1986b). Note that these *Manduca* studies utilized a partially purified PTTH, but identical results were later obtained with a recombinant PTTH (Gilbert *et al.*, 2000). Similar results have been obtained from *Bombyx* studies (Gu *et al.*, 1996, 1998; Dedos and Fugo, 1999b). Furthermore, *Bombyx* prothoracic glands stimulated with PTTH under Ca^{2+} -replete conditions continued to secrete ecdysteroids at elevated levels even after transfer to a Ca^{2+} -free medium (Birkenbeil and Dedos, 2002). Thus, if sufficient cAMP has been synthesized, extracellular Ca^{2+} is apparently no longer needed, at least under short-term *in vitro* conditions; any Ca^{2+} -dependent events that occur subsequent to cAMP generation must depend on $[\text{Ca}^{2+}]_i$ for time intervals of at least 2 h, a common *in vitro* incubation period.

The importance of cAMP in PTTH-stimulated ecdysteroid synthesis has been demonstrated in several studies. In *Manduca*, multiple analyses demonstrated that cell-permeable, excitatory cAMP analogs stimulated ecdysteroid synthesis (Smith *et al.*, 1984, 1985; Smith and Gilbert, 1986; Rybczynski and Gilbert, 1994). Furthermore, an antagonist form of cAMP (Rp-cAMP) substantially inhibited PTTH-stimulated ecdysteroidogenesis (Smith *et al.*, 1996). Excitatory cAMP analogs stimulated ecdysteroid synthesis in *Bombyx* prothoracic glands as well (Gu *et al.*, 1996, 1998; Dedos and Fugo, 1999c).

Adenylate cyclase, which synthesizes cAMP from ATP, is found in multiple forms in vertebrates. The activity of these forms may be stimulated or inhibited by, or be insensitive to, G proteins or Ca^{2+} -calmodulin. In *Drosophila*, there appear to be nine isoforms of adenylate cyclase, although five of these may be restricted to the male germline (Cann *et al.*, 2000). Meller *et al.* (1988, 1990) found that the *Manduca* prothoracic gland expresses Ca^{2+} /calmodulin-dependent adenylate cyclase activity that exhibits a developmental shift in some characteristics. Either of two calmodulin antagonists inhibited nearly all cyclase activity in membrane preparations (Meller *et al.*, 1988), suggesting that the vast majority, if not entirety, of gland adenylate cyclases are calmodulin-dependent. GTP- γ -S, a poorly hydrolyzable GTP analog, stimulated cyclase activity in both larval and pupal membrane preparations; however, in larval glands, GTP- γ -S was not effective without the simultaneous presence of calmodulin. This developmental switch in the characteristics of gland cyclase activity occurred about day 5 of the fifth larval instar, just prior to the beginning of the large premolt ecdysteroid peak. The switch was also characterized by changes in cyclase sensitivity to cholera toxin and NaF, two factors that interact with G proteins (Meller *et al.*, 1990). It is not known if the change in cyclase characteristics represents a change in cyclase isoform, a posttranslational modification, or a shift in the expression of proteins that interact with cyclase. In *Bombyx* prothoracic gland membrane preparations, calmodulin, Gpp(NH)p (a GTP analog) and NaF stimulated adenylate cyclase activity, while GDB- β -S, a GDP analog, inhibited activity (Chen *et al.*, 2001). NaF and calmodulin effects on cyclase activity were additive rather than synergistic, suggesting that both G protein- and calmodulin-dependent adenylate cyclases exist in the *Bombyx* prothoracic gland. It remains to be determined which of these putatively different adenylate cyclases is coupled to the PTTH signal transduction cascade. However, both *Bombyx* and *Manduca* prothoracic glands respond to cholera toxin with increased ecdysteroidogenesis (Girgenrath and Smith, 1996; Dedos and Fugo, 1999c); an increase in cellular cAMP levels was also measured in *Bombyx* glands. These data suggested that a $G\alpha_s$ protein might be coupled to the PTTH receptor.

Many, if not all, intracellular signaling systems exhibit downregulation after a time, with signaling intermediates returning to basal levels. During continuous presence of PTTH, $[\text{Ca}^{2+}]_i$ levels persisted above basal for at least 25 min in *Bombyx* prothoracic glands, with a peak at about 15 min after initiating PTTH stimulation; after transfer of

stimulated glands to PTTH-free conditions, $[Ca^{2+}]_i$ levels remained elevated for at least 20 min (Birkenbeil and Dedos, 2002). Similar $[Ca^{2+}]_i$ data are not available for *Manduca* prothoracic glands. Thus, how long $[Ca^{2+}]_i$ remains elevated during or after PTTH stimulation is not known. In the prothoracic glands of *Galleria* sixth and seventh (final) larval instars, Birkenbeil (1996) found developmental changes in $[Ca^{2+}]_i$, with peaks co-occurring coincident with peaks of *in vitro* ecdysteroid production. This observation suggested a relatively slow return to basal conditions and/or nonadaptation to continuous PTTH stimulation. In contrast, intracellular Ca^{2+} was essentially constant in glands of fifth instar *Manduca* larvae (Birkenbeil, 1999). The mechanisms by which prothoracic gland $[Ca^{2+}]_i$ is restored to the basal level have not been explicitly explored but presumably involve buffering by Ca^{2+} -binding proteins, reloading of internal stores and transport to the extracellular milieu.

The temporal profile of cAMP levels during PTTH stimulation of the prothoracic gland is also incomplete. PTTH elicited a rapid generation of cAMP in *Manduca* prothoracic glands, readily detectable within 2 min of applying PTTH and peaking after 5 min (Smith *et al.*, 1984). Elevated cAMP levels persisted for at least 20 min, with continuous PTTH exposure. Data from *Bombyx* prothoracic glands were similar, with a rapid rise in cAMP that reached a maximum at 10 min and continued at about twice basal for at least an hour (Gu *et al.*, 1996). Analysis of cAMP levels in *Manduca* prothoracic glands during the fifth (final) larval instar revealed a large maximum occurring at about the time of the commitment peak in ecdysteroid production (Vedeckis *et al.*, 1976), suggesting, as is the case for $[Ca^{2+}]_i$, a slow return to basal levels following stimulation and/or nonadaptation to continuous PTTH stimulation. Initially surprising were the relatively low levels of gland cAMP observed during the premolt period of high ecdysteroid production several days later (but see discussion immediately following).

Intracellular cAMP levels are a product of synthesis and breakdown. The puzzlingly low cAMP *in vivo* levels found during the premolt ecdysteroid peak (Vedeckis *et al.*, 1976), as well as in *Manduca* glands stimulated *in vitro* with PTTH at this stage and later (Smith and Gilbert, 1986; Smith and Pasquarello, 1989), appear to result chiefly from developmental differences in phosphodiesterase activity. Analysis of cAMP phosphodiesterase activity revealed a nearly six-fold increase from a minimum on days 3 and 4 to a peak just prior to pupal ecdysis; at the time of the large, premolt ecdysteroid peak,

when PTTH signaling is presumably high also, phosphodiesterase activity was ≈ 5 times the minimum (Smith and Pasquarello, 1989). This observation suggested that the importance of the cAMP pathway in PTTH signal transduction was greatly diminished in late larval and pupal glands (Vedeckis *et al.*, 1976). This proved clearly not to be the case, as demonstrated by the inhibitory action of a cAMP antagonist on PTTH-stimulated pupal ecdysteroid synthesis (Smith *et al.*, 1996).

6.4.3. Protein Kinases and Phosphatases

A very common, if not universal, response to increases in second messengers is the modulation of protein kinases and phosphatase activities. In this section, known and hypothesized kinase and phosphatase contributors to PTTH signaling are discussed. These enzymes are discussed separately, perhaps giving a linear view of their participation in the PTTH signal transduction cascade. This is clearly not the case and the enzyme-by-enzyme approach is followed merely to provide a structure to the discussion, not to imply simplicity of the pathway.

6.4.3.1. Protein kinase A Two isoforms of cAMP-dependent protein kinase A (PKA; types I and II) have been found in the *Manduca* prothoracic gland, with the majority of the activity ($\geq 95\%$) attributable to the type II isoform (Smith *et al.*, 1986b). PTTH-dependent stimulation of PKA activity was found in both larval and pupal glands, although the addition of a phosphodiesterase inhibitor was necessary to measure PTTH-dependent PKA activity in the latter. The requirement of a phosphodiesterase inhibitor to measure PTTH-dependent PKA activity in pupal glands was not surprising, given the rapid metabolism of cAMP at this stage, as discussed above (see Section 6.4.2). PTTH stimulated PKA activity rapidly, with a patent increase above controls following 3 min of exposure, and with elevated levels still evident after 90 min (Smith *et al.*, 1986b). PKA is composed of two subunits: one catalytic and the other regulatory. In prothoracic glands of early fifth larval instars of *Manduca*, the catalytic subunit remains relatively constant per mg protein for the first 4 days while the regulatory subunit increases approximately three-fold (Smith *et al.*, 1993). This may insure that PKA is only activated when a fairly high threshold of cAMP has accumulated in response to PTTH (Smith *et al.*, 1993), but this hypothesis is assuredly tentative, pending information not currently available, on the relative abundances of the catalytic and regulatory subunits at other stages of development.

The rapid activation of PKA in response to PTTH stimulation suggests that PKA plays a proximate role in the control of PTTH-stimulated steroidogenesis. The effects of agonist and antagonist cAMP homologs is supportive but not conclusive evidence for this interpretation, since there are other cellular targets for cAMP besides PKA. For instance, in mammals, cAMP-dependent guanine nucleotide exchange factors, which activate small G proteins, have been described (Kawasaki *et al.*, 1998). These proteins can stimulate phosphorylation events, including the activation of the 70-kDa S6 kinase, a known element in PTTH action (see below), and a search of the *Drosophila* genome data base suggests that insects may possess one or more homologs to these exchange factors (data not shown). However, the PKA inhibitor H89 is effective at blocking PTTH-stimulated ecdysteroid synthesis in *Manduca* glands, albeit at fairly high doses, again implicating PKA as a regulatory factor in this pathway (Smith *et al.*, 1996). The *in vivo* substrates for PTTH-activated PKA are poorly known. PTTH, dibutyryl cAMP, and Ca^{2+} ionophore all stimulate phosphorylation of a 34-kDa protein in *Manduca* prothoracic glands (Rountree *et al.*, 1987, 1992; Combest and Gilbert, 1992; Song and Gilbert, 1994) that has been identified as the S6 ribosomal protein (Song and Gilbert, 1995). However, it is unclear that S6 is directly phosphorylated by PKA. When the (mammalian) catalytic subunit of PKA, or cAMP, was added to prothoracic gland homogenates, the phosphorylation of S6 was enhanced (Smith *et al.*, 1987a; Rountree *et al.*, 1992; Combest and Gilbert, 1992), indicating that S6 was a potential PKA substrate. However, in intact cells, the situation may be different since the immunosuppressive agent rapamycin was able to block PTTH-stimulated S6 phosphorylation (Song and Gilbert, 1994, 1995). Rapamycin acts via formation of a protein complex that inhibits p70 S6 kinase activity and PKA is neither a known substrate for rapamycin nor a component of the rapamycin–protein complex (see Section 6.4.4).

6.4.3.2. Protein kinase C The possibility that protein kinase C (PKC) was involved in the PTTH-stimulated ecdysteroid synthesis was first addressed, with negative results, by Smith *et al.* (1987a), using the potent PKC activator phorbol myristate acetate (PMA). A 90-min incubation with PMA had no effect on *Manduca* steroidogenesis, either alone or in conjunction with PTTH (see also Smith, 1993). However, in *Bombyx*, PMA boosted prothoracic gland ecdysteroid synthesis after 5 h of treatment, but no effect was apparent after 2 h (Dedos and

Fugo, 2001), suggesting that PKC did not play an acute role in steroidogenesis. Furthermore, studies on inositol metabolism in both *Manduca* and *Bombyx* failed to find PTTH-related generation of PIP_2 metabolites (see Section 6.4.2). Diacyl glycerol (DAG), one of these metabolites, is an activator of PKCs and is formed in a 1:1 ratio with IP_3 . The apparent absence of IP_3 suggested that DAG was also absent and hence that PKCs were not involved in PTTH action. Nevertheless, three lines of evidence do indicate that a PKC is part of the signal transduction network of PTTH, and that it occupies an early and important position in regulating prothoracic gland ecdysteroidogenesis. First, the PKC inhibitor chelerythrine Cl inhibited PTTH-stimulated ecdysteroid synthesis by both *Bombyx* (Dedos and Fugo, 2001) and *Manduca* prothoracic glands (Figure 9a) (Rybczynski and Gilbert, unpublished data); PTTH stimulated ERK phosphorylation was also inhibited (Figure 9a). Second, PTTH-stimulated the phosphorylation of several (unidentified) prothoracic gland proteins, as recognized by an antibody against phosphorylated PKC substrate sequences (Figure 9b), and chelerythrine Cl inhibited these phosphorylations (data not shown) (Rybczynski and Gilbert, unpublished data). Third, although measurable changes in IP_3 or other PIP_2 metabolites have not been detected following PTTH stimulation, phospholipase C inhibitors blocked PTTH-stimulated ecdysteroid synthesis in *Bombyx* (2-nitro-4-carboxyphenyl-N, N-diphenylcarbamate: Dedos and Fugo, 2001) and *Manduca* (U73122: Fellner *et al.*, unpublished data) prothoracic glands (Figure 9c). U73122 also inhibited PTTH-stimulated ERK phosphorylation (Figure 9c) and the release of Ca^{2+} from IP_3 -sensitive stores (see Section 6.4.2). These data suggest that PTTH stimulates inositol metabolism and activates PKCs, but the inositol increases are too small or transient to have been detected. Intracellular compartmentalization of early acting components of the signal transduction system might make small changes biologically meaningful and eliminate the need for large-scale intracellular increases in IP_3 or DAG.

The identity and function of proteins phosphorylated by PKC in response to PTTH stimulation of the prothoracic gland are currently unknown. PKCs can modulate the function of a wide variety of proteins including ion channels (see Shearman *et al.*, 1989), MAP kinases (Suarez *et al.*, 2003), plasma membrane receptors (Medler and Bruch, 1999), cytoskeletal elements (Gujdar *et al.*, 2003), and nuclear proteins (Martelli *et al.*, 2003). The observation that some putative PKC phosphorylations took place only after several hours of PTTH stimulation

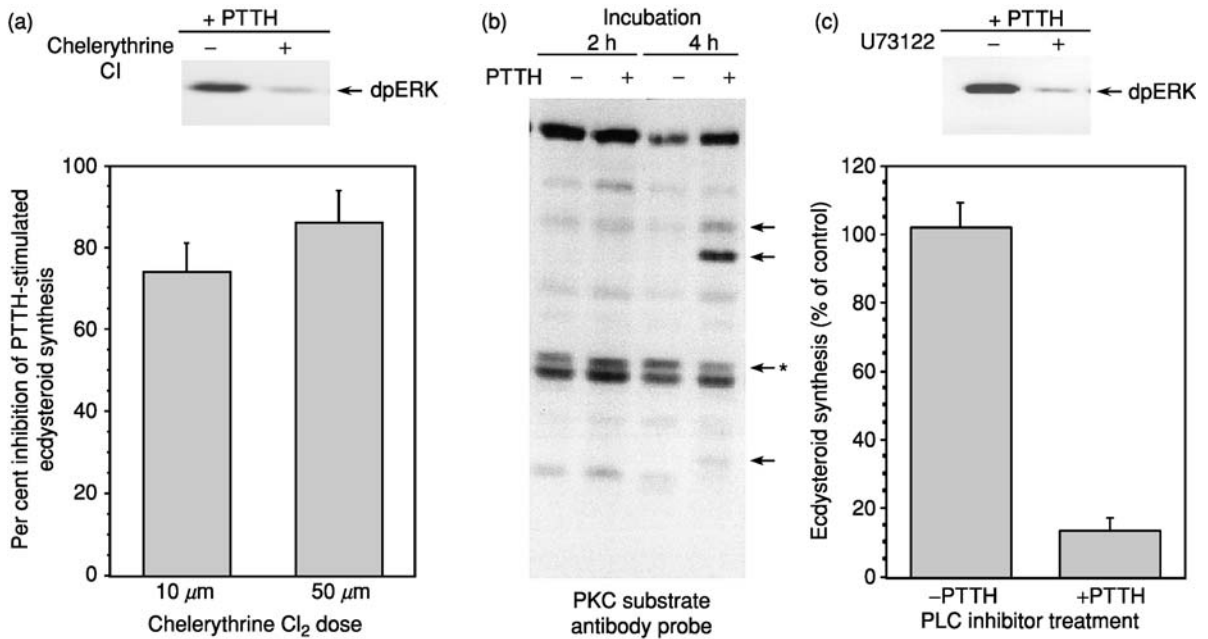


Figure 9 Evidence for the involvement of PKC and PLC in PTH-stimulated events in the larval prothoracic gland of *Manduca sexta*. Ecdysteroids were measured with a radioimmunoassay and protein phosphorylation detected following SDS-PAGE of gland proteins, using phospho-specific antibodies and chemiluminescence reagents. (a) Chelerythrine Cl, an inhibitor of PKC, blocks PTH-stimulated ecdysteroid synthesis (lower panel) and ERK phosphorylation (upper panel). Chelerythrine Cl had no effect on basal ecdysteroid synthesis and ERK phosphorylation (data not shown). (b) PTH stimulates the phosphorylation of proteins recognized by an antibody against putative PKC phosphorylation substrate sites. Glands were incubated for up to 4 h \pm PTH. (c) U73122, an inhibitor of PLC β , blocks PTH-stimulated ecdysteroid synthesis, without affecting basal synthesis (lower panel). U73122 also inhibits PTH-stimulated ERK phosphorylation (upper panel) but basal ERK phosphorylation is unaffected (data not shown). (Data from Rybczynski, Fellner, and Gilbert, unpublished data.)

(Figure 9b) suggested that PKC might be involved in the long-term effects of PTH, such as transcription, as well as in the acute regulation of steroid synthesis. The position of PKC in the PTH signaling hierarchy has not been determined but, given a probable requirement for DAG, it seems likely that PKC is among the first intracellular components to be called into action. This hypothesis is supported by the observations that (1) inhibition of PKC activity prevented PTH-dependent ecdysteroid synthesis and (2) inhibition of PKC activity prevented PTH-dependent MAP kinase phosphorylation (see below), i.e., PKC is upstream of MAP kinases (Rybczynski and Gilbert, unpublished data). The time course of PKC activity in the PTH signal transduction network may be extended given the observed late appearance of some putative PKC phosphorylation events (Figure 9b).

6.4.3.3. Extracellular signal-regulated kinase

The mitogen-activated protein kinases (MAP kinases) are a family of related proteins that are activated by a wide variety of extracellular signals, ranging from growth factors and peptide hormones to heat and osmotic stresses. MAP kinases, in turn, regulate

a wide variety of intracellular processes from microtubule stability and protein synthesis to transcription. There are several families of MAP kinases, which are organized into three-level signaling modules, i.e., a MAP kinase, a MAP kinase kinase (MEK), and a MAP kinase kinase kinase (MEKK or MAP3K) (see Lewis *et al.*, 1998; Kyriakis, 2000). Perhaps the most studied of the MAP kinase modules is the extracellular signal-regulated kinase (ERK) cascade. This module typically starts with a protein called RAF, and proceeds through a MEK to the ERK protein(s); in mammals there are two ERKs while *Drosophila* and *Manduca* appear to express a single ERK (see Lewis *et al.*, 1998; Biggs and Zipursky, 1992; Rybczynski *et al.*, 2001). A number of proteins lie upstream from RAF, the small G protein RAS is a frequent but not ubiquitous activator of RAF, which itself may lie downstream of G protein-coupled receptors, tyrosine kinase receptors, or cytokine receptors (see Lewis *et al.*, 1998). MEKs and MEKKs are activated by single phosphorylations while ERKs require a dual phosphorylation on threonine and tyrosine residues for activity.

A PTH stimulation of the ERK pathway in larval *Manduca* prothoracic glands was shown

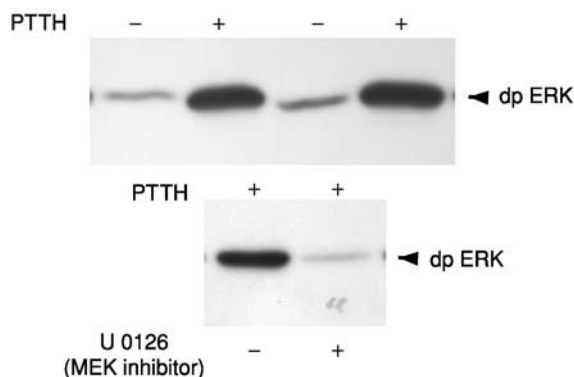


Figure 10 PTTH stimulates ERK phosphorylation in the larval prothoracic gland of *Manduca sexta* (upper panel) that is blocked by the MAPK/ERK kinase inhibitor U0126 (lower panel). ERK phosphorylation was detected following SDS-PAGE of gland proteins, using an antiphosphorylated ERK antibody and chemiluminescence reagents. (Adapted from Rybczynski, R., Bell, S.C., Gilbert, L.I. 2001. Activation of an extracellular signal-regulated kinase (ERK) by the insect prothoracicotropic hormone. *Mol. Cell. Endocrinol.* 184, 1–11.)

recently (Rybczynski *et al.*, 2001) using activity assays, inhibitors of ERK phosphorylation, and antibodies that recognize the dually phosphorylated state (see **Figure 10**). This study revealed that PTTH caused a rapid but transient increase in ERK phosphorylation and that inhibitors of ERK activation blocked both basal and PTTH-stimulated ecdysteroid syntheses. The data suggested that ERKs might be involved in regulating some aspects of the acute PTTH response in prothoracic glands. Further examination showed that PTTH-stimulated ERK phosphorylation was primarily a larval gland response. As *Manduca* larvae progressed through the fifth instar, PTTH-stimulated phosphorylation in the prothoracic gland declined until it was undetectable in pupal glands (Rybczynski and Gilbert, 2003). Concurrently, ERK levels declined in the glands but the basal levels of phosphorylation increased in response to unknown factors. These two phenomena combined to cause the aforementioned decline in PTTH-stimulated ERK phosphorylation. Rybczynski and Gilbert (2003) also found that PTTH-stimulated ERK phosphorylation occurred via a Ca^{2+} -dependent pathway but not one that was cAMP-dependent. Some PKC isoforms require Ca^{2+} for activation and thus are candidates for the regulation of the ERK cascade. As discussed above, PKC inhibition resulted in a block of PTTH-stimulated ERK phosphorylation (**Figure 8c**). In fact, PKC may directly activate RAF, the first component of the ERK phosphorylation module, without the participation of RAS (see Liebman, 2001). However, PKC might control ERK phosphorylation

in a less specific manner if PKC regulates PTTH-stimulated Ca^{2+} channel opening. Of note in this regard is the observation that PKC modulation of dihydropyridine-sensitive Ca^{2+} channels occurs in *Drosophila* tissues (Gu and Singh, 1997). The protein kinase Pyk2, which can be activated by increases in $[Ca^{2+}]_i$ and by PKC (Lev *et al.*, 1995), is also a reasonable candidate to be an upstream regulator of the ERK cascade.

The list of potential targets of ERK phosphorylation is long and encompasses transcription factors, steroid and growth factor receptors, various membrane-associated proteins, cytoskeletal proteins, kinases, and proteins involved in regulating translation (see Grewal *et al.*, 1999). Studies to discover PTTH-related ERK phosphorylation targets in the prothoracic gland are currently underway but no conclusive identifications can be made at this time. In most cell types, some fraction of dually phosphorylated ERKs migrate into the nucleus where they modulate transcription; no conclusive information about the prothoracic gland on this topic is presently available. Preliminary cell fractionation experiments suggested that dually phosphorylated ERKs do indeed move into the prothoracic gland cells' nuclei following PTTH stimulation (Rybczynski and Gilbert, unpublished data); however, the basal lamina surrounding the gland and the very large nuclei make clean subcellular fractions difficult to obtain and the conclusion must be considered tentative.

6.4.3.4. p70 S6 kinase The S6 protein is a prominent ribosomal phosphoprotein that can be phosphorylated by PKA and a 70-kDa S6 kinase (p70S6K) (Palen and Traugh, 1987). S6 phosphorylation is a frequent and presumably important event in various intercellular signaling pathways (see Erickson, 1991; Volarevic and Thomas, 2001). Phosphorylation of S6 by p70S6K, but apparently not by PKA, can increase the rate of translation of some mRNAs (Palen and Traugh, 1987). PTTH has long been known to induce the phosphorylation of a ≈ 34 -kDa protein in prothoracic glands of *Manduca*, which was hypothesized to be S6 (see Gilbert *et al.*, 1988); this identification proved to be correct (see Section 6.4.4).

Song and Gilbert (1994) showed that p70S6K was responsible for PTTH-stimulated S6 phosphorylation in *Manduca* prothoracic glands. Using the macrolide immunosuppressant rapamycin, which blocks p70S6K phosphorylation-dependent kinase activity (see Dufner and Thomas, 1999), they found that PTTH-stimulated phosphorylation of an S6 peptide substrate was inhibited by rapamycin,

when tested with an S6K1-enriched protein fraction prepared from glands treated with both PTTH and rapamycin. Furthermore, rapamycin inhibited PTTH-stimulated ecdysteroid synthesis at low (nM) concentrations. These observations also implicate the participation of at least two additional proteins in the PTTH transduction cascade, although direct proof of their activity remains to be obtained. One is the phosphoinositide 3OH-dependent kinase (PI3K) and the second is a protein termed, among others, TOR (target of rapamycin; the *Drosophila* gene is called *dTOR*) and *frap* (FKBP12-rapamycin-associated protein). TORs are proteins that contain a kinase domain similar to that of PI3K; their activity is inhibited when rapamycin binds to the small protein FKBP, forming an inhibitory complex that interacts with TORs (see Fumagalli and Thomas, 2000). Both PI3K and TOR positively regulate p70S6K activity. It is likely that another level of proteins is interposed between p70S6K and PI3K, such as the atypical PKCs (see Fumagalli and Thomas, 2000). TORs probably interact directly with p70S6K and also inhibit phosphatases that downregulate p70S6K activity (see Fumagalli and Thomas, 2000). Activation of PI3K by PTTH might take place via several intermediary proteins, such as RAS, or the $\beta\gamma$ subunit of G proteins (see Coffey, 2000). The regulation of TOR function, in the context of PTTH, is more problematic. It may be activated by PI3K, perhaps via an intermediate kinase, but the regulation of TORs is incompletely understood (see Raught *et al.*, 2001; Abraham, 2002).

6.4.3.5. Tyrosine kinases Many intercellular signaling molecules evoke a series of protein phosphorylations on tyrosine residues within the target tissues. Tyrosine kinases include both intracellular proteins as well as membrane-spanning receptors. The possibility that PTTH signal transduction involves tyrosine kinase has been addressed in part by Smith *et al.* (2003). Their work revealed that PP1, an inhibitor of the Src tyrosine kinase, repressed PTTH-stimulated cAMP and ecdysteroid synthesis in *Manduca* prothoracic glands. PP1 did not appear to interact with adenylate cyclase, since forskolin was still able to stimulate cAMP production in the presence of PP1. This result suggested that a Src kinase might act very early in PTTH signaling, perhaps at the level of Ca^{2+} mobilization. Indeed, tyrosine kinases can activate phospholipases, generating IP_3 and raising $[\text{Ca}^{2+}]_i$ via the IP_3 receptor/ Ca^{2+} channel (see Rhee, 2001); tyrosine kinases can also directly activate the IP_3 receptor/ Ca^{2+} channel through phosphorylation (Jayaraman *et al.*, 1996). Another intriguing possibility is that

stimulation of the PTTH receptor, if it is indeed a GPCR, might transactivate a tyrosine receptor kinase via G proteins and other intermediates, and consequently recruit some or all of the signaling pathway of a completely different receptor class (see Luttrell *et al.*, 1999; Wetzker and Bohmer, 2003). Perhaps relevant to this scenario is the observation that an antibody against the vertebrate insulin receptor, a tyrosine kinase receptor, cross-reacted with a protein of appropriate molecular weight in the prothoracic gland (Smith *et al.*, 1997) and a second antibody against tyrosine-phosphorylated proteins indicated that PTTH stimulated the phosphorylation of a protein of the same molecular weight (Smith *et al.*, 2003). However, care must be taken in interpreting the effects of this single kinase inhibitor, since PP1, at the micromolar concentrations employed, might inhibit other kinases, including those in the ERK pathway (see Bain *et al.*, 2003).

6.4.4. Ribosomal Protein S6

Ribosomal protein S6 is a small (≈ 34 kDa) basic protein, present at one copy per 40S ribosome, that is located near the tRNA/mRNA binding site (see Fumagalli and Thomas, 2000). Phosphorylation of S6 is associated with mitogenic stimulation of non-dividing cells and occurs in vertebrate steroidogenic cells in response to factors such as FSH and TSH (Das *et al.*, 1996; Suh *et al.*, 2003); of course, FSH and TSH have mitogenic effects as well as effects on hormone synthesis. S6 phosphorylation at six serine residues near the C-terminal of the protein has been correlated with the selective increase of certain mRNAs, especially those containing polypyrimidine tracts near the transcription initiation site (see Erickson, 1991; Fumagalli and Thomas, 2000; Meyuhau and Hornstein, 2000; Volarevic and Thomas, 2001).

Rountree *et al.* (1987) and others (see Section 6.4.3.1) discovered that PTTH stimulates the phosphorylation of a ≈ 34 kDa-protein in *Manduca* prothoracic glands that was hypothesized to be the S6 ribosomal protein. Song and Gilbert (1994, 1995, 1997) confirmed this identification by using the p70S6K inhibitor rapamycin and, more rigorously, by two-dimensional analysis of ribosomal proteins (see Figure 11). As discussed earlier, they found that rapamycin blocked S6 phosphorylation and ecdysteroid synthesis; furthermore, rapamycin inhibited the accumulation of specific, newly synthesized proteins, including a heatshock 70-kDa cognate protein (Song and Gilbert, 1995), which had been hypothesized to be necessary for PTTH-stimulated ecdysteroid synthesis (Rybczynski and Gilbert, 1994, 1995a; see below).

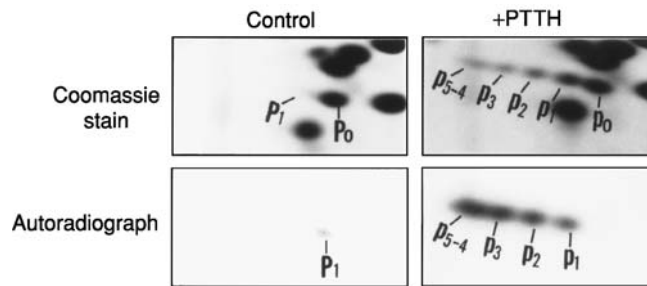


Figure 11 PTTH stimulates the phosphorylation of ribosomal protein S6 in the prothoracic gland of *Manduca sexta*. The upper two panels show the effect of PTTH on the migration pattern of the S6 protein (labeled P_0 to P_{5-4}) in two-dimensional gel analyses of ribosomal proteins, as visualized with Coomassie Blue staining. The lower two panels show the effect of PTTH on the migration pattern of the S6 protein, as visualized with autoradiography of ^{32}P -labeled proteins, following two-dimensional gel analyses of ribosomal proteins. The data reveal that up to five sites on S6 are phosphorylated following the stimulation of prothoracic glands with PTTH. (Adapted from Gilbert, L.I., Song, Q., Rybczynski, R. 1997. Control of ecdysteroidogenesis: activation and inhibition of prothoracic gland activity. *Invert. Neurobiol.* 3, 205–216.)

cDNA cloning of the *Manduca* S6 mRNA revealed that the sequence coded for a polypeptide of $\approx 29\,000$ MW, with an estimated isoelectric point of 11.5 (Song and Gilbert, 1997). Like the mammalian S6 proteins, the *Manduca* sequence contained a cluster of six serines in the final (C-terminal) 20 amino acids. Phosphoamino acid analysis indicated that, following PTTH stimulation, the *Manduca* S6 protein was phosphorylated solely on serines. The two-dimensional analysis of ribosomal proteins showed that PTTH-stimulation resulted in phosphorylation of up to five sites; nonstimulated glands yielded an S6 with an apparent single phosphorylation (Song and Gilbert, 1995, 1997). The location of the PTTH-stimulated serine phosphorylations is not known since, in addition to the six terminal serines, the *Manduca* protein was deduced to contain an additional 10 residues. The combination of Northern blotting, using the S6 cDNA as a probe, and immunoblotting, using polyclonal antibodies against the S6 protein terminus, showed that S6 RNA and protein levels in the prothoracic gland were not correlated (Song and Gilbert, 1997). S6 RNA per unit total RNA was highest in the first 3 days of the fifth instar and decreased linearly to a much lower level by day 7; this low level persisted through day 4 of pupal life, interrupted only by a minor increase on the first day following pupal ecdysis. In contrast, S6 proteins showed two peaks of abundance. The first occurred on days 6 and 7 of the fifth instar, when the prothoracic gland is beginning the large surge of ecdysteroid synthesis that precedes the larval–pupal molt. The second peak fell on days 3 and 4 of pupal–adult development, the beginning of the large pupal–adult period of ecdysteroid synthesis. As a control, S6 protein levels in the fat body were assayed. These showed no relationship to S6 in the prothoracic gland or to hemolymph

ecdysteroid titers. Rather, S6 protein in fat body showed a steady decline from the beginning to the end of the fifth instar and then fairly constant low levels through day 4 of pupal–adult development. These data suggested that S6 levels might be a controlling factor in supporting high levels of ecdysteroid synthesis.

Although these composite data strongly support the notion that the PI3K/TOR–p70S6K–S6 axis is essential for PTTH-stimulated ecdysteroid synthesis to occur, there are some puzzling data that do not readily fit this scheme. Both Rountree *et al.* (1987) and Smith *et al.* (1987a) discovered that, like PTTH, a cAMP analog (dibutyryl cAMP (dbcAMP)) stimulated S6 phosphorylation and Rountree *et al.* (1987) found that JH and a JH analog inhibited S6 phosphorylation in glands studied *in vitro* (Rountree *et al.*, 1987). However, under the same *in vitro* conditions, JH had no effect on PTTH-stimulated cAMP synthesis and ecdysteroid synthesis (Rountree and Bollenbacher, 1986). A second puzzling observation, revolving again around cAMP, stems from the study of the effects of rapamycin on steroidogenesis. As described above, rapamycin inhibits both PTTH-stimulated S6 phosphorylation, and ecdysteroidogenesis. However, dbcAMP was able to override the rapamycin block and stimulate ecdysteroid synthesis to the usual levels above basal, even though rapamycin blocked the dbcAMP-dependent S6 phosphorylation (Song and Gilbert, 1994). These two sets of data suggest that a vigorous, six-site S6 phosphorylation response may not be required to support PTTH-stimulated ecdysteroid synthesis. In this respect, it would be interesting to use the more sensitive two-dimensional gel analysis of ribosomal proteins to determine if JH and rapamycin blocked all phosphorylations above the basal level; it might be that the single-dimension gel

analysis used was not sensitive enough to detect partial S6 phosphorylation in these experiments. It is also possible that there are alternate pathways by which PTTH stimulates ecdysteroid synthesis and that these pathways can substitute for the presumably default S6-dependent one. For instance, activation of the ERK pathway can also upregulate protein translation in some cells (see Grewal *et al.*, 1999).

6.4.5. Translation and Transcription

In vertebrate steroidogenic tissues, the final signal transduction step in peptide-regulated steroid hormone production is the rapid translation of one or more proteins. In these taxa, two proteins, StAR (steroidogenic acute regulatory protein) and DBI (diazepam-binding inhibitor), facilitate the delivery of cholesterol across the mitochondrial membrane to the side-chain cleavage P450 enzyme, which converts cholesterol to pregnenolone (see Stocco and Clark, 1997; Papadopoulos *et al.*, 1997). This step is rate limiting and is alleviated by rapid translation of StAR and/or DBI that is stimulated by a peptide hormone such as adrenocorticotrophic hormone (ACTH). A *Manduca* DBI homolog has been cloned and evidence suggests a role for DBI in basal ecdysteroid synthesis (Snyder and Feyereisen, 1993; Snyder and Van Antwerpen, 1998), but experiments to explicitly test its role in acute PTTH-stimulated ecdysteroidogenesis have yet to be performed. Analysis of the *Drosophila* and *Anopheles* genome databases indicates that an insect protein with close homology to the StAR-like vertebrate protein MLN64 exists but the similarity of the insect protein to StAR itself is lower. MLN64 contains a region homologous to the cholesterol-binding region of StAR but the insect protein sequence contains a large interior region that has no vertebrate counterpart and *in situ* hybridizations in *Drosophila* indicate that expression is not limited to the ring gland.

In insects, it is clear that PTTH-stimulated ecdysteroid synthesis also requires translation, as revealed by the ability of translation inhibitors like cycloheximide and puromycin to block such ecdysteroid synthesis (Keightley *et al.*, 1990; Kulesza *et al.*, 1994; Rybczynski and Gilbert, 1995a); translation inhibitors have no effect on basal ecdysteroid synthesis, under standard *in vitro* protocols. In *Drosophila*, the movement of an ecdysteroid precursor(s) between intracellular compartments (endoplasmic reticulum and mitochondrion) probably involves a carrier protein, although the necessity of translation in this species has not been demonstrated (Warren and Gilbert, 1996; Warren *et al.*, 1996).

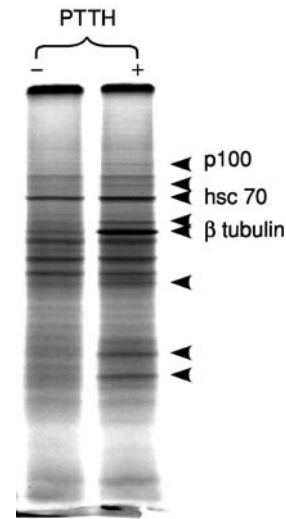


Figure 12 PTTH stimulates the synthesis of specific proteins in the prothoracic gland of *Manduca sexta*. Glands were incubated with ^{35}S -methionine \pm PTTH for 1 h and then subjected to SDS-PAGE and autoradiography. The arrowheads indicate newly synthesized proteins whose accumulation in the prothoracic gland is upregulated by PTTH. Hsc 70, β tubulin and p100 were identified in the first studies on this system (Rybczynski and Gilbert 1994, 1995a; 1995b); the additional PTTH-regulated translation products were only detected after further refinement of the SDS-PAGE conditions (Rybczynski and Gilbert, unpublished data).

Analysis of protein synthesis in *Manduca* prothoracic glands, using ^{35}S -methionine to label newly synthesized proteins, revealed that PTTH stimulated overall translation at most developmental stages and that some proteins, perhaps as many as 10, were differentially translated above this general increase (see Figure 12) (Keightley *et al.*, 1990; Rybczynski and Gilbert, 1994; Gilbert *et al.*, 1997). Two of these proteins have been identified and cloned: a β tubulin (Rybczynski and Gilbert, 1995a, 1998) and a 70-kDa heatshock cognate protein (hsc 70) (Rybczynski and Gilbert, 1995b, 2000). PTTH-stimulated synthesis of hsc 70 rose slowly and was prolonged, suggesting that hsc 70 might function as a chaperone in supporting translation, and in other long-term effects of PTTH not directly connected to acute changes in ecdysteroidogenesis, e.g., an hsc 70 protein appears to be necessary for proper function of the ecdysone receptor (Arbeitman and Hogness, 2000). PTTH-stimulated hsc 70 synthesis above basal at all stages examined during the fifth instar and pupal–adult development; peaks occurred on the third day of the fifth instar (V_3) and the first day of pupal–adult development (P_1) (Rybczynski and Gilbert, 1995b). In contrast, PTTH-stimulated β tubulin synthesis occurred

rapidly and was transitory, suggesting a more direct role in ecdysteroidogenesis for this cytoskeletal protein (Rybczynski and Gilbert, 1995a). Peaks in PTTH-stimulated β tubulin synthesis occurred on V_3 and P_1 (Rybczynski and Gilbert, 1995a), as observed for hsc 70. Indeed, treatment of pupal prothoracic glands with microtubule-disrupting drugs inhibited PTTH-stimulated ecdysteroid synthesis (Watson *et al.*, 1996) but this effect was not seen in larval glands (Rybczynski and Gilbert, unpublished data). Furthermore, the pupal inhibition effect was associated with translation inhibition that was overridden by treatment of glands with a cAMP analog, indicating that physical coupling between the PTTH receptor and a protein acting early in the transducing cascade was disrupted by such drugs (Rybczynski and Gilbert, unpublished data). The reason for the larval-pupal difference in microtubule effects on ecdysteroid synthesis is unknown. Note that neither study (Watson *et al.*, 1996; Rybczynski and Gilbert, unpublished data) explicitly monitored the effects of the microtubule disrupting drugs on cytoskeletal structures or cell shape. Note also that the role of the microtubule cytoskeleton in vertebrate steroidogenic cells is not clear. Basal steroidogenesis seems to be generally immune to microtubule-acting drugs while peptide hormone-stimulated steroidogenesis is variably inhibited or not, depending on the cell type and drug concentration (see Feuilletoy and Vaudry, 1996). In systems where peptide hormone-stimulated steroidogenesis is inhibited, the data suggest that the pharmacological disruption of microtubules disturbs early events in the signal transduction cascade, e.g., receptor-G protein-cyclase interactions (see Feuilletoy and Vaudry, 1996), a hypothesis consistent with the above-mentioned ability of a cAMP analog to override such a disruption.

The identities of other PTTH-stimulated proteins remain unknown. Attempts to use antivertebrate StAR antibodies against *Manduca* prothoracic gland proteins were not interpretable, due to multiple protein cross-reactions, at a range of MWs, in immunoblot analyses (Rybczynski and Gilbert, unpublished data). Note that the predicted molecular weight of the *Drosophila* MLN64/StAR homolog is considerably larger (≈ 61 kDa) than the vertebrate molecule (≈ 30 kDa). PTTH-stimulated protein synthesis is not limited to specific proteins but can also be detected in overall rates of translation (Keightley *et al.*, 1990; Rybczynski and Gilbert, 1994; Kulesza *et al.*, 1994). The amount by which PTTH stimulates overall translation above basal appears to vary with the stage, with V_3 glands responding at about six times the rate of V_1 glands (Rybczynski and

Gilbert, 1994). The incubation of nonstimulated prothoracic glands with ^{35}S -methionine revealed that the pattern of translated proteins varied among several stages in the fifth larval instar (Lee *et al.*, 1995); how these changes relate to changes in the ecdysteroidogenic capability of the prothoracic gland, or to PTTH signaling, is unknown. Unfortunately, a more detailed developmental analysis of protein synthesis, both basal and PTTH stimulated, by larval and pupal prothoracic glands remains to be done. As mentioned above, phosphorylation of ribosomal protein S6 preferentially increases the translation of mRNAs containing a 5'-polypyrimidine tract. The identification of these RNAs is undoubtedly incomplete but recent evidence reveals that in vertebrate cells many such RNAs code for ribosomal proteins (see Pearson and Thomas, 1995), suggesting that some of the unidentified PTTH-dependent translation products are also components of the ribosome.

PTTH stimulates transcription in prothoracic glands, based on the incorporation of radiolabeled uridine into acid-precipitable RNA in response to dibutyryl cAMP (Keightley *et al.*, 1990). Inhibition of transcription using actinomycin D resulted in a partial inhibition of both PTTH- and dibutyryl cAMP-stimulated ecdysteroid synthesis (Keightley *et al.*, 1990; Rybczynski and Gilbert, 1995b). These data suggested that transcription may be necessary for a maximal ecdysteroidogenic response to PTTH. However, actinomycin D had a larger effect on translation ($\approx 50\%$ inhibition in a 45-min incubation) than might be expected unless an unprecedented fraction of prothoracic gland proteins was translated from mRNAs with very short half-lives (Rybczynski and Gilbert, 1995b). In other words, the possibility that actinomycin D interfered with translation, as well as with transcription, cannot be ruled out, thus making it difficult to determine the role of transcription in acute increases of ecdysteroidogenesis.

cDNA clones for *Manduca* $\beta 1$ tubulin and hsc 70 were obtained, with the hope that the expression pattern of these two genes might shed some light on their functions in PTTH signaling. In the prothoracic gland, $\beta 1$ tubulin mRNA exhibited a peak in the fifth instar just before wandering (Rybczynski and Gilbert, 1998). β tubulin protein levels paralleled the mRNA profile during the fifth instar but in contrast to the mRNA profile, β tubulin protein abundance exhibited a second peak on day 2 (P_2) of pupal-adult development. These data might indicate that β tubulin protein levels are important to glands that are preparing to synthesize large amounts of ecdysteroids but do little to explain

the very rapid synthesis that occurs upon PTTH stimulation (Rybczynski and Gilbert, 1995b). Hsc 70 mRNA levels also showed a peak in the fifth instar, just before wandering (V_4), with lesser peaks on V_7 and P_2 (Rybczynski and Gilbert, 2000). Hsc 70 protein levels showed peaks on V_3 , V_7 , and P_0 levels, yielding a pattern of expression that does not clearly relate to either prothoracic gland growth or ecdysteroid synthesis. Although the profiles of β tubulin and hsc 70 mRNAs and proteins did not lend themselves to understanding the roles these gene products play in PTTH-stimulated ecdysteroid synthesis, analyses of brain and fat body indicated that the expression patterns of these products were tissue specific (Rybczynski and Gilbert, 1998, 2000). Complicating the question of determining what special roles β tubulin and hsc 70 might play in PTTH signaling in the prothoracic gland is the fact that both these proteins are very abundant and participate in various housekeeping duties in the cell. Discerning a PTTH-specific function against this background is difficult. Clearly, the topic of transcription in PTTH-stimulated ecdysteroid synthesis has not been exhausted and is worthy of further scrutiny.

6.4.6. Protein Phosphatases

The stimulation of ecdysteroidogenesis *in vivo* by PTTH is transient, and with varying biosynthetic rates, depending on the developmental stage. Given that PTTH stimulates the phosphorylation of a number of kinases and an unknown number of target proteins, it is reasonable to assume that downregulation of PTTH-stimulated ecdysteroid synthesis involves the activity of protein phosphatases. This topic has received scant attention in regard to the action of PTTH. Song and Gilbert (1996) used okadaic acid and calyculin, highly effective inhibitors of the serine/threonine-specific protein phosphatases 1 and 2A, to address this topic. They found that treatment of *Manduca* prothoracic glands with either of these inhibitors enhanced the phosphorylation of S6, and of several unidentified proteins, but that PTTH-stimulated phosphorylation of S6 was not augmented by a cotreatment with the PP inhibitors. However, although okadaic acid and calyculin increased S6 phosphorylation, these drugs inhibited both basal and PTTH-stimulated ecdysteroid and protein synthesis; included in the latter effect was the apparent inhibition of the syntheses of both β tubulin and hsc 70. Finally, Song and Gilbert (1996) found that PTTH stimulated phosphatase activity capable of dephosphorylating S6, and perhaps other proteins, and that this activity was inhibited by okadaic acid,

implicating PP1 and/or PP2A in the downregulation of PTTH effects. The combined data indicated that the multiple phosphorylation of S6, by itself, was not sufficient to drive increased ecdysteroidogenesis. They also suggested that the nonphosphorylated state of one or more proteins was necessary for normal ecdysteroid synthesis, assuming that neither okadaic acid nor calyculin had effects on proteins other than phosphatases. That PTTH both stimulated S6 phosphorylation and dephosphorylation processes were expected given the likelihood that negative feedback loops exist in the prothoracic gland to restore its ecdysteroidogenic activity to basal levels.

The MAP kinases, like the ERK activated by PTTH stimulation (see Section 6.4.3), can be dephosphorylated by a number of phosphatases (see Camps *et al.*, 2000). Dephosphorylation of ERKs at either the threonine or tyrosine sites results in loss of ERK kinase activity; PP2A and several tyrosine phosphatases, e.g., PTP-SL, STEP, and HePTP, have all been shown to inactivate vertebrate ERKs (see Camps *et al.*, 2000). In addition, ERKs and other MAP kinases are dephosphorylated by unique dual-specificity phosphatases (DSPs) that remove phosphates from both threonine and tyrosine residues. These DSPs are probably the major agents of vertebrate ERK inactivation (see Camps *et al.*, 2000). Mammals express at least 9 DSP genes and at least one homolog is found in *Drosophila* (D-mkp) (Cornelius and Engel, 1995). Currently, the contributions of various protein phosphatases to the regulation of prothoracic gland ERK phosphorylation are unknown. Serine/threonine phosphatases are likely regulators of proteins, like MEK, that are upstream from ERK and their role can be assessed, in part, by using well-characterized inhibitors like okadaic acid. Understanding the role of DSPs is more difficult and will require, *inter alia*, the further characterization of DSP-specific inhibitors that are currently under development (Vogt *et al.*, 2003).

6.5. The Prothoracic Gland

The summary view of the PTTH signaling system presented in Figure 7 is no doubt incomplete and will be subject to future additions and amendments. Among the factors deliberately left out of this scheme are those that cause changes in the prothoracic gland that are either independent of PTTH or that are PTTH dependent but not linked to the rapid regulation of ecdysteroidogenesis. In this section, an overview of these factors, both demonstrated and hypothesized, is presented.

6.5.1. Developmental Changes in the Prothoracic Gland

Current evidence indicates that the cells of the prothoracic gland either do not divide after embryonic life or do so very infrequently (see discussion in Rybczynski and Gilbert, 2000). Nevertheless, the gland is far from being a static tissue. A number of components in the PTH transduction cascade change as the fifth larval instar progresses, as discussed in the previous section. These include an increase in cAMP phosphodiesterase activity, decrease in the ERK phosphorylation response, an increase in the amount of the regulatory subunit of PKA, and a change in calmodulin sensitivity, rendering pupal adenylate cyclase less responsive than the larval enzyme. In addition to these events, a number of additional developmental differences have been described. First, while cell number is constant, cell size changes dynamically and rapidly, accompanied by a number of structural changes. In *Manduca* and *Spodoptera*, cell size, i.e., diameter, is roughly correlated with ecdysteroidogenic capacity during the last larval instar, with large-celled glands being the most productive (Sedlak *et al.*, 1983; Zimowska *et al.*, 1985; Hanton *et al.*, 1993). As expected, as cell size increases, the protein content of the gland also increases. In *Manduca*, total extractable and particulate protein increases from a minimum at the beginning of the fifth instar of $\approx 3 \mu\text{g/gland}$ to $\pm 20 \mu\text{g/gland}$ on V_4 ; protein levels stay high through V_7 before reaching a low of 10–12 $\mu\text{g/gland}$ on P_0 (Smith and Pasquarello, 1989; Meller *et al.*, 1990; Rybczynski and Gilbert, 1995b). Protein content rises again through at least P_4 (Rybczynski and Gilbert, 1995b). Though size matters, the ecdysteroidogenic potential of prothoracic glands is not a simple function of cell size or protein content. An analysis of protein content and ecdysteroidogenesis in V_1 to V_3 *Manduca* fifth instar prothoracic glands revealed that basal and PTH-stimulated synthesis per milligram of protein increased during this period; that is, ecdysteroid synthesis per gland increased faster than did the gland's total protein content (Rybczynski and Gilbert, 1994). A reanalysis of data gathered throughout the fifth instar of *Manduca* (provided by Smith and Pasquarello, 1989) confirms this observation, and revealed that a peak of basal ecdysteroid synthesis per unit protein occurred on V_4 . Levels after V_4 declined but never reached the low seen on V_1 . PTH-stimulated synthesis per milligram of protein exhibited a different pattern. A peak was again seen on V_4 but levels were high and relatively similar from V_2 through V_7 , followed by a dip on V_8 and V_9 , and

then a sharp rise to near V_4 levels on P_0 . Note that PTH-stimulated synthesis per milligram of protein was essentially the same on V_2 and V_3 using the data from Smith and Pasquarello (1989), while Rybczynski and Gilbert (1994) found V_2 levels to be considerably lower than V_3 levels.

An additional developmental change in prothoracic glands has been described for *Bombyx*. *Bombyx* prothoracic glands from the early fifth instar do not respond to PTH stimulation with either increased ecdysteroid or cAMP synthesis, and are also distinguished by an exceedingly low basal level of steroid synthesis *in vitro* (Sakurai, 1983; Gu *et al.*, 1996). However, these glands apparently respond with increased ecdysteroid synthesis to dibutyryl cAMP, suggesting that there is an upstream developmental "lesion" in one or more components of the PTH signaling pathway (Gu *et al.*, 1996, 1997). In contrast, Takaki and Sakurai (2003) found that dibutyryl cAMP did not elicit ecdysteroid synthesis in early fifth instar glands. The reason for the discrepancy in results is not known but might lie in methodological or strain-specific differences among the studies. Further work indicated that early fifth instar glands from *Bombyx* possess an adenylate cyclase that is much less responsive to Ca^{2+} /calmodulin stimulation than that characterized later in the instar when the gland is responsive to PTH (Chen *et al.*, 2001). It is not known if this cyclase difference is sufficient to explain fully the refractory nature of early fifth instar glands, nor is it known what underlies the cyclase behavior, i.e., are different adenylate cyclases present at different developmental stages? The larger question – why are *Bombyx* glands refractory while *Manduca* glands are not – is conjectural. The possibility that the *Bombyx* condition is a consequence of its domestication cannot be excluded.

These observations showing developmental changes in the signaling and biosynthetic components of the prothoracic gland raise an obvious question. What factor(s) controls the growth and differentiation state of the gland? At present, we can only speculate. PTH is, of course, a candidate. Functionally homologous vertebrate peptide hormones, acting alone or in concert with other factors, often have a differentiation and mitogenic effect on their target tissues in addition to their steroidogenic activity (see Adashi, 1994; Richards, 1994; Richards *et al.*, 2002). Presently, two lines of evidence suggest that PTH may have nonecdysteroidogenic effects on the prothoracic gland. First, PTH hemolymph titers in *Bombyx* showed a pattern not completely consonant with ecdysteroidogenesis, indicating other roles for this peptide

hormone (see Section 6.3.4). Second, PTTH stimulates two categories of protein synthesis in the *Manduca* prothoracic gland (Rybczynski and Gilbert, 1994). One class of proteins comprised a small group of molecules whose translation appears to be linked to PTTH-stimulated ecdysteroidogenesis and presumably includes one or more proteins that are necessary for PTTH-stimulated increases in steroid synthesis (see also Song and Gilbert, 1995). The second class of translation products was less well defined and might include most, if not all, house-keeping mRNAs in the prothoracic gland. Translation of this group of proteins was detected by increases in the acid-precipitable, radiolabeled proteins and not by particular pattern changes discernible in protein bands following SDS-PAGE. This second class of translation products has been considered to reflect a trophic, nonsteroidogenic effect of PTTH (Rybczynski and Gilbert, 1994) and does not appear to be an obligate result of PTTH stimulation of prothoracic glands. For instance, PTTH stimulation of *Manduca* V₁ glands did not measurably increase overall protein synthesis while the increases in translation of specific proteins and in ecdysteroid synthesis were readily seen (Rybczynski and Gilbert, 1994). Furthermore, the Ca²⁺ ionophore A23187 stimulated ecdysteroid and specific protein synthesis in V₂ and V₃ glands but had no effect (V₂) or an inhibitory effect (–50%; V₃) on general, trophic protein synthesis. Simultaneous stimulation with both A23187 and dibutyryl cAMP, admittedly a pharmacologically complex situation, likewise stimulated both ecdysteroid and specific protein synthesis while decreasing general protein synthesis below basal on all 3 days studied (V₁–V₃) (Rybczynski and Gilbert, 1994). Whether the trophic effect of PTTH occurs beyond stages V₂ and V₃ is currently unknown; it would not be surprising if the effect was minimal during periods when the prothoracic gland is not growing appreciably, e.g., V₅ to P₀.

It seems likely that PTTH also stimulates transcription in the prothoracic gland that is not linked to acute regulation of ecdysteroid synthesis. Again, if parallels between vertebrate and insect steroidogenic tissues hold true, it is expected that PTTH stimulation of prothoracic glands would upregulate transcription of mRNAs that code for the enzymes that synthesize ecdysteroids from cholesterol, as well as ancillary, supportive proteins like adrenodoxin reductase (see Simpson and Waterman, 1988; Orme-Johnson, 1990). The recent cloning of several of the cytochrome P450s that are involved in ecdysteroid synthesis in *Drosophila* should allow this issue to be addressed in the near future. This

transcriptional trophic effect, like that of translation, may be limited to certain developmental periods. A lack of effect would not be surprising in the period immediately preceding prothoracic gland apoptosis in those species where such programmed cell death occurs (see Section 6.5.4).

6.5.2. Regulators of the Prothoracic Gland other than PTTH

The prothoracic gland, like other internal organs, is bathed in hemolymph and is potentially regulated by any number of the hormones found in the hemolymph, in addition to PTTH. The influence of the juvenile hormones has been discussed (see Section 6.3.3; and Chapters 8 and 9) but it bears repeating that the evidence is weak for direct regulation of prothoracic gland physiology by JHs. Aside from JHs and the ecdysteroids that are covered in Section 6.5.3, most, if not all, of these hormones are peptides or small proteins.

Several prothoracicotropic factors, which were partially purified from lepidopteran proctodea, are discussed briefly in Section 6.2.5. Rather more far afield was the observation that an ovarian-derived dipteran oostatic factor (trypsin modulating oostatic factor or TMOF) appears to exert dose-dependent stimulatory and inhibitory effects on ring gland and prothoracic gland basal and PTTH-stimulated ecdysteroid production (Hua and Koolman, 1995; Hua *et al.*, 1999; Gelman and Borovsky, 2000). The ecdysiostatic effect of TMOF, a hexapeptide, on *C. vicina* ring glands was attributed to a TMOF-dependent rise in cAMP, with the latter being found to be inhibitory of basal ecdysteroidogenesis (Hua and Koolman, 1995). However, an earlier study showed that dibutyryl cAMP stimulated *Calliphora* ring gland ecdysteroidogenesis (Richard and Saunders, 1987) and it is not currently possible to reconcile these conflicting observations about cAMP. How TMOF might exert a prothoracicotropic effect rather than an ecdysiostatic effect under some conditions is also not known. Thus, these observations are intriguing but the *in vivo* significance of TMOF in regard to prothoracic gland function remains to be rigorously determined.

Another small molecule, a nonapeptide termed prothoracicotropic peptide (PTSP), has been purified from *Bombyx* brain (Hua *et al.*, 1999). This peptide, identical in sequence to a myoinhibitory peptide first isolated from *Manduca* ventral nerve cord (Mas-MIP I: Blackburn *et al.*, 1995), was able to inhibit basal (Hua *et al.*, 1999; Dedos *et al.*, 2001) and PTTH-stimulated (Hua *et al.*, 1999) ecdysteroidogenesis *in vitro* in a dose-dependent manner. PTSP

appears to block “basal” Ca^{2+} influx via dihydropyridine-sensitive channels (Dedos *et al.*, 2001; Dedos and Birkenbeil, 2003) but PTSP did not block PTHH-stimulated Ca^{2+} influx (Dedos and Birkenbeil, 2003). It is difficult to reconcile the latter observation with the finding that PTSP blocked PTHH-stimulated ecdysteroid synthesis (Hua *et al.*, 1999), given that PTHH-stimulated ecdysteroid synthesis requires Ca^{2+} influx. Furthermore, rather large doses were required (≥ 100 nM) to achieve an ecdysteroidogenic inhibition relative to the dose needed for myoinhibition (1 nM; Blackburn *et al.*, 1995). Davis *et al.* (2003), using immunohistochemical techniques, have found that an apparent release of MIP I (PTSP) from *Manduca* epitracheal glands appears to coincide with the rapid drop in ecdysteroid hemolymph titer that occurs just prior to larval ecdysis. This intriguing observation deserves confirmation, e.g., via studies under long-day conditions, where this ecdysteroid decline occurs approximately 24 h before ecdysis, or in the fifth instar, where ecdysteroid levels begin a fall several days before pupal ecdysis (Bollenbacher *et al.*, 1981; Grieneisen *et al.*, 1993). Given these data calling into question the *in vivo* function of this peptide, it seems safest to consider PTSP a candidate prothoracostatic factor, pending further studies. If PTSP does occur *in vivo* at high nanomolar concentrations, it is likely to have wide effects beyond the prothoracic gland, i.e., many cell types undoubtedly express dihydropyridine-sensitive Ca^{2+} channels the inhibition of which would affect cellular function throughout the organism.

The changing electrical properties of prothoracic glands during diapause were discussed above in regard to *Manduca* PTHH (see Section 6.3.6). However, the electrical properties of prothoracic glands may be important in another context, i.e., as a factor in species wherein the prothoracic gland is innervated. Innervation of prothoracic glands is widely distributed among taxa, but it is not clear that it is a universal feature within any given insect group, e.g., the Lepidoptera contain species with and without innervation (see Sedlak, 1985; references in Okajima and Watanabe, 1989). The role of innervation in chronic or acute regulation of ecdysteroid synthesis has not been well studied. In *P. americana*, the activity of a nerve originating from the prothoracic ganglion was correlated positively with the secretion of ecdysteroids from the prothoracic gland (Richter and Gersch, 1983) but experimental discharge of this nerve did not have a significant effect on ecdysteroidogenesis (Richter, 1985). Sectioning the prothoracic gland nerve of last instar larvae prevented the normal ecdysteroid peak seen

between days 20 and 24 but the subsequent larger peak seen after day 26 was unaffected. These observations suggest a bimodal control of *Periplaneta* ecdysteroid synthesis in this instar, with the second peak presumed to be controlled solely by PTHH. However, a role for PTHH in the first peak cannot be ruled out, i.e., neuronal input could control the reactive state of the gland, in regard to PTHH, without directly regulating ecdysteroidogenesis.

In the moth *Mamestra*, the neurons innervating the prothoracic gland are inhibitory in regard to basal ecdysteroid synthesis but their effect on PTHH-stimulated synthesis is not known (Okajima and Watanabe, 1989; Okajima and Kumagai, 1989; Okajima *et al.*, 1989). Conflicting data on the role of innervation complicates any attempt to generalize at this time. For instance, Alexander (1970) proposed an inhibitory role in *Galleria* for the prothoracic gland nerve originating in the subesophageal ganglion while Mala and Sehnal (1978) argued for an excitatory (steroidogenic) influence. Neuronal input to the prothoracic gland may also originate in the brain. In both *S. crassipalpis* and *Drosophila*, species possessing the compound endocrine structure termed the ring gland, histochemical studies using back-filling techniques and reporter genes revealed that cells of the lateral protocerebrum directly innervate the ring gland (Giebultowicz and Denlinger, 1985; Siegmund and Korge, 2001). In the case of *Drosophila*, the identified cells appear to be neurosecretory, based on size and distinctive morphology. In *Sarcophaga*, several of these cells, presumably also neurosecretory, terminated in the corpus allatum region of the ring gland, as found for the PTHH-producing cells of lepidopterans. *Drosophila*, like *Sarcophaga*, contained several cells in the lateral protocerebrum that innervated the corpus allatum region. However, *Drosophila* also contained a pair of cells that innervated the prothoracic gland region of the ring gland. This observation suggests that if the neuronal cells in question regulate ecdysteroid synthesis, such regulation may not proceed as in the Lepidoptera, i.e., a soluble PTHH, released into the general circulation, might not be needed. In fact, a *Drosophila* PTHH with homology to the lepidopteran molecules might be completely lacking and its function taken over by another signaling molecule, perhaps even one that is considered a conventional neurotransmitter.

Clearly, the part that prothoracic gland innervation plays in affecting steroid synthesis deserves further attention. At present, based on structural studies, it seems likely that direct nervous input from ganglia is primarily via peptides rather than

neurotransmitters (see Sedlak, 1985). Peptidergic input may reflect a chronic or modulatory role for neuronal input in the control of prothoracic gland function; the possibility that such peptides may serve as trophic factors important for gland survival as seen for vertebrate nerve growth factor (see Levi-Montalcini *et al.*, 1996) is an intriguing and unexplored topic.

The changes in prothoracic gland cell size, protein content, and ecdysteroidogenic potential that have been documented during larval and pupal–adult development may be influenced heavily by the trophic influences of PTTH and, in some species, neurotrophic factors, as discussed above. Additional strong candidates for the regulation of prothoracic gland biology during development are the insect insulin-like proteins (ILPs). As discussed earlier (see Section 6.2.5), insects have a number of such genes (seven in *Drosophila*; Brogiolo *et al.*, 2001) and at least four lines of evidence suggest that one or more ILPs might affect prothoracic gland biology. First, it has been known for decades that insulin is helpful or even necessary for the growth (division) of some insect cells in culture (Mosna and Barigozzi, 1975; Davis and Shearn, 1977). Second, the cells of the prothoracic gland undergo large changes in size during development and the insect insulin pathway is involved in regulating cell size, not only cell cycling. For instance, a mutation in the *Drosophila* insulin receptor substrate gene *Chico* yields flies that are small because their cells are small (see Goberdhan and Wilson, 2003). Third, antibody studies suggest that the prothoracic gland expresses an insulin receptor (Smith *et al.*, 1997; see also Section 6.4.3). Fourth, there is precedence for insulin control of ecdysteroid synthesis, although in the mosquito ovary and not the prothoracic gland (Graf *et al.*, 1997; Riehle and Brown, 1999). The idea that insulin affects prothoracic glands in some way must be tested rigorously. Especially useful will be the presumed future availability of authentic insect insulin-like molecules, their use in well-characterized *in vitro* experimental regimes, and the determination of the titers of insulin-like proteins in the hemolymph during larval and pupal–adult development.

6.5.3. Ecdysteroid Feedback on Ecdysteroidogenesis in the Prothoracic Gland

The rise and fall of circulating hormone levels is characteristic of many endocrine signaling events, including the coordination of larval and pupal–adult development of insects by ecdysteroids. Several processes contribute to the regulation of circulating levels of active hormone, e.g., secretion

rates, metabolism to inactive forms, sequestration, and excretion. The secretion rate is itself the product of multiple variables, namely: substrate availability, maximum biochemical synthesis rates, stimulation of the producing tissue by a trophic factor, product storage, presence of inhibitory factors, product feedback, etc.

Ecdysteroid feedback on the prothoracic gland, both positive and negative, can proceed through at least two paths. First, ecdysteroids might directly interact with the prothoracic gland, potentially resulting in rapid or slow changes in the PTTH signaling cascade or the ecdysteroid biosynthetic pathway. Second, ecdysteroids might act indirectly, by modifying the behavior of another tissue, which then, through a nonecdysteroid pathway, modulates the biology of the prothoracic gland.

Only a limited amount of data has been gathered to test the hypothesis that the prothoracic gland is subject to both rapid and delayed feedback from ecdysteroids. Sakurai and Williams (1989) showed that incubating larval *Manduca* prothoracic glands for 24 h with low concentrations of 20E (e.g., 0.20 μM) resulted in an inhibition of 20% to 60% of basal ecdysteroid synthesis. In contrast, pupal glands generally experienced a stimulation of synthesis. This was true of prothoracic glands from both nondiapausing and diapausing pupae but the effect of PTTH on these glands was not evaluated. A study of the effect of shorter 20E incubations on basal and PTTH-stimulated *Manduca* glands revealed that when a higher dose (10 μM) of 20E was employed a very significant inhibition of basal ecdysteroidogenesis ($\approx 75\%$) was seen after only 1 h of incubation (Song and Gilbert, 1998); note that in these experiments, prothoracic glands were preincubated with exogenous 20E but their ecdysteroid production was measured subsequently in medium lacking the supplemental 20E. Somewhat surprisingly, this regime of pretreatment with 20E had no effect on PTTH-stimulated ecdysteroid synthesis, relative to controls not incubated with 10 μM 20E, unless the preincubation was for more than 3 h. These observations suggest that the repression of basal ecdysteroid synthesis after short incubations was due to a reversible block in the steroidogenic pathway that could be overcome by PTTH stimulation. What constitutes this block is open to speculation. The data currently in hand suggest that the PTTH signal transduction pathway is not measurably active in regulating steroidogenesis in the absence of PTTH. Perhaps the rapid 20E effect on basal steroidogenesis was due to direct, competitive inhibition by 20E of some step in ecdysteroid

synthesis. This possibility could be tested by using lower concentrations of 20E and non-steroidal compounds that bind to the ecdysone receptor but that would not be expected to interact with the biosynthetic enzymes involved in ecdysteroidogenesis. In fact, Jiang and Koolman (1999) used the non-steroidal ecdysone agonist RH-5849 in such a test, using *C. vicina* ring glands. Their results revealed that RH-5849 inhibited basal ecdysone secretion rapidly, with more than 50% inhibition seen within 30 min of incubation. These data suggest that ecdysteroid feedback on the prothoracic gland involved regulation of transcription or nongenomic effects, perhaps mediated through second messenger signaling initiated via a plasma membrane ecdysteroid receptor (see Chapter 8). How such signaling might interact with the basal synthesis pathway is unknown; it is possible that RH-5849 interferes with the synthesis pathway in a manner unrelated to its ecdysone agonist effect.

Longer incubations (>3 h) with 20E, resulted in the downregulation of steroidogenesis which was not fully reversible with PTTH (brain extract) treatment (Song and Gilbert, 1998). This effect seems likely to be due to changes in transcription and translation, and this hypothesis should be testable using newly, or soon-to-be, available molecular probes against the transcripts coding for the ecdysteroidogenic enzymes. Incubation of *Manduca* prothoracic glands with 10 μ M 20E altered the expression pattern of USP proteins within 6 h (Song and Gilbert, 1998). USP is an obligate partner to the EcR protein in forming the functional ecdysteroid receptor (see Chapter 7), and this change might be a sign of an altered transcription pattern in the gland. The possibility that components of the PTTH signaling pathway were also downregulated cannot be ruled out and this supposition could also be tested using molecular probes, or by using commercially available antibodies to assess protein levels and the phosphorylation states thereof.

The above data suggested that 20E levels directly regulated basal and PTTH-stimulated ecdysteroid synthesis by the prothoracic gland. An indirect type of 20E feedback on prothoracic gland activity, involving one or more intermediate tissues, is also a possibility. In *Bombyx*, early fifth instar glands are refractory to PTTH stimulation *in vitro* (see Sections 6.3.3 and 6.5.1). Injection of 0.5–4.0 μ g 20E into intact *Bombyx* larvae also resulted in partial inhibition of basal ecdysteroid synthesis at the higher doses, when glands from treated animals were later assayed *in vitro* (Takaki and Sakurai, 2003), but a consistent effect of 20E injection on PTTH-stimulated ecdysteroid synthesis was not

seen. However, when the brain and corpora allata were removed from larvae prior to control (water) or 20E injections, somewhat different results were obtained. First, at stages where basal ecdysteroid synthesis was expected to have declined between the treatment and sampling periods in response to exogenous 20E, such declines were absent or blunted. Second, when such glands were challenged with PTTH, the gland activation was larger than expected. These results suggested that 20E might downregulate prothoracic gland activity via stimulating the release of an inhibitory brain or corpus allatum factor (see Section 6.5.2). Whether or not both direct and indirect feedback of 20E on prothoracic glands occurs in nonmanipulated animals is an open question. The effects seen upon removal of the brain and corpora allata are suggestive, but this operation removes a Pandora's box of cell types, neuronal connections, and hormones, and the results stemming from such an operation must be interpreted with care.

6.5.4. Apoptosis of the Prothoracic Gland

Programmed cell death or apoptosis is a normal component of development in multicellular organisms, including insects, and involves a complex, multistep cascade of intracellular events (see Vernooy *et al.*, 2000). The only demonstrated function of the prothoracic glands is to produce ecdysteroids and thus to control and coordinate the molting process. It is not surprising, therefore, that prothoracic glands undergo apoptosis during pupal–adult development or early in adult life, once sufficient ecdysteroids have been produced to accomplish this last molt. Programmed cell death of the prothoracic glands occurs in insects that possess a structurally distinct gland, like Lepidoptera, as well as in species possessing a ring gland, where the prothoracic gland is part of a composite, multi-tissue organ.

In *Drosophila*, the degeneration of the prothoracic gland portion of the ring gland during pupal–adult development takes place at a time when whole animal ecdysteroid titers are high and production of ecdysteroids *in vitro* is low (Dai and Gilbert, 1991). This observation led to the suggestion that the source of ecdysteroids at this time was either another tissue, e.g., oenocytes, or the conversion of previously produced hormone from an inactive, conjugate to an active form, with the conjugate not being recognized by the radioimmunoassay employed to assess steroid hormone levels (Dai and Gilbert, 1991). Given the uncertainties about the control of ecdysteroid synthesis in *Drosophila*

discussed earlier in this chapter, it is also possible that the production *in vitro* is not an accurate index of steroidogenesis *in vivo*; however, this hypothesis presupposes that a prothoracic gland that is undergoing apoptosis is still capable of considerable ecdysteroid synthesis.

The structural and hormonal correlates of the programmed cell death of the *Manduca* prothoracic gland, a “free-standing” tissue, have been addressed by Dai and Gilbert (1997, 1998, 1999). In this species, apoptosis of the gland was initiated on day 6 (P_6) of pupal–adult development (Dai and Gilbert, 1997). P_6 is approximately 2 days after the pupal–adult peak of ecdysteroidogenesis and is a time when fat body and hemocytes begin to envelop the gland (Dai and Gilbert, 1997). Ecdysteroid synthesis fell rapidly after P_6 , as the glands continued to degenerate, and by P_{14} , only cellular debris was present.

The external factors controlling apoptosis of the prothoracic gland have been partially determined. The absence of juvenile hormones at the beginning of pupal–adult development was originally believed to be an enabling condition (Wigglesworth, 1955; Gilbert, 1962) and subsequent data supported this proposal, i.e., injection of JH into pupae at this stage prevented gland death (Gilbert, 1962; Dai and Gilbert, 1997). The absence of JH was not sufficient to initiate apoptosis because prothoracic glands extirpated from early pupal stages and placed in JH-free culture conditions do not undergo apoptosis (Dai and Gilbert, 1999). Nevertheless, a hormonal basis for gland apoptosis was indicated because the addition of 20E to these cultures efficiently promoted programmed cell death, if the steroid exposure was ≥ 24 h. The 20E doses found to be effective *in vitro* were well within the physiological range seen in the days preceding normal, *in vivo* cell death. These cultured-gland experiments also revealed an extra level of complexity in the control of prothoracic gland apoptosis, namely, that JH did not protect glands *in vitro* against 20E-induced cell death (Dai and Gilbert, 1999). Thus, once again, the role of JHs in modulating prothoracic gland physiology was indirect, involving another tissue and probably another hormone. One might also conclude that 20E-induced apoptosis of the prothoracic gland represents the ultimate negative feedback loop for this steroid hormone-producing gland.

6.5.5. Parasitoids and Ecdysteroidogenesis

Insect parasitoids lay their eggs on or in host insects. Following hatching, the parasitoid develops until finally emerging, with the host generally dying as a

result of the infection. Parasitoids manipulate the physiology of the host organism to their advantage and this often includes disrupting the normal function of the host’s endocrine system (see Beckage, 1985, 1997, 2002). It must also be pointed out that parasitoid infections are biologically complex, involving not only the parasitoid, but also ovarian proteins, symbiotic viruses, and venoms. These additional factors can have major effects on the host’s physiology, even in the absence of the parasitoids.

Parasitoids utilize a wide variety of tactics to increase the suitability of their hosts as growth chambers and even a modest review of the subject is beyond the constraints of this chapter (see Beckage, 1985, 1997, 2002). However, several endocrine effects are frequent enough to deserve mention in the context of considering PTTH. One of the most common effects of parasitoid infestation appears to be elevated JH levels. These high titers result in developmental arrest of the host and stem from multiple factors, including increased secretion by the host, decreased degradation, and JH secretion by the parasitoid and perhaps by parasitoid-derived teratocytes (Cole *et al.*, 2002). Elevated levels of JH may act partly through blocking PTTH release since increased levels of PTTH in brains of parasitized *Manduca* have been observed (Žitňan *et al.*, 1995). In some Lepidoptera–parasitoid interactions, the prothoracic gland disintegrates, e.g., *H. virescens* parasitized by *Campoletis sonorensis*, an ichneumonid wasp (Dover and Vinson, 1990; Dover *et al.*, 1995), while in other species, the gland persists. In prothoracic glands from *Manduca* parasitized by *Cotesia congregata*, a braconid wasp, both basal and brain extract-stimulated ecdysteroid synthesis *in vitro* were lower than synthesis from glands of nonparasitized larvae (Kelly *et al.*, 1998). This is strongly reminiscent of the effect of experimentally augmented JH levels on prothoracic glands discussed in Section 6.3.3, and is consistent with a view that this parasitism interaction, which blocks development in the fifth instar, is essentially a juvenilizing event. Evidence from *in vitro* studies suggested that a portion of the elevated JH in this example was secreted by the parasitoid and did not simply result from decreased JH catabolism (Cole *et al.*, 2002). *In vivo* ecdysteroid levels at this time were not strongly suppressed relative to control early fifth instar larvae, but the high premolt peak seen normally in the second half of the fifth instar was absent. Ligation experiments revealed that it was likely that some ecdysteroids were secreted by the parasitoid *Cotesia* (Gelman *et al.*, 1998). A stronger suppression of ecdysteroid hemolymph titer has been described for precocious prepupae of

Trichoplusia ni parasitized by the wasp *Chelonus* near *curvimaculatus* (Jones *et al.*, 1992); in this interaction, an ecdysteroid peak observed shortly before the emergence of the parasitoid larvae was also believed to originate with the parasitoid.

Evidence that parasitism has a direct effect on the functioning of the prothoracic gland comes primarily from work on *H. virescens* parasitized by the braconid wasp *Cardiochiles nigriceps*. In this interaction, basal ecdysteroid synthesis by the glands was considerably depressed when measured *in vitro* (Pennacchio *et al.*, 1997, 1998a); JH levels were elevated and JH metabolism depressed in parasitized larvae during the first 4 days of the final larval instar (Li *et al.*, 2003). Such glands showed no activation when challenged with a crude PTTH extract or forskolin (an adenylate cyclase activator) or dibutyryl cAMP; but all three reagents stimulated ecdysteroid synthesis in control glands. Further investigation revealed several biochemical lesions downstream from cAMP generation (Pennacchio *et al.*, 1997, 1998a), including depressed basal protein synthesis and phosphorylation and a failure to increase protein phosphorylation and synthesis upon PTTH stimulation. No difference in RNA synthesis was found between control and parasite-derived glands (Pennacchio *et al.*, 1998a). Surprisingly, the effects of *Cardiochiles* parasitism on the prothoracic gland proved to be largely the result of coinfection with the *C. nigriceps* polydnavirus that is injected into the host along with the *Cardiochiles* eggs and venom (Pennacchio *et al.*, 1998b). Venoms also play a role in parasitoid manipulation of host prothoracic glands. The venom of the ectoparasitic wasp *Eulophus pennicornis*, when injected into larvae of the tomato moth (*Lacanobia oleraceae*) results in depressed basal ecdysteroid synthesis by prothoracic glands when assayed 48 h later and, furthermore, such glands failed to respond to forskolin with increased steroidogenesis (Marris *et al.*, 2001). However, incubation of prothoracic glands with venom extract for 3 h had no effect. This observation suggests that either venom requires a longer time to disrupt the PTTH transduction cascade, or that an intermediate tissue is involved in the venom effect.

These and similar observations suggest that the roles of viruses and venoms in manipulating host endocrine systems can be great. How parasitoids and their coevolved, symbiotic viruses influence the PTTH-prothoracic gland axes of their hosts is far from resolved and might well involve a number of tactics that include altering hormone synthesis and prohormone conversion by their hosts (Beckage, 1985), hormone release, and feedback systems.

6.6. PTTH: The Future

At the end of a review article, it is both a cliché and a truism to write phrases like “While we have learned a great deal about this topic in the last few years, there is clearly much left to be understood,” and “Although we have obtained the answers to many questions about this topic, the answers have raised even more questions.” These comments are certainly true about PTTH, and many unanswered and new questions about PTTH have been raised in this review. Finally, a few of these questions will be iterated and a few new ones added. The answers to these questions will require not only new methodologies and model systems, but also new ways of looking at familiar topics.

What we know about PTTHs derives chiefly from studies of Lepidoptera. Are there closely related molecules in all or most other taxa, or do other pathways function, e.g., total neuronal control of ecdysteroid synthesis? Similarly, our knowledge of PTTH signaling within the prothoracic gland comes almost entirely from lepidopteran studies. Are other, significantly different control mechanisms used? Certainly, the work on putative PTTH-containing extracts from *Drosophila* suggests that the control of ecdysteroid synthesis by hormones originating outside the prothoracic gland might proceed by novel means.

Even within the scope of lepidopteran studies, a great deal of the control pathway activated by PTTH remains to be determined. We have little knowledge of negative feedback mechanisms, and the function of the developmental changes seen in prepupal prothoracic glands is currently mainly a source of speculation. The necessity for new protein synthesis in PTTH-stimulated ecdysteroidogenesis is well established but the role of such protein(s) has not been determined. If one of these proteins facilitates steroid precursor movement, as in vertebrate steroidogenesis, what do the other proteins do? Another question concerns the complexity of PTTH signal transduction (see Figure 7). If the prothoracic gland's only function is to make ecdysteroids, why is the PTTH signaling cascade, which we know imperfectly, so complex? Are the multiple kinase networks simply cellular fossils that reflect a lack of selection to simplify, with layer upon layer of control mechanisms, as evolution sharpened the control of steroid synthesis?

If there might be proposed one final “big” question about PTTH, perhaps the best candidate would be “What else does PTTH do?” *Bombyx* PTTH was first purified from the heads of adult animals, long

after the prothoracic glands have disintegrated via programmed cell death. Is this PTTH reservoir an artifact of the domestication of *Bombyx*, or does PTTH modulate the physiology of other cell types, steroidogenic or not?

Insects comprise a very large fraction of the animal world with which mankind must coexist and compete with for the world's resources. We can only hope that the human resources to study these questions keep pace with our need to understand and adapt to this amazing class of animals.

References

- Abraham, R.T., 2002. Identification of TOR signaling complexes: more TORC for the cell growth engine. *Cell* 111, 9–12.
- Adachi-Yamada, T., Iwami, M., Kataoka, H., Suzuki, A., Ishizaki, H., 1994. Structure and expression of the gene for the prothoracicotropic hormone of the silkworm *Bombyx mori*. *Eur. J. Biochem.* 220, 633–643.
- Adashi, E.Y., 1994. Endocrinology of the ovary. *Hum. Reprod.* 9, 815–827.
- Agui, N., 1989. *In vitro* release of prothoracicotropic hormone (PTTH) from the cultured brain of *Mamestra brassicae* L.; effects of neurotransmitters on PTTH release. In: Mitsuhashi, J. (Ed.), *Invertebrate Cell System Applications*, vol. 1. CRC, Press Boca Raton, pp. 111–119.
- Agui, N., Bollenbacher, W.E., Gilbert, L.I., 1983. *In vitro* analysis of prothoracicotropic hormone specificity and prothoracic gland sensitivity in Lepidoptera. *Experientia* 39, 984–988.
- Agui, N., Bollenbacher, W.E., Granger, N.A., Gilbert, L.I., 1980. Corpus allatum is release site for insect prothoracicotropic hormone. *Nature* 285, 669–670.
- Agui, N., Granger, N.A., Bollenbacher, W.E., Gilbert, L.I., 1979. Cellular localization of the insect prothoracicotropic hormone: *In vitro* assay of a single neurosecretory cell. *Proc. Natl Acad. Sci. USA* 76, 5694–5698.
- Aizono, Y., Endo, Y., Sattelle, D.B., Shirai, Y., 1997. Prothoracicotropic hormone-producing neurosecretory cells in the silkworm, *Bombyx mori*, express a muscarinic acetylcholine receptor. *Brain Res.* 763, 131–136.
- Alexander, N.J., 1970. A regulatory mechanism of ecdysone release in *Galleria mellonella*. *J. Insect Physiol.* 16, 271–276.
- Allegret, P., 1964. Interrelationship of larval development, metamorphosis and age in a pyralid lepidopteran, *Galleria mellonella* (L.), under the influence of dietetic factors. *Exp. Gerontol.* 1, 49–66.
- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. *J. Mol. Evol.* 215, 403–410.
- Arbeitman, M.N., Hogness, D.S., 2003. Molecular chaperones activate the *Drosophila* ecdysone receptor, an RXR heterodimer. *Cell* 101, 67–77.
- Bain, J., McLauchlan, H., Elliott, M., Cohen, P., 2003. The specificities of protein kinase inhibitors: an update. *Biochem. J.* 371, 199–204.
- Baker, F.C., Tsai, L.W., Reuter, C.C., Schooley, D.A., 1987. *In vivo* fluctuation of JH, JH acid, and ecdysteroid titer, and JH esterase activity during development of fifth stadium *Manduca sexta*. *Insect Biochem.* 17, 989–996.
- Bean, D.W., Beck, S.D., 1983. Haemolymph ecdysteroid titres in diapause and nondiapause larvae of the European corn borer, *Ostrinia nubilalis*. *J. Insect Physiol.* 29, 687–693.
- Beckage, N.E., 1985. Endocrine interactions between endoparasitic insects and their hosts. *Annu. Rev. Entomol.* 30, 371–413.
- Beckage, N.E., 1997. New insights: how parasites and pathogens alter the endocrine physiology and development of insect hosts. In: Beckage, N.E. (Ed.), *Parasites and Pathogens: Effects on Host Hormones and Behavior*. Chapman and Hall, New York, pp. 3–36.
- Beckage, N.E., 2002. Parasite- and pathogen-mediated manipulation of host hormones and behavior. In: Pfaff, D., Arnold, A., Etgen, A., Fahrbach, S., Rubin, R. (Eds.), *Hormones, Brain, and Behavior*, vol. 3. Academic Press, New York, pp. 281–315.
- Beckel, W.E., Friend, W., 1964. The relation of abdominal distension and nutrition to molting in *Rhodnius prolixus* (Stahl) (Hemiptera). *Can. J. Zool.* 42, 71–78.
- Berridge, M.J., Lipp, P., Bootman, M.D., 2000. The versatility and universality of calcium signalling. *Nat. Rev.: Mol. Cell Biol.* 1, 11–21.
- Biggs, III, W.H., Zipursky, S.L., 1992. Primary structure, expression, and signal-dependent tyrosine phosphorylation of a *Drosophila* homolog of extracellular signal-regulated kinase. *Proc. Natl Acad. Sci. USA* 89, 6295–6299.
- Birkenbeil, H., 1996. Involvement of calcium in prothoracicotropic stimulation of ecdysone synthesis in *Galleria mellonella*. *Arch. Insect Biochem. Physiol.* 33, 39–52.
- Birkenbeil, H., 1999. Intracellular calcium in PTTH-stimulated prothoracic glands of *Manduca sexta* (Lepidoptera: Sphingidae). *Eur. J. Entomol.* 96, 295–298.
- Birkenbeil, H., 2000. Pharmacological study of signal transduction during stimulation of prothoracic glands from *Manduca sexta*. *J. Insect Physiol.* 46, 1409–1414.
- Birkenbeil, H., Dedos, S.G., 2002. Ca²⁺ as second messenger in PTTH-stimulated prothoracic glands of the silkworm, *Bombyx mori*. *Insect Biochem. Mol. Biol.* 32, 1625–1634.
- Blackburn, M.B., Wagner, R.M., Kochansky, J.P., Harrison, D.J., Thomas-Laemont, P., et al., 1995. The identification of two myoinhibitory peptides, with sequence similarities to the galanins, isolated from the ventral nerve cord of *Manduca sexta*. *Regulat. Pept.* 57, 213–219.
- Böhm, S.K., Grady, E.F., Bunnnett, N.W., 1997. Regulatory mechanisms that modulate signalling by G-protein-coupled receptors. *Biochem. J.* 322, 1–18.

- Bollenbacher, W.E., Agui, N., Granger, N.A., Gilbert, L.I., 1979. *In vitro* activation of insect prothoracic glands by the prothoracicotropic hormone. *Proc. Natl Acad. Sci. USA* 76, 5148–5152.
- Bollenbacher, W.E., Gilbert, L.I., 1981. Neuroendocrine control of postembryonic development in insects. In: Farner, D.S., Lederer, K. (Eds.), *Neurosecretion*. Plenum, New York, pp. 361–370.
- Bollenbacher, W.E., Granger, N.A., 1985. Endocrinology of the prothoracicotropic hormone. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 7. Pergamon, New York, pp. 109–151.
- Bollenbacher, W.E., Granger, N.A., Katahira, E.J., O'Brien, M.A., 1987. Developmental endocrinology of larval moulting in the tobacco hornworm, *Manduca sexta*. *J. Exp. Biol.* 128, 175–192.
- Bollenbacher, W.E., Katahira, E.J., O'Brien, M.A., Gilbert, L.I., Thomas, M.K., et al., 1984. Insect prothoracicotropic hormone: evidence for two molecular forms. *Science* 224, 1243–1245.
- Bollenbacher, W.E., O'Brien, M.A., Katahira, E.J., Gilbert, L.I., 1983. A kinetic analysis of the action of the insect prothoracicotropic hormone. *Mol. Cell. Endocrinol.* 32, 27–46.
- Bollenbacher, W.E., Smith, S.L., Goodman, W., Gilbert, L.I., 1981. Ecdysteroid titer during larval-pupal development of the tobacco hornworm, *Manduca sexta*. *Gen. Comp. Endocrinol.* 44, 302–306.
- Bowen, M.F., Bollenbacher, W.E., Gilbert, L.I., 1984a. *In vitro* studies on the role of the brain and prothoracic glands in the pupal diapause of *Manduca sexta*. *J. Exp. Biol.* 108, 9–24.
- Bowen, M.F., Gilbert, L.I., Bollenbacher, W.E., 1986. Endocrine control of insect diapause: an *in vitro* analysis. *In Vitro Invert. Horm. Genes* C210, 1–14.
- Bowen, M.F., Saunders, D.S., Bollenbacher, W.E., Gilbert, L.I., 1984b. *In vitro* reprogramming of the photoperiodic clock in an insect brain-retrocerebral complex. *Proc. Natl Acad. Sci. USA* 81, 5881–5884.
- Boyer, J.L., Graber, S.G., Waldo, G.L., Harden, T.K., Garrison, J.C., 1994. Selective activation of phospholipase C by recombinant G-protein α - and $\beta\gamma$ -subunits. *J. Biol. Chem.* 269, 2814–2819.
- Broggiolo, W., Stocker, H., Ikeya, T., Rintelen, F., Fernandez, R., et al., 2001. An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control. *Curr. Biol.* 11, 213–221.
- Burt, E.T., 1938. On the corpora allata of dipterous insects II. *Proc. Royal Soc. Lond. B126*, 210–223.
- Camps, M., Nichols, A., Arkinstall, S., 2000. Dual specificity phosphatases: a gene family for control of MAP kinase function. *FASEB J.* 14, 6–16.
- Cann, M.J., Chung, E., Levin, L.R., 2000. A new family of adenylyl cyclase genes in the male germline of *Drosophila melanogaster*. *Devel. Genes Evol.* 210, 200–206.
- Carrow, G., Calabrese, R.L., Williams, C.M., 1981. Spontaneous and evoked release of prothoracicotropin from multiple neurohemal organs of the tobacco hornworm. *Proc. Natl Acad. Sci. USA* 78, 5866–5870.
- Chen, C.H., Gu, S.H., Chow, Y.S., 2001. Adenylyl cyclase in prothoracic glands during the last larval instar of the silkworm, *Bombyx mori*. *Insect Biochem. Mol. Biol.* 31, 659–664.
- Chippendale, G.M., 1984. Environmental signals, the neuroendocrine system, and the regulation of larval diapause in the southwestern corn borer, *Diatraea grandiosella*. *Ciba Found. Symp.* 104, 259–276.
- Chippendale, G.M., Yin, C.M., 1973. Endocrine activity retained in diapause insect larvae. *Nature* 246, 511–513.
- Chippendale, G.M., Yin, C.M., 1976. Diapause of the southwestern corn borer, *Diatraea grandiosella* Dyar (Lepidoptera: Pyralidae): effects of a juvenile hormone mimic. *Bull. Entomol. Res.* 66, 75–79.
- Clementi, E., Meldolesi, J., 1996. Pharmacological and functional properties of voltage-independent Ca^{2+} channels. *Cell Calcium* 19, 269–279.
- Coffer, P.J., 2000. Phosphatidylinositol 3-kinase signaling: a tale of two kinase activities. In: Woodgett, J. (Ed.), *Protein Kinase Functions*. Oxford University Press, Oxford, pp. 1–39.
- Cole, T.J., Beckage, N.E., Tan, F.F., Srinivasan, A., Ramaswamy, S.B., 2002. Parasitoid–host endocrine relations: self-reliance or co-optation? *Insect Biochem. Mol. Biol.* 32, 1673–1679.
- Combost, W.L., Gilbert, L.I., 1992. Polyamines modulate multiple protein phosphorylation pathways in the insect prothoracic gland. *Mol. Cell. Endocrinol.* 83, 11–19.
- Combettes-Souverain, M., Issad, T., 1998. Molecular basis of insulin action. *Diab. Metab.* 24, 477–489.
- Cornelius, G., Engel, M., 1995. Stress causes induction of MAP kinase-specific phosphatase and rapid repression of MAP kinase activity in *Drosophila*. *Cellular Sig.* 7, 611–615.
- Dai, J.-D., Gilbert, L.I., 1991. Metamorphosis of the corpus allatum and degeneration of the prothoracic gland during larval-pupal-adult transformation of *Drosophila melanogaster*: a cytophysiological analysis of the ring gland. *Devel. Biol.* 144, 309–326.
- Dai, J.-D., Gilbert, L.I., 1997. Programmed cell death of the prothoracic glands of *Manduca sexta* during pupal-adult metamorphosis. *Insect Biochem. Mol. Biol.* 27, 69–78.
- Dai, J.-D., Gilbert, L.I., 1998. Juvenile hormone prevents the onset of programmed cell death in the prothoracic glands of *Manduca sexta*. *Gen. Comp. Endocrinol.* 109, 155–165.
- Dai, J.-D., Gilbert, L.I., 1999. An *in vitro* analysis of ecdysteroid-elicited cell death in the prothoracic gland of *Manduca sexta*. *Cell Tissue Res.* 297, 319–327.
- Dai, J.-D., Mizoguchi, A., Gilbert, L.I., 1994. Immunoreactivity of neurosecretory granules in the brain-retrocerebral complex of *Manduca sexta* to heterologous antibodies against *Bombyx* prothoracicotropic hormone and bombyxin. *Invert. Reprod. Devel.* 26, 187–196.

- Dai, J.-D., Mizoguchi, A., Satake, S., Ishizaki, H., Gilbert, L.I., 1995. Developmental changes in the prothoracicotropic hormone content of the *Bombyx mori* brain-retrocerebral complex and hemolymph: analysis by immunogold electron microscopy, quantitative image analysis, and time-resolved fluoroimmunoassay. *Devel. Biol.* 171, 212–223.
- Danks, H.V., 1987. Insect dormancy: an ecological perspective. Biological Survey of Canada (Terrestrial Arthropods), Monograph Series I. Ottawa, pp. 439.
- Das, S., Maizels, E.T., DeManno, D., St. Clair, E., Adam, S.A., et al., 1996. A stimulatory role of cyclic adenosine 3',5'-monophosphate in follicle-stimulating hormone-activated mitogen-activated protein kinase signaling pathway in rat ovarian granulosa cells. *Endocrinology* 137, 967–974.
- Davis, N.T., Blackburn, M.B., Golubeva, E.G., Hildebrand, J.G., 2003. Localization of myoinhibitory peptide immunoreactivity in *Manduca sexta* and *Bombyx mori*, with indications that the peptide has a role in molting and ecdysis. *J. Exp. Biol.* 206, 1449–1460.
- Davis, K.T., Shearn, A., 1977. *In vitro* growth of imaginal disks from *Drosophila melanogaster*. *Science* 196, 438–440.
- Dedos, S.G., Birkenbeil, H., 2003. Inhibition of cAMP signalling cascade-mediated Ca^{2+} influx by a prothoracicotropic peptide (Mas-MIP I) via dihydropyridine-sensitive Ca^{2+} channels in the prothoracic glands of the silkworm, *Bombyx mori*. *Insect Biochem. Mol. Biol.* 33, 219–228.
- Dedos, S.G., Fugo, H., 1996. Effects of fenoxycarb on the secretory activity of the prothoracic glands in the fifth instar of the silkworm, *Bombyx mori*. *Gen. Comp. Endocrinol.* 104, 213–224.
- Dedos, S.G., Fugo, H., 1999a. Disturbance of adult ecdysis by fenoxycarb in the silkworm, *Bombyx mori*. *J. Insect Physiol.* 45, 257–264.
- Dedos, S.G., Fugo, H., 1999b. Interactions between Ca^{2+} and cAMP in ecdysteroid secretion from the prothoracic glands of *Bombyx mori*. *Mol. Cell. Endocrinol.* 154, 63–70.
- Dedos, S.G., Fugo, H., 1999c. Downregulation of the cAMP signal transduction cascade in the prothoracic glands is responsible for the fenoxycarb-mediated induction of permanent 5th instar larvae in *Bombyx mori*. *Insect Biochem. Mol. Biol.* 29, 723–729.
- Dedos, S.G., Fugo, H., 2001. Involvement of calcium, inositol-1,4,5 trisphosphate and diacylglycerol in the prothoracicotropic hormone-stimulated ecdysteroid synthesis and secretion in the prothoracic glands of *Bombyx mori*. *Zool. Sci.* 18, 1245–1251.
- Dedos, S.G., Fugo, H., Nagata, S., Takamiya, M., Kataoka, H., 1999. Differences between recombinant PTTH and crude brain extracts in cAMP-mediated ecdysteroid secretion from the prothoracic glands of the silkworm, *Bombyx mori*. *J. Insect Physiol.* 45, 415–422.
- Dedos, S.G., Nagata, S., Ito, J., Takamiya, M., 2001. Action kinetics of a prothoracicotropic peptide from *Bombyx mori* and its possible signaling pathway. *Gen. Comp. Endocrinol.* 122, 98–108.
- Dover, B.A., Tanaka, T., Vinson, S.B., 1995. Stadium-specific degeneration of host prothoracic glands by *Campoletis sonorensis* calyx fluid and its association with host ecdysteroid titers. *J. Insect Physiol.* 41, 947–955.
- Dover, B.A., Vinson, S.B., 1990. Stage-specific effects of *Campoletis sonorensis* parasitism on *Heliothis virescens* development and prothoracic glands. *Physiol. Entomol.* 15, 405–414.
- Dufner, A., Thomas, G., 1999. Ribosomal S6 kinase signaling and the control of translation. *Exp. Cell Res.* 253, 100–109.
- Endo, K., Fujimoto, Y., Kondo, M., Yamanaka, A., Watanabe, M., et al., 1997. Stage-dependent changes of the prothoracicotropic hormone (PTTH) activity of brain extracts and of the PTTH sensitivity of the prothoracic glands in the cabbage armyworm, *Mamestra brassicae*, before and during winter and aestival pupal diapause. *Zool. Sci.* 14, 127–133.
- Endo, K., Fujimoto, Y., Masaki, T., Kumagai, K., 1990. Stage-dependent changes in the activity of the prothoracicotropic hormone (PTTH) in the brain of the Asian comma butterfly, *Polygonia c-aureum* L. *Zool. Sci.* 7, 695–702.
- Erickson, R.L., 1991. Structure, expression, and regulation of protein kinases involved in the phosphorylation of ribosomal protein S6. *J. Biol. Chem.* 266, 6007–6010.
- Eusebio, E.J., Moody, W.J., 1986. Calcium-dependent action potentials in the prothoracic gland of *Manduca sexta*. *J. Exp. Biol.* 126, 531–536.
- Fain, M.J., Riddiford, L.M., 1976. Reassessment of the critical periods for prothoracicotropic hormone and juvenile hormone secretion in the larval molt of the tobacco hornworm *Manduca sexta*. *Gen. Comp. Endocrinol.* 30, 131–141.
- Fescemyer, H.W., Masler, E.P., Kelly, T.J., Lusby, W.R., 1995. Influence of development and prothoracicotropic hormone on the ecdysteroids produced *in vitro* by the prothoracic glands of female gypsy moth (*Lymantria dispar*) pupae and pharate adults. *J. Insect Physiol.* 41, 489–500.
- Feuilloley, M., Vaudry, H., 1996. Role of the cytoskeleton in adrenocortical cells. *Endocrine Rev.* 17, 269–288.
- Fujimoto, Y., Endo, K., Watanabe, M., Kumagai, K., 1991. Species-specificity in the action of big and small prothoracicotropic hormones (PTTHs) of four species of lepidopteran insects, *Mamestra brassicae*, *Bombyx mori*, *Papilio xuthus* and *Polygonia c-aureum*. *Zool. Sci.* 8, 351–358.
- Fujishita, M., Ishizaki, H., 1982. Temporal organization of endocrine events in relation to the circadian clock during larval-pupal development in *Samia cynthia ricini*. *J. Insect Physiol.* 28, 77–84.
- Fukuda, S., 1941. Role of the prothoracic gland in differentiation of the imaginal characters in the silkworm pupa. *Annotationes Zool. Japon.* 20, 9–13.

- Fullbright, G., Lacy, E.R., Bullesbach, E.E., 1997. The prothoracicotropic hormone bombyxin has specific receptors on insect ovarian cells. *Eur. J. Biochem.* 245, 774–780.
- Fumagalli, S., Thomas, G., 2000. S6 phosphorylation and signal transduction. In: Sonenberg, N., Hershey, J.W.B., Mathews, M.B. (Eds.), *Translational Control of Gene Expression*. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 695–717.
- Gazi, L., Lopez-Gimenez, J.F., Strange, P.G., 2002. Formation of oligomers by G protein-coupled receptors. *Curr. Opin. Drug Discov. Devel.* 5, 756–763.
- Gelman, D.B., Borovsky, D., 2000. *Aedes aegypti* TMOF modulates ecdysteroid production by prothoracic glands of the gypsy moth, *Lymantria dispar*. *Arch. Insect Biochem. Physiol.* 45, 60–68.
- Gelman, D.B., Beckage, N.E., 1995. Low molecular weight ecdysiotropins in proctodea of fifth instars of the tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphingidae), and hosts parasitized by the braconid wasp *Cotesia congregata* (Hymenoptera: Braconidae). *Eur. J. Entomol.* 92, 123–129.
- Gelman, D.B., Reed, D.A., Beckage, N.E., 1998. Manipulation of fifth-instar host (*Manduca sexta*) ecdysteroid levels by the parasitoid wasp *Cotesia congregata*. *J. Insect Physiol.* 44, 833–843.
- Gelman, D.B., Thyagaraja, B.S., Kelly, T.J., Masler, E.P., Bell, R.A., et al., 1991. The insect gut: a new source of ecdysiotropic peptides. *Experientia* 47, 77–80.
- Gelman, D.B., Thyagaraja, B.S., Kelly, T.J., Masler, E.P., Bell, R.A., et al., 1992. Prothoracicotropic hormone levels in brains of the European corn borer, *Ostrinia nubilalis*: diapause vs. the non-diapause state. *J. Insect Physiol.* 38, 383–395.
- Gibbs, D., Riddiford, L.M., 1977. Prothoracicotropic hormone in *Manduca sexta*. Localization by a larval assay. *J. Exp. Biol.* 66, 255–266.
- Giebultowicz, J.M., 2000. Molecular mechanism and cellular distribution of insect circadian clocks. *Annu Rev. Entomol.* 45, 769–793.
- Giebultowicz, J.M., Denlinger, D.L., 1985. Identification of neurons innervating the ring gland of the flesh fly larva, *Sarcophaga crassipalis* Macquart (Diptera: Sarcophagidae). *Int. J. Insect Morphol. Embryol.* 14, 155–161.
- Gilbert, L.I., 1962. Maintenance of the prothoracic gland by the juvenile hormone in insects. *Nature* 193, 1205–1207.
- Gilbert, L.I., Bollenbacher, W.E., Agui, N., Granger, N.A., Sedlak, B.J., et al., 1981. The prothoracicotropes: source of the prothoracicotropic hormone. *Am. Zool.* 21, 641–653.
- Gilbert, L.I., Combest, W.L., Smith, W.A., Meller, V.H., Rountree, D.B., 1988. Neuropeptides, second messengers and insect molting. *BioEssays* 8, 153–157.
- Gilbert, L.I., Rybczynski, R., Song, Q., Mizoguchi, A., Morreale, R., et al., 2000. Dynamic regulation of prothoracic gland ecdysteroidogenesis: *Manduca sexta* recombinant prothoracicotropic hormone and brain extracts have identical effects. *Insect Biochem. Mol. Biol.* 30, 1079–1089.
- Gilbert, L.I., Rybczynski, R., Tobe, S., 1996. Regulation of endocrine function leading to insect metamorphosis. In: Gilbert, L.I., Tata, J., Atkinson, B.R. (Eds.), *Metamorphosis: Post-Embryonic Reprogramming of Gene Expression in Insect and Amphibian Cells*. Academic Press, NY, pp. 59–107.
- Gilbert, L.I., Rybczynski, R., Warren, J.T., 2002. Control and biochemical nature of the ecdysteroidogenic pathway. *Annu Rev. Entomol.* 47, 883–916.
- Gilbert, L.I., Song, Q., Rybczynski, R., 1997. Control of ecdysteroidogenesis: activation and inhibition of prothoracic gland activity. *Invert. Neurobiol.* 3, 205–216.
- Girgenrath, S., Smith, W.A., 1996. Investigation of presumptive mobilization pathways for calcium in the steroidogenic action of big prothoracicotropic hormone. *Insect Biochem. Mol. Biol.* 26, 455–463.
- Goberdhan, D.C., Wilson, C., 2003. The functions of insulin signaling: size isn't everything, even in *Drosophila*. *Differentiation* 71, 375–397.
- Goltzene, F., Holder, F., Charlet, M., Meister, M., Oka, T., 1992. Immunocytochemical localization of Bombyx-PTTH-like molecules in neurosecretory cells of the brain of the migratory locust, *Locusta migratoria*. A comparison with neuroparsin and insulin-related peptide. *Cell Tissue Res.* 269, 133–140.
- Graf, R., Neuenschwander, S., Brown, M.R., Ackermann, U., 1997. Insulin-mediated secretion of ecdysteroids from mosquito ovaries and molecular cloning of the insulin receptor homologue from ovaries of bloodfed *Aedes aegypti*. *Insect Mol. Biol.* 6, 151–163.
- Granger, N.A., Bollenbacher, W.E., 1981. Hormonal control of insect metamorphosis. In: Gilbert, L.I., Frieden, E. (Eds.), *Metamorphosis*. 2nd edn. Plenum, New York, pp. 105–137.
- Gray, R.S., Muehleisen, D.P., Katahira, E.J., Bollenbacher, W.E., 1994. The prothoracicotropic hormone (PTTH) of the commercial silkworm, *Bombyx mori*, in the CNS of the tobacco hornworm, *Manduca sexta*. *Peptides* 15, 777–782.
- Grewal, S.S., York, R.D., Stork, P.J., 1999. Extracellular-signal-regulated kinase signalling in neurons. *Curr. Opin. Neurobiol.* 9, 544–553.
- Grieneisen, M.L., Warren, J.T., Gilbert, L.I., 1993. Early steps in ecdysteroid biosynthesis: evidence for the involvement of cytochrome P-450 enzymes. *Insect Biochem. Mol. Biol.* 23, 13–23.
- Gruetzmacher, M.C., Gilbert, L.I., Granger, N.A., Goodman, W., Bollenbacher, W.E., 1984a. The effect of juvenile hormone on prothoracic gland function during larval-pupal development of the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol.* 30, 331–340.
- Gu, S.-H., Chow, Y.-S., 1996. Regulation of juvenile hormone biosynthesis by ecdysteroid levels during the early stages of the last two larval instars of *Bombyx mori*. *J. Insect Physiol.* 42, 625–632.

- Gu, S.-H., Chow, Y.-S., Lin, F.-J., Wu, J.-L., Ho, R.-J., 1996. A deficiency in prothoracicotropic hormone transduction pathway during the early last larval instar of *Bombyx mori*. *Mol. Cell. Endocrinol.* 120, 99–105.
- Gu, S.-H., Chow, Y.-S., O'Reilly, D.R., 1998. Role of calcium in the stimulation of ecdysteroidogenesis by recombinant prothoracicotropic hormone in the prothoracic glands of the silkworm, *Bombyx mori*. *Insect Biochem. Mol. Biol.* 28, 861–867.
- Gu, S.-H., Chow, Y.-S., Yin, C.-M., 1997. Involvement of juvenile hormone in regulation of prothoracicotropic hormone transduction during the early last larval instar of *Bombyx mori*. *Mol. Cell. Endocrinol.* 127, 109–116.
- Gu, G.-G., Singh, S., 1997. Modulation of the dihydropyridine-sensitive calcium channels in *Drosophila* by a phospholipase C-mediated pathway. *J. Neurobiol.* 33, 265–275.
- Gujdar, A., Sipeki, S., Bander, E., Buday, L., Farago, A., 2003. Phorbol ester-induced migration of HepG2 cells is accompanied by intensive stress fibre formation, enhanced integrin expression and transient down-regulation of p21-activated kinase 1. *Cell. Signall.* 15, 307–318.
- Haluska, P., Adjei, A.A., 2001. Receptor tyrosine kinase inhibitors. *Curr. Opin. Invest. Drugs* 2, 280–286.
- Hamilton, S.E., McKinnon, L.A., Jackson, D.A., Goldman, P.S., Migeon, J.C., et al., 1995. Molecular analysis of the regulation of muscarinic receptor expression and function. *Life Sci.* 56, 939–943.
- Hanton, W.K., Watson, R.D., Bollenbacher, W.E., 1993. Ultrastructure of prothoracic glands during larval-pupal development of the tobacco hornworm, *Manduca sexta*: a reappraisal. *J. Morphol.* 216, 95–112.
- Hartfelder, K., Hanton, W.K., Bollenbacher, W.E., 1994. Diapause-dependent changes in prothoracicotropic hormone-producing neurons of the tobacco hornworm, *Manduca sexta*. *Cell Tissue Res.* 277, 69–78.
- Hayes, G.C., Muehleisen, D.P., Bollenbacher, W.E., Watson, R.D., 1995. Stimulation of ecdysteroidogenesis by small prothoracicotropic hormone: role of calcium. *Mol. Cell. Endocrinol.* 115, 105–112.
- Helfrich-Förster, C., Tauber, M., Park, J.H., Muhlig-Versen, M., Schneuwly, S., et al., 2000. Ectopic expression of the neuropeptide pigment-dispersing factor alters behavioral rhythms in *Drosophila melanogaster*. *J. Neurosci.* 20, 3339–3353.
- Henrich, V.C., 1995. Comparison of ecdysteroid production in *Drosophila* and *Manduca*: pharmacology and cross-species neural reactivity. *Arch. Insect Biochem. Physiol.* 30, 239–254.
- Henrich, V.C., Pak, M.D., Gilbert, L.I., 1987a. Neural factors that stimulate ecdysteroid synthesis by the larval ring gland of *Drosophila melanogaster*. *J. Comp. Physiol. B* 157, 543–549.
- Henrich, V.C., Rybczynski, R., Gilbert, L.I., 1999. Peptide hormones, steroid hormones and puffs: mechanisms and models in insect development. *Vit. Horm.* 55, 73–125.
- Henrich, V.C., Tucker, R.L., Maroni, G., Gilbert, L.I., 1987b. The ecdysoneless (*ecd1ts*) mutation disrupts ecdysteroid synthesis autonomously in the ring gland of *Drosophila melanogaster*. *Devel. Biol.* 120, 50–55.
- Hua, Y.-J., Koolman, J., 1995. An ecdysiostatin from flies. *Regul. Pept.* 57, 263–271.
- Hua, Y.-J., Tanaka, Y., Nakamura, K., Sakakibara, M., Nagata, S., et al., 1999. Identification of a prothoracicotropic peptide in the larval brain of the silkworm, *Bombyx mori*. *J. Biol. Chem.* 274, 31169–31173.
- Huang, E.J., Reichardt, L.F., 2003. Trk receptors: roles in neuronal signal transduction. *Annu. Rev. Biochem.* 72, 609–642.
- Hynes, R.O., Zhao, Q., 2000. The evolution of cell adhesion. *J. Cell Biol.* 150, F89–F95.
- Ichikawa, M., Ishizaki, H., 1961. Brain hormone of the silkworm, *Bombyx mori*. *Nature* 191, 933–934.
- Ichikawa, M., Ishizaki, H., 1963. Protein nature of the brain hormone of insects. *Nature* 198, 308–309.
- Inoue, T., Thomas, J.H., 2001. Targets of TGF- β signaling in *Caenorhabditis elegans* dauer formation. *Devel. Biol.* 217, 192–204.
- Ishibashi, J., Kataoka, H., Isogai, A., Kawakami, A., Saegusa, H., et al., 1994. Assignment of disulfide bond location in prothoracicotropic hormone of the silkworm, *Bombyx mori*: a homodimeric protein. *Biochemistry* 33, 5912–5919.
- Ishizaki, H., Mizoguchi, A., Fujishita, M., Suzuki, A., Moriya, I., et al., 1983b. Species specificity of the insect prothoracicotropic hormone (PTTH): the presence of *Bombyx*- and *Samia*-specific PTTHs in the brain of *Bombyx mori*. *Devel. Growth Different.* 25, 593–600.
- Ishizaki, H., Suzuki, A., 1980. Prothoracicotropic hormone. In: Miller, T.A. (Ed.), *Neurohormonal Techniques in Insects*. Springer, New York, pp. 244–276.
- Ishizaki, H., Suzuki, A., 1984. Prothoracicotropic hormone of *Bombyx mori*. In: Hoffman, J., Porchet, M. (Eds.), *Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones*. Springer, New York, pp. 63–77.
- Ishizaki, H., Suzuki, A., 1992. Brain secretory peptides of the silkworm *Bombyx mori*: prothoracicotropic hormone and bombyxin. In: Joose, J., Buijs, R.M., Tilders, F.J.H. (Eds.), *Progress in Brain research*, vol. 92. Elsevier, Amsterdam, pp. 1–14.
- Ishizaki, H., Suzuki, A., 1994. The brain secretory peptides that control moulting and metamorphosis of the silkworm, *Bombyx mori*. *Int. J. Devel. Biol.* 38, 301–310.
- Ishizaki, H., Suzuki, A., Moriya, I., Mizoguchi, A., Fujishita, M., et al., 1983a. Prothoracicotropic hormone bioassay: pupal-adult *Bombyx* assay. *Devel. Growth Different.* 25, 585–592.
- Jayaraman, T., Ondrias, K., Ondriasova, E., Marks, A.R., 1996. Regulation of the inositol 1,4,5-trisphosphate

- receptor by tyrosine phosphorylation. *Science* 272, 1492–1494.
- Jenkins, S.P., Brown, M.R., Lea, A.O., 1992. Inactive prothoracic glands in larvae and pupae of *Aedes aegypti*: ecdysteroid release by tissues in the thorax and abdomen. *Insect Biochem. Mol. Biol.* 22, 553–559.
- Jhoti, H., McLeod, A.N., Blundell, T.L., Ishizaki, H., Nagasawa, H., *et al.*, 1987. Prothoracicotrophic hormone has an insulin-like tertiary structure. *FEBS Lett.* 219, 419–425.
- Jiang, R.-J., Koolman, J., 1999. Feedback inhibition of ecdysteroids: evidence for a short feedback loop repressing steroidogenesis. *Arch. Insect Biochem. Physiol.* 41, 54–59.
- Jones, D., Gelman, D., Loeb, M., 1992. Hemolymph concentrations of host ecdysteroids are strongly suppressed in precocious prepupae of *Trichoplusia ni* parasitized and pseudoparasitized by *Chelonus near curvimaculatus*. *Arch. Insect Biochem. Physiol.* 21, 155–165.
- Kataoka, H., Nagasawa, H., Isogai, A., Ishizaki, H., Suzuki, A., 1991. Prothoracicotropic hormone of the silkworm, *Bombyx mori*: amino acid sequence and dimeric structure. *Agric. Biol. Chem.* 55, 73–86.
- Kataoka, H., Nagasawa, H., Isogai, A., Tamura, S., Mizoguchi, A., *et al.*, 1987. Isolation and partial characterization of prothoracicotropic hormone of the silkworm, *Bombyx mori*. *Agric. Biol. Chem.* 51, 1067–1076.
- Kawakami, A., Kataoka, H., Oka, T., Mizoguchi, A., Kimura-Kawakami, M., *et al.*, 1990. Molecular cloning of the *Bombyx mori* prothoracicotropic hormone. *Science* 247, 1333–1335.
- Kawasaki, H., Springett, G.M., Mochizuki, N., Toki, S., Nakaya, M., *et al.*, 1998. A family of cAMP-binding proteins that directly activate Rap1. *Science* 282, 2275–2279.
- Keightley, D.A., Lou, K.J., Smith, W.A., 1990. Involvement of translation and transcription in insect steroidogenesis. *Mol. Cell. Endocrinol.* 74, 229–237.
- Kelly, T.J., Gelman, D.B., Reed, D.A., Beckage, N.E., 1998. Effects of parasitization by *Cotesia congregata* on the brain-prothoracic gland axis of its host, *Manduca sexta*. *J. Insect Physiol.* 44, 323–332.
- Kelly, T.J., Kingan, T.G., Masler, C.A., Robinson, C.H., 1996. Analysis of the ecdysiotropic activity in larval brains of the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol.* 42, 873–880.
- Kelly, T.J., Masler, E.P., Thyagaraja, B.S., Bell, R.A., Imberski, R.B., 1992. Development of an *in vitro* assay for prothoracicotropic hormone of the gypsy moth, *Lymantria dispar* (L.) following studies on identification, titers and synthesis of ecdysteroids in last-instar females. *J. Comp. Physiol. B* 162, 81–87.
- Kiguchi, K., Agui, N., 1981. Ecdysteroid levels and developmental events during larval moulting in the silkworm, *Bombyx mori*. *J. Insect Physiol.* 27, 805–812.
- Kim, A.-J., Cha, G.-H., Kim, K., Gilbert, L.I., Lee, C.C., 1997. Purification and characterization of the prothoracicotropic hormone of *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA* 94, 1130–1135.
- Kingan, T.G., 1981. Purification of the prothoracicotropic hormone from the tobacco hornworm *Manduca sexta*. *Life Sci.* 28, 2585–2594.
- Kiriishi, S., Nagasawa, H., Kataoka, H., Suzuki, A., Sakurai, S., 1992. Comparison of the *in vivo* and *in vitro* effects of bombyxin and prothoracicotropic hormone on prothoracic glands of the silkworm, *Bombyx mori*. *Zool. Sci.* 9, 149–155.
- Kiriishi, S., Rountree, D.B., Sakurai, S., Gilbert, L.I., 1990. Prothoracic gland synthesis of 3-dehydroecdysone and its hemolymph 3b-reductase mediated conversion to ecdysone in representative insects. *Experientia* 46, 716–721.
- Kobayashi, M., Kimura, J., 1958. The “brain” hormone in the silkworm, *Bombyx mori* L. *Nature* 181, 1217.
- Kobayashi, M., Yamazaki, M., 1966. The proteinic brain hormone in an insect, *Bombyx mori* L. (Lepidoptera: Bombycidae). *Appl. Entomol. Zool.* 12, 53–60.
- Kondo, H., Ino, M., Suzuki, A., Ishizaki, H., Iwami, M., 1996. Multiple gene copies for bombyxin, an insulin-related peptide of the silkworm *Bombyx mori*: structural signs for gene rearrangement and duplication responsible for generation of multiple molecular forms of bombyxin. *J. Mol. Evol.* 259, 926–937.
- Kopeć, S., 1922. Studies on the necessity of the brain for the inception of insect metamorphosis. *Biol. Bull.* 42, 323–342.
- Krishnan, B., Levine, J.D., Lynch, M.K., Dowse, H.B., Funes, P., *et al.*, 2001. A new role for cryptochrome in a *Drosophila* circadian oscillator. *Nature* 411, 313–317.
- Kulesza, P., Lee, C.Y., Watson, R.D., 1994. Protein synthesis and ecdysteroidogenesis in prothoracic glands of the tobacco hornworm (*Manduca sexta*): stimulation by big prothoracicotropic hormone. *Gen. Comp. Endocrinol.* 93, 448–458.
- Kyriakis, J.M., 2000. Mammalian MAP kinase pathways. In: Woodgett, J. (Ed.), Protein Kinase Functions. Oxford University Press, Oxford, pp. 40–156.
- LaRochelle, W.J., Pierce, J.H., May-Siroff, M., Giese, N., Aaronson, S.A., 1992. Five PDGF B amino acid substitutions convert PDGF A to a PDGF B-like transforming molecule. *J. Biol. Chem.* 267, 17074–17077.
- Lee, C.Y., Lee, K.J., Chumley, P.H., Watson, C.J., Abdur-Rahman, A., *et al.*, 1995. Capacity of insect (*Manduca sexta*) prothoracic glands to secrete ecdysteroids: relation to glandular growth. *Gen. Comp. Endocrinol.* 100, 404–412.
- Leonardi, M.G., Cappellozza, S., Ianne, P., Cappellozza, L., Parenti, P., *et al.*, 1996. Effects of topical application of an insect growth regulator (fenoxycarb) on some physiological parameters on the fifth instar larvae of the silkworm *Bombyx mori*. *Comp. Biochem. Physiol.* 113B, 361–365.
- Lester, D.S., Gilbert, L.I., 1986. Developmental changes in choline uptake and acetylcholine metabolism in the

- larval brain of the tobacco hornworm, *Manduca sexta*. *Brain Res.* 391, 201–209.
- Lester, D.S., Gilbert, L.I., 1987. Characterization of acetylcholinesterase activity in the larval brain of *Manduca sexta*. *Insect Biochem.* 17, 99–109.
- Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., *et al.*, 1995. Protein tyrosine kinase PYK2 involved in Ca^{2+} -induced regulation of ion channel and MAP kinase functions. *Nature* 376, 737–745.
- Levi-Montalcini, R., Skaper, S.D., Dal Toso, R., Petrelli, L., Leon, A., 1996. Nerve growth factor: from neurotrophin to neurokinine. *Trends Neurosci.* 19, 514–520.
- Lewis, T.S., Shapiro, P.S., Ahn, N.G., 1998. Signal transduction through MAP kinase cascades. *Adv. Cancer Res.* 74, 49–139.
- Li, S., Falabella, P., Kuriachan, I., Vinson, S.B., Borst, D.W., *et al.*, 2003. Juvenile hormone synthesis, metabolism, and resulting haemolymph titre in *Heliothis virescens* larvae parasitized by *Toxoneuron nigriceps*. *J. Insect Physiol.* 49, 1021–1030.
- Liebmann, C., 2001. Regulation of MAP kinase activity by peptide signalling pathway: paradigms of multiplicity. *Cell. Signall.* 13, 777–785.
- Loeb, M.J., 1982. Diapause and development in the tobacco budworm, *Heliothis virescens*: a comparison of haemolymph ecdysteroid titres. *J. Insect Physiol.* 28, 667–673.
- Loh, Y.P., Brownstein, M.J., Gainer, H., 1984. Proteolysis in neuropeptide processing and other neural functions. *Annu. Rev. Neurosci.* 7, 189–222.
- Lonard, D.M., Bhaskaran, G., Dahm, K.H., 1996. Control of prothoracic gland activity by juvenile hormone in fourth instar *Manduca sexta* larvae. *J. Insect Physiol.* 42, 205–213.
- Luttrell, L.M., Daaka, Y., Lefkowitz, R.J., 1999. Regulation of tyrosine kinase cascades by G-protein-coupled receptors. *Curr. Opin. Cell Biol.* 11, 177–183.
- Mains, R.E., Dickerson, I.M., May, V., Stoffers, D.A., Perkins, S.N., *et al.*, 1990. Cellular and molecular aspects of peptide hormone biosynthesis. *Front. Neuroendocrinol.* 11, 52–89.
- Mala, J., Sehna, F., 1978. Role of the nerve cord in the control of prothoracic glands in *Galleria mellonella*. *Experientia* 34, 1233–1235.
- Mansfield, S.G., Cammer, S., Alexander, S.C., Muehleisen, D.P., Gray, R.S., *et al.*, 1998. Molecular cloning and characterization of an invertebrate cellular retinoic acid binding protein. *Proc. Natl Acad. Sci. USA* 95, 6825–6830.
- Marris, G.C., Weaver, R.J., Bell, J., Edwards, J.P., 2001. Venom from the ectoparasitoid wasp *Eulophus pennicornis* disrupts host ecdysteroid production by regulating host prothoracic gland activity. *Physiol. Entomol.* 26, 229–238.
- Martelli, A.M., Faenza, I., Billi, A.M., Fala, F., Cocco, L., *et al.*, 2003. Nuclear protein kinase C isoforms: key players in multiple cell functions? *Histol. and Histopathol.* 18, 1301–1312.
- Massagué, J., 1998. TGF- β signal transduction. *Annu. Rev. Biochem.* 67, 753–791.
- Masumura, M., Satake, S., Saegusa, H., Mizoguchi, A., 2000. Glucose stimulates the release of bombyxin, an insulin-related peptide of the silkworm *Bombyx mori*. *Gen. Comp. Endocrinol.* 118, 393–399.
- McDaniel, C.N., 1979. Hemolymph ecdysone concentrations in *Hyalophora cecropia* pupae, dauer pupae, and adults. *J. Insect Physiol.* 25, 143–145.
- Medler, K.F., Bruch, R.C., 1999. Protein kinase C β and δ selectively phosphorylate odorant and metabotropic glutamate receptors. *Chem. Senses* 24, 295–299.
- Meller, V.H., Combest, W.L., Smith, W.A., Gilbert, L.I., 1988. A calmodulin-sensitive adenylate cyclase in the prothoracic glands of the tobacco hornworm, *Manduca sexta*. *Mol. Cell. Endocrinol.* 59, 67–76.
- Meller, V.H., Gilbert, L.I., 1990. Occurrence, quaternary structure and function of G protein subunits in an insect endocrine gland. *Mol. Cell. Endocrinol.* 74, 133–141.
- Meller, V.H., Sakurai, S., Gilbert, L.I., 1990. Developmental regulation of calmodulin-dependent adenylate cyclase in an insect endocrine gland. *Cell Regul.* 1, 771–780.
- Mesce, K.A., Fahrback, S.E., 2002. Integration of endocrine signals that regulate insect ecdysis. *Front. Neuroendocrinol.* 23, 179–199.
- Meyuhaus, O., Hornstein, E., 2000. Translational control of TOP mRNAs. In: Sonenberg, N., Hershey, J.W.B., Mathews, M.B. (Eds.), *Translational Control of Gene Expression*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 671–693.
- Mizoguchi, A., 2001. Effects of juvenile hormone on the secretion of prothoracicotropic hormone in the last- and penultimate-instar larvae of the silkworm *Bombyx mori*. *J. Insect Physiol.* 47, 767–775.
- Mizoguchi, A., Dedos, S.G., Fugo, H., Kataoka, H., 2002. Basic pattern of fluctuation in hemolymph PTTH titers during larval-pupal and pupal-adult development of the silkworm, *Bombyx mori*. *Gen. Comp. Endocrinol.* 127, 181–189.
- Mizoguchi, A., Ishizaki, H., 1982. Prothoracic glands of the saturniid moth *Samia cynthia ricini* possess a circadian clock controlling gut purge timing. *Proc. Natl Acad. Sci. USA* 79, 2726–2730.
- Mizoguchi, A., Ohashi, Y., Hosoda, K., Ishibashi, J., Kataoka, H., 2001. Developmental profile of the changes in the prothoracicotropic hormone titer in hemolymph of the silkworm *Bombyx mori*: correlation with ecdysteroid secretion. *Insect Biochem. Mol. Biol.* 31, 349–358.
- Mizoguchi, A., Oka, T., Kataoka, H., Nagasawa, H., Suzuki, A., *et al.*, 1990. Immunohistochemical localization of prothoracicotropic hormone-producing cells in the brain of *Bombyx mori*. *Devel. Growth Different.* 32, 591–598.
- Monconduit, H., Mauchamp, B., 1998. Effects of ultra-low doses of fenoxycarb on juvenile hormone-regulated physiological parameters in the silkworm, *Bombyx mori* L. *Arch. Insect Biochem. Physiol.* 37, 178–189.

- Morfis, M., Christopoulos, A., Sexton, P.M., 2003. RAMPs: 5 years on, where to now? *Trends Pharmacol. Sci.* 24, 596–601.
- Morris, A.J., Scarlata, S., 1997. Regulation of effectors by G-protein α - and $\beta\gamma$ -subunits. Recent insights from studies of the phospholipase c- β isoenzymes. *Biochem. Pharmacol.* 54, 429–435.
- Mosna, G., Barigozzi, C., 1975. Stimulation of growth by insulin in *Drosophila* embryonic cells *in vitro*. *Experientia* 32, 855–856.
- Muehleisen, D.P., Gray, R.S., Katahira, E.J., Thomas, M.K., Bollenbacher, W.E., 1993. Immunoaffinity purification of the neuropeptide prothoracicotropic hormone from *Manduca sexta*. *Peptides* 14, 531–541.
- Mulye, H., Gordon, R., 1993. Effects of fenoxycarb, a juvenile hormone analogue, on lipid metabolism of the eastern spruce budworm, *Choristoneura fumiferana*. *J. Insect Physiol.* 39, 721–727.
- Myers, E.M., Yu, J., Sehgal, A., 2003. Circadian control of eclosion: interaction between a central and peripheral clock in *Drosophila melanogaster*. *Curr. Biol.* 13, 526–533.
- Nagata, K., Maruyama, K., Kojima, K., Yamamoto, M., Tanaka, M., *et al.*, 1999. Prothoracicotropic activity of SBRPs, the insulin-like peptides of the saturniid silkworm *Samia cynthia ricini*. *Biochem. Biophys. Res. Comm.* 266, 575–578.
- Nielsen, H., Engelbrecht, J., Brunak, S., von Heijne, G., 1997. A neural network method for identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Int. J. Neural Syst.* 8, 581–599.
- Nijhout, H.F., 1979. Stretch-induced molting in *Oncopeltus fasciatus*. *J. Insect Physiol.* 25, 277–281.
- Nijhout, H.F., 1981. Physiological control of molting in insects. *Am. Zool.* 21, 631–640.
- Nijhout, H.F., 1994. *Insect Hormones*. Princeton University Press, Princeton, NJ, pp. 267.
- Nijhout, H.F., Gunnert, L.W., 2002. Bombyxin is a growth factor for wing imaginal disks in Lepidoptera. *Proc. Natl Acad. Sci. USA* 99, 15446–15450.
- Nimi, S., Sakurai, S., 1997. Development changes in juvenile hormone and juvenile hormone acid titers in the hemolymph and *in-vitro* juvenile hormone synthesis by corpora allata of the silkworm, *Bombyx mori*. *J. Insect Physiol.* 43, 875–884.
- Noguti, T., Adachi-Yamada, T., Katagiri, T., Kawakami, A., Iwami, M., *et al.*, 1995. Insect prothoracicotropic hormone: a new member of the vertebrate growth factor superfamily. *FEBS Lett.* 376, 251–256.
- O'Brien, M.A., Granger, N.A., Agui, N., Gilbert, L.I., Bollenbacher, W.E., 1986. Prothoracicotropic hormone in the developing brain of the tobacco hornworm *Manduca sexta*: Relative amounts of two molecular forms. *J. Insect Physiol.* 32, 719–725.
- O'Brien, M.A., Katahira, E.J., Flanagan, T.R., Arnold, L.W., Haughton, G., *et al.*, 1988. A monoclonal antibody to the insect prothoracicotropic hormone. *J. Neurosci.* 8, 3247–3257.
- Offermanns, S., 2003. G-proteins as transducers in transmembrane signalling. *Prog. Biophys. Mol. Biol.* 83, 101–130.
- Okajima, A., Kumagai, K., 1989. The inhibitory control of prothoracic gland activity by the neurosecretory neurones in a moth, *Mamestra brassicae*. *Zool. Sci.* 6, 851–858.
- Okajima, A., Kumagai, K., Watanabe, M., 1989. The involvement of afferent chemoreceptive activity in the nervous regulation of the prothoracic gland in a moth, *Mamestra brassicae*. *Zool. Sci.* 6, 859–866.
- Okajima, A., Watanabe, M., 1989. Electrophysiological identification of neuronal pathway to the prothoracic gland and the change in electrical activities of the prothoracic gland innervating neurones during larval development of a moth, *Mamestra brassicae*. *Zool. Sci.* 6, 459–468.
- Okuda, M., Sakurai, S., Ohtaki, T., 1985. Activity of the prothoracic gland and its sensitivity to prothoracicotropic hormone in the penultimate and last-larval instar of *Bombyx mori*. *J. Insect Physiol.* 31, 455–461.
- O'Leary, P.D., Hughes, R.A., 2003. Design of potent peptide mimetics of brain-derived neurotrophic factor. *J. Biol. Chem.* 278, 25738–25744.
- Orikasa, C., Yamauchi, H., Nagasawa, H., Suzuki, A., Nagata, M., 1993. Induction of oocyte-nurse cell differentiation in the ovary by the brain during the initial stage of oogenesis in the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae). *Appl. Entomol. Zool.* 28, 303–311.
- Orme-Johnson, N.R., 1990. Distinctive properties of adrenal cortex mitochondria. *Biochim. Biophys. Acta* 1020, 213–231.
- Pak, J.-W., Chung, K.W., Lee, C.C., Kim, K., Namkoong, Y., *et al.*, 1992. Evidence for multiple forms of the prothoracicotropic hormone in *Drosophila melanogaster* and indication of a new function. *J. Insect Physiol.* 38, 167–176.
- Pak, M.D., Gilbert, L.I., 1987. A developmental analysis of ecdysteroids during the metamorphosis of *Drosophila melanogaster*. *J. Liq. Chromatog.* 10, 2591–2611.
- Palen, E., Traugh, J.A., 1987. Phosphorylation of ribosomal protein S6 by cAMP-dependent protein kinase and mitogen-stimulated S6 kinase differentially alters translation of globin mRNA. *J. Biol. Chem.* 262, 3518–3523.
- Papadopoulos, V., Amri, H., Boujrad, N., Cascio, C., Culty, M., *et al.*, 1997. Peripheral benzodiazepine receptor in cholesterol transport and steroidogenesis. *Steroids* 62, 21–28.
- Pearson, R.B., Thomas, G., 1995. Regulation of p70s6k/p85s6k and its role in the cell cycle. *Progr. Cell Cycle Res.* 1, 21–32.
- Pelc, D., Steel, C.G.H., 1997. Rhythmic steroidogenesis by the prothoracic glands of the insect *Rhodnius prolixus* in the absence of rhythmic neuropeptide input: implications for the role of prothoracicotropic hormone. *Gen. Comp. Endocrinol.* 108, 358–365.

- Pennacchio, F., Falabella, P., Sordetti, R., Varricchio, P., Malva, C., *et al.*, 1998a. Prothoracic gland inactivation in *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) larvae parasitized by *Cardiochiles nigriceps* Viereck (Hymenoptera: Braconidae). *J. Insect Physiol.* 44, 845–857.
- Pennacchio, F., Falabella, P., Vinson, S.B., 1998b. Regulation of *Heliothis virescens* prothoracic glands by *Cardiochiles nigriceps* polydnavirus. *Arch. Insect Biochem. Physiol.* 38, 1–10.
- Pennacchio, F., Sordetti, R., Falabella, P., Vinson, S.B., 1997. Biochemical and ultrastructural alterations in prothoracic glands of *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) last instar larvae parasitized by *Cardiochiles nigriceps* Viereck (Hymenoptera: Braconidae). *Insect Biochem. Mol. Biol.* 27, 439–450.
- Pieroni, J.P., Jacobowitz, O., Chen, J., Iyengar, R., 1993. Signal recognition and integration by Gs-stimulated adenylyl cyclases. *Curr. Opin. Neurobiol.* 3, 345–351.
- Raught, B., Gingras, A.C., Sonenberg, N., 2001. The target of rapamycin (TOR) proteins. *Proc. Natl Acad. Sci. USA* 98, 7037–7044.
- Redfern, C.P.F., 1983. Ecdysteroid synthesis by the ring gland of *Drosophila melanogaster* during late-larval, pre-pupal and pupal development. *J. Insect Physiol.* 29, 65–71.
- Rhee, S.G., 2001. Regulation of phosphoinositide-specific phospholipase C. *Annu. Rev. Biochem.* 70, 281–312.
- Richard, D.S., Gilbert, L.I., 1991. Reversible juvenile hormone inhibition of ecdysteroid and juvenile hormone synthesis by the ring gland of *Drosophila melanogaster*. *Experientia* 47, 1063–1066.
- Richard, D.S., Saunders, D.S., 1987. Prothoracic gland function in diapause and non-diapause destined *Sarcophaga argyrostoma* and *Calliphora vicina*. *J. Insect Physiol.* 33, 385–392.
- Richard, D.S., Warren, J.T., Saunders, D.S., Gilbert, L.I., 1987. Haemolymph ecdysteroid titres in diapause and non-diapause destined larvae and pupae of *Sarcophaga argyrostoma*. *J. Insect Physiol.* 33, 115–122.
- Richards, J.S., 1994. Hormonal control of gene expression in the ovary. *Endocr. Rev.* 15, 725–751.
- Richards, J.S., Russell, D.L., Ochsner, S., Hsieh, M., Doyle, K.H., *et al.*, 2002. Novel signaling pathways that control ovarian follicular development, ovulation, and luteinization. *Rec. Progr. Horm. Res.* 57, 195–220.
- Richter, K., 1985. Physiological investigations on the role of the innervation in the regulation of the prothoracic gland in *Periplaneta americana*. *Arch. Insect Biochem. Physiol.* 2, 319–329.
- Richter, K., 2001. Daily changes in neuroendocrine control of moulting hormone secretion in the prothoracic gland of the cockroach *Periplaneta americana* (L.). *J. Insect Physiol.* 47, 333–338.
- Richter, K., Gersch, M., 1983. Electrophysiological evidence of nervous involvement in the control of the prothoracic gland in *Periplaneta americana*. *Experientia* 39, 917–918.
- Richter, K., Peschke, E., Peschke, D., 2000. A neuroendocrine releasing effect of melatonin in the brain of an insect, *Periplaneta americana* (L.). *J. Pineal Res.* 28, 129–135.
- Riddiford, L.M., 1976. Hormonal control of insect epidermal cell commitment *in vitro*. *Nature* 259, 115–117.
- Riddiford, L.M., 1994. Cellular and molecular actions of juvenile hormone. I. General considerations and premetamorphic actions. *Adv. Insect Physiol.* 24, 213–274.
- Riddiford, L.M., 1996. Juvenile hormone: the status of its “status quo” action. *Arch. Insect Biochem. Physiol.* 32, 271–286.
- Riehle, M.A., Brown, M.R., 1999. Insulin stimulates ecdysteroid production through a conserved signaling cascade in the mosquito *Aedes aegypti*. *Insect Biochem. Mol. Biol.* 29, 855–860.
- Roberts, B., 1984. Photoperiodic regulation of prothoracicotrophic hormone release in late larval, prepupal and pupa stages of *Sarcophaga bullata*. *Ciba Found. Symp.* 104, 164–187.
- Roberts, B., Gilbert, L.I., 1986. Ring gland and prothoracic gland sensitivity to interspecific prothoracicotrophic hormone extracts. *J. Comp. Physiol. B* 156, 767–771.
- Roberts, B., Henrich, V., Gilbert, L.I., 1987. Effects of photoperiod on the timing of larval wandering in *Drosophila melanogaster*. *Physiol. Entomol.* 12, 175–180.
- Rountree, D.B., Bollenbacher, W.E., 1986. The release of the prothoracicotrophic hormone in the tobacco hornworm, *Manduca sexta*, is controlled intrinsically by juvenile hormone. *J. Exp. Biol.* 120, 41–58.
- Rountree, D.B., Combest, W.L., Gilbert, L.I., 1987. Protein phosphorylation in the prothoracic glands as a cellular model for juvenile hormone-prothoracicotrophic hormone interactions. *Insect Biochem.* 17, 943–948.
- Rountree, D.B., Combest, W.L., Gilbert, L.I., 1992. Prothoracicotrophic hormone regulates the phosphorylation of a specific protein in the prothoracic glands of the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol.* 22, 353–362.
- Rybczynski, R., Bell, S.C., Gilbert, L.I., 2001. Activation of an extracellular signal-regulated kinase (ERK) by the insect prothoracicotrophic hormone. *Mol. Cell. Endocrinol.* 184, 1–11.
- Rybczynski, R., Gilbert, L.I., 1994. Changes in general and specific protein synthesis that accompany ecdysteroid synthesis in stimulated prothoracic glands of *Manduca sexta*. *Insect Biochem. Mol. Biol.* 24, 175–189.
- Rybczynski, R., Gilbert, L.I., 1995a. Prothoracicotrophic hormone elicits a rapid, developmentally specific synthesis of β tubulin in an insect endocrine gland. *Devel. Biol.* 169, 15–28.
- Rybczynski, R., Gilbert, L.I., 1995b. Prothoracicotrophic hormone-regulated expression of a hsp 70 cognate protein in the insect prothoracic gland. *Mol. Cell. Endocrinol.* 115, 73–85.
- Rybczynski, R., Gilbert, L.I., 1998. Cloning of a β 1 tubulin cDNA from an insect endocrine gland: Developmental

- and hormone-induced changes in mRNA expression. *Mol. Cell. Endocrinol.* 141, 141–151.
- Rybczynski, R., Gilbert, L.I., 2000. cDNA cloning and expression of a hormone-regulated heat shock protein (hsc 70) from the prothoracic gland of *Manduca sexta*. *Insect Biochem. Mol. Biol.* 30, 579–589.
- Rybczynski, R., Gilbert, L.I., 2003. Prothoracicotrophic hormone stimulated extracellular signal-regulated kinase (ERK) activity: the changing roles of Ca^{2+} - and cAMP-dependent mechanisms in the insect prothoracic glands during metamorphosis. *Mol. Cell. Endocrinol.* 205, 159–168.
- Rybczynski, R., Mizoguchi, A., Gilbert, L.I., 1996. *Bombyx* and *Manduca* prothoracicotrophic hormones: an immunologic test for relatedness. *Gen. Comp. Endocrin.* 102, 247–254.
- Safranek, L., Cymborowski, B., Williams, C.M., 1980. Effects of juvenile hormone on ecdysone-dependent development in the tobacco hornworm, *Manduca sexta*. *Biol. Bull.* 158, 248–256.
- Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425.
- Sakurai, S., 1983. Temporal organization of endocrine events underlying larval–larval ecdysis in the silkworm, *Bombyx mori*. *J. Insect Physiol.* 29, 919–932.
- Sakurai, S., 1984. Temporal organization of endocrine events underlying larval–pupal metamorphosis in the silkworm, *Bombyx mori*. *J. Insect Physiol.* 30, 657–664.
- Sakurai, S., Gilbert, L.I., 1990. Biosynthesis and secretion of ecdysteroids by the prothoracic glands. In: Ohnishi, E., Ishizaki, H. (Eds.), *Molting and Metamorphosis*. Springer, Berlin, pp. 83–106.
- Sakurai, S., Kaya, M., Satake, S., 1998. Hemolymph ecdysteroid titer and ecdysteroid-dependent developmental events in the last-larval stadium of the silkworm, *Bombyx mori*: role of low ecdysteroid titer in larval–pupal metamorphosis and a reappraisal of the head critical period. *J. Insect Physiol.* 44, 867–881.
- Sakurai, S., Okuda, M., Ohtaki, T., 1989. Juvenile hormone inhibits ecdysone secretion and responsiveness to prothoracicotrophic hormone in prothoracic glands of *Bombyx mori*. *Gen. Comp. Endocrinol.* 75, 222–230.
- Sakurai, S., Williams, C.M., 1989. Short-loop negative and positive feedback on ecdysone secretion by prothoracic gland in the tobacco hornworm, *Manduca sexta*. *Gen. Comp. Endocrinol.* 75, 204–216.
- Sampath, T.K., Rashka, K.E., Doctor, J.S., Tucker, R.F., Hoffmann, F.M., 1993. *Drosophila* transforming growth factor β superfamily proteins induce endochondral bone formation in mammals. *Proc. Natl Acad. Sci. USA* 90, 6004–6008.
- Satake, S., Masumura, M., Ishizaki, H., Nagata, K., Kataoka, H., et al., 1997. Bombyxin, an insulin-related peptide of insects, reduces the major storage carbohydrates in the silkworm *Bombyx mori*. *Comp. Biochem. Physiol. B* 118, 349–357.
- Sauman, I., Reppert, S.M., 1996a. Molecular characterization of prothoracicotrophic hormone (PTTH) from the giant silkworm *Antheraea pernyi*: developmental appearance of PTTH-expressing cells and relationship to circadian clock cells in central brain. *Devel. Biol.* 178, 418–429.
- Sauman, I., Reppert, S.M., 1996b. Circadian clock neurons in the silkworm *Antheraea pernyi*: novel mechanisms of *Period* protein regulation. *Neuron* 17, 889–900.
- Saunders, D.S., 2002. *Insect Clocks*. Elsevier, Amsterdam, p. 560.
- Schneiderman, H.A., Gilbert, L.I., 1964. Control of growth and development in insects. *Science* 143, 325–333.
- Sedlak, B.J., 1985. Structure of endocrine glands. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 7. Pergamon, New York, pp. 109–151.
- Sedlak, B.J., Marchione, L., Devorkin, B., Davino, R., 1983. Correlations between endocrine gland ultrastructure and hormone titers in the fifth larval instar of *Manduca sexta*. *Gen. Comp. Endocrinol.* 52, 291–310.
- Sehnal, F., 1983. Juvenile hormone analogues. In: Downer, R., Laufer, H. (Eds.), *Endocrinology of Insects*. Allan R Liss, pp. 657–672.
- Sehnal, F., Hansen, I., Scheller, K., 2002. The cDNA structure of the prothoracicotrophic hormone (PTTH) of the silkworm *Hyalophora cecropia*. *Insect Biochem. Mol. Biol.* 32, 233–237.
- Sehnal, F., Svacha, P., Zrzavy, J., 1996. Evolution of insect metamorphosis. In: Gilbert, L.I., Tata, J., Atkinson, B.R. (Eds.), *Metamorphosis: Post-Embryonic Reprogramming of Gene Expression in Insect and Amphibian Cells*. Academic Press, NY, pp. 3–58.
- Seidah, N.G., Prat, A., 2002. Precursor convertases in the secretory pathway, cytosol and extracellular milieu. *Essays Biochem.* 38, 79–94.
- Shearman, M.S., Sekiguchi, K., Nishizuka, Y., 1989. Modulation of ion channel activity: a key function of the protein kinase C enzyme family. *Pharmacol. Rev.* 41, 211–237.
- Shionoya, M., Matsubayashi, H., Asahina, M., Kuniyoshi, H., Nagata, S., et al., 2003. Molecular cloning of the prothoracicotrophic hormone from the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol.* 33, 795–801.
- Shirai, Y., Aizono, Y., Iwasaki, T., Yanagida, A., Mori, H., et al., 1993. Prothoracicotrophic hormone is released five times in the 5th-larval instar of the silkworm *Bombyx mori*. *J. Insect Physiol.* 39, 83–88.
- Shirai, Y., Iwasaki, T., Matsubara, F., Aizono, Y., 1994. The carbachol-induced release of prothoracicotrophic hormone from brain–corpus cardiacum–corpus allatum complex of the silkworm, *Bombyx mori*. *J. Insect Physiol.* 40, 469–473.
- Shirai, Y., Shimazaki, K., Iwasaki, T., Matsubara, F., Aizono, Y., 1995. The in vitro release of prothoracicotrophic hormone (PTTH) from the brain–corpus cardiacum–corpus allatum complex of silkworm,

- Bombyx mori*. *Comp. Biochem. Physiol. C* 110, 143–148.
- Shirai, Y., Uno, T., Aizono, Y., 1998. Small GTP-binding proteins in the brain-corpus cardiacum-corpus allatum complex of the silkworm, *Bombyx mori*: involvement in the secretion of prothoracicotropic hormone. *Arch. Insect Biochem. Physiol.* 38, 177–184.
- Siegmund, T., Korge, G., 2001. Innervation of the ring gland of *Drosophila melanogaster*. *J. Comp. Neurol.* 431, 481–491.
- Simpson, E.R., Waterman, M.R., 1988. Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH. *Annu. Rev. Physiol.* 50, 427–440.
- Smith, W.A., 1993. Second messengers and the action of prothoracicotropic hormone in *Manduca sexta*. *Am. Zool.* 33, 330–339.
- Smith, W.A., 1995. Regulation and consequences of cellular changes in the prothoracic glands of *Manduca sexta* during the last larval instar: a review. *Arch. Insect Biochem. Physiol.* 30, 271–293.
- Smith, W.A., Bowen, M.F., Bollenbacher, W.E., Gilbert, L.I., 1986a. Cellular changes in the prothoracic glands of *Manduca sexta* during pupal diapause. *J. Exp. Bio.* 120, 131–142.
- Smith, W.A., Combest, W.L., Gilbert, L.I., 1986b. Involvement of cyclic AMP-dependent protein kinase in prothoracicotropic hormone-stimulated ecdysone synthesis. *Mol. Cell. Endocrinol.* 47, 25–33.
- Smith, W.A., Combest, W.L., Rountree, D.B., Gilbert, L.I., 1987a. Neuropeptide control of ecdysone biosynthesis. In: Law, J.H. (Ed.), *Molecular Entomology*. Alan R. Liss, New York, pp. 129–139.
- Smith, W.A., Gilbert, L.I., 1986. Cellular regulation of ecdysone synthesis by the prothoracic glands of *Manduca sexta*. *Insect Biochem.* 16, 143–147.
- Smith, W.A., Gilbert, L.I., 1989. Early events in peptide-stimulated ecdysteroid secretion by the prothoracic glands of *Manduca sexta*. *J. Exp. Zool.* 252, 262–270.
- Smith, W.A., Gilbert, L.I., Bollenbacher, W.E., 1984. The role of cyclic AMP in the regulation of ecdysone synthesis. *Mol. Cell. Endocrinol.* 37, 285–294.
- Smith, W.A., Gilbert, L.I., Bollenbacher, W.E., 1985. Calcium-cyclic AMP interactions in prothoracicotropic hormone stimulation of ecdysone synthesis. *Mol. Cell. Endocrinol.* 39, 71–78.
- Smith, W.A., Koundinya, M., McAllister, T., Brown, A., 1997. Insulin receptor-like tyrosine kinase in the tobacco hornworm, *Manduca sexta*. *Arch. Insect Biochem. Physiol.* 35, 99–110.
- Smith, W.A., Pasquarello, T.J., 1989. Developmental changes in phosphodiesterase activity and hormonal response in prothoracic glands of *Manduca sexta*. *Mol. Cell. Endocrinol.* 63, 239–246.
- Smith, W.A., Priester, J., Morais, J., 2003. PTTH-stimulated ecdysone secretion is dependent upon tyrosine phosphorylation in the prothoracic glands of *Manduca sexta*. *Insect Biochem. Mol. Biol.* 33, 1317–1325.
- Smith, W.A., Rountree, D.B., Bollenbacher, W.E., Gilbert, L.I., 1987b. Dissociation of the prothoracic glands of *Manduca sexta* into hormone-responsive cells. In: Borkovec, A., Gelman, D. (Eds.), *Progress in Insect Neurochemistry and Neurophysiology*. Humana Press, Clifton, NJ, pp. 319–322.
- Smith, W.A., Varghese, A.H., Lou, K.J., 1993. Developmental changes in cyclic AMP-dependent protein kinase associated with increased secretory capacity of *Manduca sexta* prothoracic glands. *Mol. Cell. Endocrinol.* 90, 187–195.
- Smith, W.A., Varghese, A.H., Healy, M.S., Lou, K.J., 1996. Cyclic AMP is a requisite messenger in the action of big PTTH in the prothoracic glands of pupal *Manduca sexta*. *Insect Biochem. Mol. Biol.* 26, 161–170.
- Snyder, M.J., Feyereisen, R., 1993. A diazepam binding inhibitor (DBI) homolog from the tobacco hornworm, *Manduca sexta*. *Mol. Cell. Endocrinol.* 94, R1–R4.
- Snyder, M.J., Van Antwerpen, R., 1998. Evidence for a diazepam-binding inhibitor (DBI) benzodiazepine receptor-like mechanism in ecdysteroidogenesis by the insect prothoracic gland. *Cell Tissue Res.* 294, 161–168.
- Song, Q., Gilbert, L.I., 1994. S6 phosphorylation results from prothoracicotropic hormone stimulation of insect prothoracic glands: a role for S6 kinase. *Devel. Genet.* 15, 332–338.
- Song, Q., Gilbert, L.I., 1995. Multiple phosphorylation of ribosomal protein S6 and specific protein synthesis are required for prothoracicotropic hormone-stimulated ecdysteroid biosynthesis in the prothoracic glands of *Manduca sexta*. *Insect Biochem. Mol. Biol.* 25, 591–602.
- Song, Q., Gilbert, L.I., 1996. Protein phosphatase activity is required for prothoracicotropic hormone-stimulated ecdysteroidogenesis in the prothoracic glands of the tobacco hornworm, *Manduca sexta*. *Arch. Insect. Biochem. Physiol.* 31, 465–480.
- Song, Q., Gilbert, L.I., 1997. Molecular cloning, developmental expression, and phosphorylation of ribosomal protein S6 in the endocrine gland responsible for insect molting. *J. Biol. Chem.* 272, 4429–4435.
- Song, Q., Gilbert, L.I., 1998. Alterations in ultraspiracle (USP) content and phosphorylation state accompany feedback regulation of ecdysone synthesis in the insect prothoracic gland. *Insect Biochem. Mol. Biol.* 28, 849–860.
- Stanewsky, R., 2002. Clock mechanisms in *Drosophila*. *Cell Tissue Res.* 309, 11–26.
- Stanewsky, R., 2003. Genetic analysis of the circadian system in *Drosophila melanogaster* and mammals. *J. Neurobiol.* 54, 111–147.
- Steel, C.G.H., Vafopoulou, X., 2002. Physiology of circadian systems. In: Saunders, D.S., with Steel, C.G.H., Vafopoulou, X., Lewis, R.D. (Eds.), *Insect Clocks*. 3rd edn. Elsevier, Amsterdam, pp. 115–188.
- Stengl, M., Homberg, U., 1994. Pigment-dispersing hormone-immunoreactive neurons in the cockroach

- Leucophaea maderae* share properties with circadian pacemaker neurons. *J. Comp. Physiol. A* 175, 203–213.
- Stocco, D.M., Clark, B.J., 1997. The role of the steroidogenic acute regulatory protein in steroidogenesis. *Steroids* 62, 29–36.
- Suarez, C., Diaz-Torga, G., Gonzalez-Iglesias, A., Vela, J., Mladovan, A., et al., 2003. Angiotensin II phosphorylation of extracellular signal-regulated kinases in rat anterior pituitary cells. *Am. J. Physiol.: Endocrinol. Metab.* 85, E645–E653.
- Suh, J.M., Song, J.H., Kim, D.W., Kim, H., Chung, H.K., et al., 2003. Regulation of the phosphatidylinositol 3-kinase, Akt/protein kinase B, FRAP/mammalian target of rapamycin, and ribosomal S6 kinase 1 signaling pathways by thyroid-stimulating hormone (TSH) and stimulating type TSH receptor antibodies in the thyroid gland. *J. Biol. Chem.* 278, 21960–21971.
- Takaki, K., Sakurai, S., 2003. Regulation of prothoracic gland ecdysteroidogenic activity leading to pupal metamorphosis. *Insect Biochem. Mol. Biol.* 33, 1189–1199.
- Tanaka, M., Kataoka, H., Nagata, K., Nagasawa, H., Suzuki, A., 1995. Morphological changes of BM-N4 cells induced by bombyxin, an insulin-related peptide of *Bombyx mori*. *Regul. Pept.* 57, 311–318.
- Tauber, M.J., Tauber, C.A., Masaki, S., 1986. Seasonal Adaptations of Insects. Oxford University Press, Oxford, pp. 411.
- Tissenbaum, H.A., Hawdon, J., Perregaux, M., Hotez, P., Guarente, L., et al., 2000. A common muscarinic pathway for diapause recovery in the distantly related nematode species *Caenorhabditis elegans* and *Ancylostoma caninum*. *Proc. Natl Acad. Sci. USA* 97, 460–465.
- Tomioka, K., Agui, N., Bollenbacher, W.E., 1995. Electrical properties of the prothoracicotropic hormone cells in diapausing and non-diapausing pupae of the tobacco hornworm, *Manduca sexta*. *Zool. Sci.* 12, 165–173.
- Truman, J.W., 1972. Physiology of insect rhythms. II. The silkworm brain as the location of the biological clock controlling eclosion. *J. Comp. Physiol.* 81, 99–114.
- Truman, J.W., Riddiford, L.M., 1974. Physiology of insect rhythms. 3. The temporal organization of the endocrine events underlying pupation of the tobacco hornworm. *J. Exp. Biol.* 60, 371–382.
- Truman, J.W., Riddiford, L.M., 2002. Endocrine insights into the evolution of metamorphosis in insects. *Annu. Rev. Entomol.* 47, 467–500.
- Vafopoulou, X., Sim, C.-H., Steel, C.G.H., 1996. Prothoracicotropic hormone in *Rhodnius prolixus*: *in vitro* analysis and changes in amounts in the brain and retrocerebral complex during larval-adult development. *J. Insect Physiol.* 42, 407–415.
- Vafopoulou, X., Steel, C.G.H., 1996. The insect neuropeptide prothoracicotropic hormone is released with a daily rhythm: re-evaluation of its role in development. *Proc. Natl Acad. Sci. USA* 93, 3368–3372.
- Vafopoulou, X., Steel, C.G., 1997. Ecdysteroidogenic action of *Bombyx* prothoracicotropic hormone and bombyxin on the prothoracic glands of *Rhodnius prolixus* *in vitro*. *J. Insect Physiol.* 43, 651–656.
- Vafopoulou, X., Steel, C.G., 1998. A photosensitive circadian oscillator in an insect endocrine gland: photic induction of rhythmic steroidogenesis *in vitro*. *J. Comp. Physiol. A* 182, 343–349.
- Vafopoulou, X., Steel, C.G.H., 1999. Daily rhythm of responsiveness to prothoracicotropic hormone in prothoracic glands of *Rhodnius prolixus*. *Arch. Insect. Biochem. Physiol.* 41, 117–123.
- Vafopoulou, X., Steel, C.G.H., 2001. Induction of rhythmicity in prothoracicotropic hormone and ecdysteroids in *Rhodnius prolixus*: roles of photic and neuroendocrine Zeitgebers. *J. Insect Physiol.* 47, 935–941.
- Vafopoulou, X., Steel, C.G.H., 2002. Prothoracicotropic hormone of *Rhodnius prolixus*: partial characterization and rhythmic release of neuropeptides related to *Bombyx* PTTH and bombyxin. *Invert. Reprod. Devel.* 42, 111–120.
- Vázquez-Prado, J., Casas-Gonzalez, P., Gracia-Sáinz, J.A., 2003. G protein-coupled receptor cross-talk: pivotal roles of protein phosphorylation and protein-protein interactions. *Cell. Signall.* 15, 549–557.
- Vedeckis, W.V., Gilbert, L.I., 1973. Production of cyclic AMP and adenosine by the brain and prothoracic glands of *Manduca sexta*. *J. Insect Physiol.* 19, 2445–2457.
- Vedeckis, W.V., Bollenbacher, W.E., Gilbert, L.I., 1974. Cyclic AMP as a possible mediator of prothoracic gland activation. *Zool. Jahrb. Physiol.* 78, 440–448.
- Vedeckis, W.V., Bollenbacher, W.E., Gilbert, L.I., 1976. Insect prothoracic glands: a role for cyclic AMP in the stimulation of α ecdysone secretion. *Mol. Cell. Endocrinol.* 5, 81–88.
- Venkatesh, K., Hasan, G., 1997. Disruption of the IP3 receptor gene of *Drosophila* affects larval metamorphosis and ecdysone release. *Curr. Biol.* 7, 500–509.
- Venkatesh, K., Siddhartha, G., Joshi, R., Patel, S., Hasan, G., 2001. Interactions between the inositol 1,4,5-trisphosphate and cyclic AMP signaling pathways regulate larval molting in *Drosophila*. *Genetics* 158, 309–318.
- Vernooy, S.Y., Copeland, J., Ghaboosi, N., Griffin, E.E., Yoo, S.J., et al., 2000. Cell death regulation in *Drosophila*: conservation of mechanism and unique insights. *J. Cell Biol.* 150, F69–F76.
- Vogt, A., Cooley, K.A., Brisson, M., Tarpley, M.G., Wipf, P., et al., 2003. Cell-active dual specificity phosphatase inhibitors identified by high-content screening. *Chem. Biol.* 10, 733–742.
- Volarevic, S., Thomas, G., 2001. Role of S6 phosphorylation and S6 kinase in cell growth. *Prog. Nucl. Acid Res. Mol. Biol.* 65, 101–127.
- Walker, G.P., Denlinger, D.L., 1980. Juvenile hormone and molting hormone titres in diapause- and non-diapause destined flesh flies. *J. Insect Physiol.* 26, 661–664.
- Warren, J.T., Bachman, J.S., Dai, J.-D., Gilbert, L.I., 1996. Differential incorporation of cholesterol and cholesterol derivatives by the larval ring glands and adult ovaries of *Drosophila melanogaster*: a putative explanation for the *(l(3)ecd¹)* mutation. *Insect Biochem. Mol. Biol.* 26, 931–943.

- Warren, J.T., Gilbert, L.I., 1996. Metabolism *in vitro* of cholesterol and 25-hydroxycholesterol by the larval prothoracic glands of *Manduca sexta*. *Insect Biochem. Mol. Biol.* 26, 917–929.
- Watson, R.D., Ackerman-Morris, S., Smith, W.A., Watson, C.J., Bollenbacher, W.E., 1996. Involvement of microtubules in prothoracicotropic hormone-stimulated ecdysteroidogenesis by insect (*Manduca sexta*) prothoracic glands. *J. Exp. Biol.* 276, 63–69.
- Watson, R.D., Bollenbacher, W.E., 1988. Juvenile hormone regulates the steroidogenic competence of *Manduca sexta* prothoracic glands. *Mol. Cell. Endocrinol.* 57, 251–259.
- Watson, R.D., Yeh, W.E., Muehleisen, D.P., Watson, C.J., Bollenbacher, W.E., 1993. Stimulation of ecdysteroidogenesis by small prothoracicotropic hormone: role of cyclic AMP. *Mol. Cell. Endocrinol.* 92, 221–228.
- Westbrook, A.L., Regan, S.A., Bollenbacher, W.E., 1993. Developmental expression of the prothoracicotropic hormone in the CNS of the tobacco hornworm, *Manduca sexta*. *J. Comp. Neurol.* 327, 1–16.
- Wetzker, R., Bohmer, F.D., 2003. Transactivation joins multiple tracks to the ERK/MAPK cascade. *Nat. Rev. Mol. Cell Biol.* 4, 651–657.
- Wigglesworth, V.B., 1933. The physiology of the cuticle and of ecdysis in *Rhodnius prolixus* (Triatomidae, Hemiptera); with special reference to the function of oenocytes and of the dermal glands. *Q. J. Microsc. Sci.* 76, 296–318.
- Wigglesworth, V.B., 1934. The physiology of ecdysis in *Rhodnius prolixus* (Hemiptera). II Factors controlling moulting and “metamorphosis.” *Q. J. Microsc. Sci.* 77, 191–222.
- Wigglesworth, V.B., 1940. The determination of characters at metamorphosis in *Rhodnius prolixus*. *J. Exp. Biol.* 17, 201–222.
- Wigglesworth, V.B., 1955. The breakdown of the thoracic gland in the adult insect, *Rhodnius prolixus*. *J. Exp. Biol.* 32, 485–491.
- Williams, C.M., 1947. Physiology of insect diapause II. Interaction between the pupal brain and prothoracic glands in the metamorphosis of the giant silkworm, *Platysamia cecropia*. *Biol. Bull.* 93, 89–98.
- Williams, C.M., 1967. The present status of the brain hormone. In: Beament, J.W.L., Treherne, J.E. (Eds.), *Insects and Physiology*. Oliver and Boyd, Edinburgh, pp. 133–139.
- Xu, W.H., Denlinger, D.L., 2003. Molecular characterization of prothoracicotropic hormone and diapause hormone in *Heliothis virescens* during diapause, and a new role for diapause hormone. *Insect Mol. Biol.* 12, 509–516.
- Xu, W.H., Rinehart, J.P., Denlinger, D.L., 2003. Structural characterization and expression analysis of prothoracicotropic hormone in the corn earworm, *Helicoverpa zea*. *Peptides* 24, 1319–1325.
- Yagi, S., Fukaya, M., 1974. Juvenile hormone as a key factor regulating larval diapause of the rice stem borer, *Chilo suppressalis*. *Appl. Ent. Zool.* 9, 247–255.
- Yagi, Y., Ishibashi, J., Nagata, K., Kataoka, H., Suzuki, A., et al., 1995. The brain neurosecretory cells of the moth *Samia cynthia ricini*: immunohistochemical localization and developmental changes of the *Samia* homologues of the *Bombyx* prothoracicotropic hormone and bombyxin. *Devel. Growth Different.* 37, 505–516.
- Yin, C.M., Chippendale, G.M., 1973. Juvenile hormone regulation of the larval diapause of the southwestern corn borer, *Diatraea grandiosella*. *J. Insect Physiol.* 19, 2403–2420.
- Yokoyama, I., Endo, K., Yamanaka, A., Kumagai, K., 1996. Species-specificity in the action of big and small prothoracicotropic hormones (PTTHs) of the swallowtail butterflies, *Papilio xuthus*, *P. machaon*, *P. bianor* and *P. helenus*. *Zool. Sci.* 13, 449–454.
- Závodská, R., Sauman, I., Sehnal, F., 2003. Distribution of PER protein, pigment-dispersing hormone, prothoracicotropic hormone, and eclosion hormone in the cephalic nervous system of insects. *J. Biol. Rhythms* 18, 106–122.
- Zimowska, G., Handler, A.M., Cymborowski, B., 1985. Cellular events in the prothoracic glands and ecdysteroid titres during the last larval instar of *Spodoptera littoralis*. *J. Insect Physiol.* 31, 331–340.
- Žitňan, D., Kingan, T.G., Kramer, S.J., Beckage, N.E., 1995. Accumulation of neuropeptides in the cerebral neurosecretory system of *Manduca sexta* larvae parasitized by the braconid wasp *Cotesia congregata*. *J. Comp. Neurol.* 356, 83–100.
- Žitňan, D., Sehnal, F., Bryant, P.J., 1993. Neurons producing specific neuropeptides in the central nervous system of normal and pupariation-delayed *Drosophila*. *Devel. Biol.* 156, 117–135.

Relevant Website

<http://www.cbs.dtu.dk> – This site provides a number of online sequence analysis tools, including programs to predict glycosylation sites in proteins.

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7 The Ecdysteroid Receptor

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7.1. Overview

7.1.1. Molecular Identity of the Insect Ecdysteroid Receptor

The morphogenetic events associated with insect development are largely triggered by the action of a single class of steroid hormones, the ecdysteroids.¹ Among all insect orders examined so far, it has been established that the ecdysteroid-induced orchestration of molting and metamorphosis is mediated by a heterodimer comprised of the ecdysone receptor (EcR; Koelle *et al.*, 1991) and Ultraspiracle (USP; Oro *et al.*, 1990; Shea *et al.*,

1990; Henrich *et al.*, 1990) that is stabilized by 20-hydroxyecdysone (20E) and which recognizes specific promoter elements in the insect genome to regulate transcription (Yao *et al.*, 1992, 1993; Thomas *et al.*, 1993). Both proteins belong to a superfamily of nuclear receptors which mediate transcriptional responses to steroids and other lipophilic molecules (Blumberg and Evans, 1998). Recently, a second ecdysteroid-signaling pathway involving USP and another nuclear receptor DHR38 (Sutherland *et al.*, 1995) has been discovered (Baker *et al.*, 2003).

The insect EcR is a distant relative of the vertebrate farnesol X receptor (FXR; Forman *et al.*, 1995) and LXR (Willy *et al.*, 1995). EcR was first identified in the fruit fly, *Drosophila melanogaster*, and has since been found in several insects, in the ixodid tick (*Amblyomma americanum*; Guo *et al.*, 1997) and in the fiddler crab (*Uca pugilator*; Durica *et al.*, 2002). No functional EcR ortholog has been reported outside the arthropod phylum (Bonneton

¹Ecdysteroids refers to the family of ecdysteroids including natural and artificial steroids. Individual forms, including the classic active “molting hormone,” 20-hydroxyecdysone (20E, formerly β -ecdysone), will be specified throughout the chapter, as will its precursor, ecdysone (formerly α -ecdysone). Ecdysteroid agonists refers to molecules which are not steroidal, but which are capable of inducing one or more insect EcR genes in a given experimental regime.

et al., 2003). The Ultraspiracle (USP) protein is an ortholog of the retinoid X receptor (RXR), which in turn is a heterodimeric partner for several other nuclear receptors (Mangelsdorf *et al.*, 1990; Oro *et al.*, 1990). Among noninsect invertebrates, an RXR ortholog has been reported from *A. americanum* (Guo *et al.*, 1998), *U. pugilator* (Chung *et al.*, 1998), and the parasitic nematode, *Dirofilaria immitis* (Shea *et al.*, 2004). RXR is widely found in humans and other vertebrates, reflecting its early evolutionary origins. Both EcR and USP have diverged evolutionarily among the insect orders, indicating that the functional properties of EcR and USP have also diverged among them.

7.1.1.2. Historical Perspective

The pursuit of the insect ecdysteroid receptor and its activities has led to a convergence of several basic and applied research disciplines. The ability of a single steroid hormone to induce widespread and coordinated changes in gene transcription has attracted basic scientists interested in the mechanistic basis of its variable gene and cellular action. For insect biologists, the diversity of developmental responses triggered by ecdysteroids among the insect orders reveals the essential importance of this process for understanding the evolutionary diversity and adaptability seen among individual insect species. In turn, the variety of responses among the insect orders has led to attempts to disrupt ecdysteroid receptor action with species-specific agonists and antagonists such as the bisacylhydrazines (Wing, 1988) for insecticidal purposes (Dhadialla *et al.*, 1998, and references therein). Finally, because ecdysteroids exert no harmful effects in humans and other organisms, the ecdysteroid receptor is now viewed as a potential inducer of beneficial transcriptional responses in plant and animal cells. Much of the work on ecdysteroid action that was reported prior to 1985 appeared in the previous edition of this series and will not be repeated here (Riddiford, 1985; O'Connor, 1985; Yund and Osterbur, 1985), except for those early events which have had an obvious bearing on continuing investigations.

The effects of ecdysteroids upon gene transcriptional activity became apparent through studies of the effects of “molting hormone,” 20E, on the polytene chromosomes of insects such as *D. melanogaster* and *Chironomus tentans* (midge). Puffing was first reported in 1933 in *D. melanogaster* by E. Heitz, and in the early 1950s Wolfgang Beermann performed seminal work on puff changes in *C. tentans*. Later, Ulrich Clever demonstrated that the

insect “molting hormone” induced puffing changes and thus reported the first evidence that steroid hormones act directly upon the transcriptional activity of specific target genes, now regarded as a central feature of steroid hormone action in all animals. A variety of biochemical studies also established the presence of a protein in cellular extracts that binds to ecdysteroids, further indicating that an intracellular receptor mediates the transcriptional response (O'Connor, 1985; Yund and Osterbur, 1985, and references therein).

Conceptually, the polytene chromosomes can be viewed as an *in situ* expression microarray (Figure 1), since the puff sites disclose the transcriptional activity of specific genes (by chromosomal location rather than by sequence). This natural array has the added and unique benefit of showing temporal changes in puff size that roughly indicate continuous changes in transcriptional rate. Hans Becker noted in 1959 that “early” puffs appear when incubated with the ecdysteroidogenic ring gland and regress as other “late puffs” appear (Becker, 1959). Ulrich Clever and Michael Ashburner later showed that blocking the protein translation of early puff RNAs with cycloheximide treatment prevents the regression of some early puffs, and simultaneously prevents the appearance of many late puffs (Ashburner *et al.*, 1974). From these findings, Ashburner postulated that an intracellular

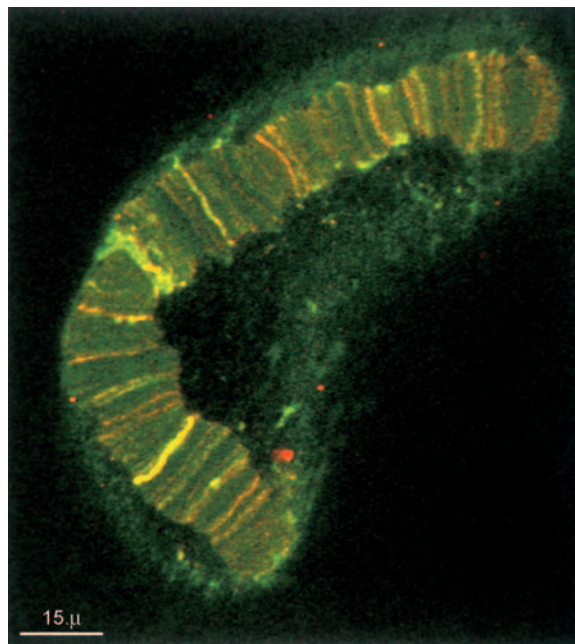


Figure 1 Prepupal chromosome III from *Chironomus tentans* labeled with antibodies against EcR (green) and RNA polymerase II (red). Yellow signals indicate colocalization of the two antibodies. Green signal is a fixation artifact. See Wegmann *et al.* (1995) for methods. (Photograph courtesy of Markus Lezzi.)

ecdysteroid receptor directs a transcriptional response at early puff sites in the presence of 20E. Further, the early puff gene products regulate the appearance of later puffs and also feedback to repress their own expression. This original model for ecdysteroid action has proven remarkably durable over the years, though it is often overlooked that several puffs described by Ashburner have not been placed within the regulatory hierarchy and that some features of the model have not yet been pursued at the molecular level.

The role of a receptor was also implicated by the identification of an ecdysone response element (EcRE) in the ecdysteroid-inducible promoter of the 27 kDa heat shock protein (*hsp27*) of *D. melanogaster* (Riddihough and Pelham, 1987). The palindromic inverted nucleotide repeat sequence of this ecdysteroid receptor target resembles the motifs that at the time of its discovery were associated with a growing class of nuclear receptors for several vertebrate hormones, including the glucocorticoids and estrogen (Hollenberg *et al.*, 1985; Green *et al.*, 1986), raising anticipation that the ecdysteroid receptor is evolutionarily conserved, just as the process of steroid action on transcriptional activity is mechanistically conserved.

Much of the progress made on the molecular genetic basis of ecdysteroid action can be traced to 1968 when, during a sabbatical at CalTech, David Hogness met Ashburner, who was a postdoctoral associate there. Hogness later visited the laboratory of Beermann at Tubingen, and decided to identify the “puff” genes targeted by ecdysteroid action. The conviction to this goal was strong enough that Hogness isolated overlapping genomic DNA segments over a span of several hundred kilobases to find them. His laboratory successfully “walked” through the chromosomal region associated with two early puff genes using this positional cloning approach (Burtis *et al.*, 1990; Segraves and Hogness, 1990). One of the early puff genes, E75,² encodes a member of the nuclear hormone receptor superfamily (Segraves and Hogness, 1990), as defined by two cysteine–cysteine zinc fingers that are responsible for the receptor protein’s recognition of specific promoter elements.

²The puff sites in *Drosophila melanogaster* were originally identified by their cytological location among the 102 subintervals arbitrarily delineated across the sex chromosome (X or chromosome 1, subintervals 1–20) and three autosomes (chromosome 2, subintervals 21–60; chromosome 3, subintervals 61–100, chromosome 4, subintervals 101–102) that comprise the *D. melanogaster* genome. The puffs are further designated as E (early or early-late) or L (late) based on their temporal appearance after 20E incubation.

E75, several other ecdysteroid-responsive gene products in *Drosophila*, and numerous other superfamily members are “orphan receptors,” that is, they carry the zinc finger configuration but do not mediate transcriptional activity through any known ligand. When E75 was used as a probe to screen a cDNA library, one of the recovered candidate clones proved to be the *D. melanogaster* ecdysone receptor gene (*DmEcR*; Koelle *et al.*, 1991), and a functional receptor was also recovered by biochemical purification from a *Drosophila* embryonic cell line (Luo *et al.*, 1991). In reality, EcR dimerizes with a second nuclear receptor, Ultraspiracle (*DmUSP*; Yao *et al.*, 1992, 1993; Thomas *et al.*, 1993), whose structural resemblance to the vertebrate RXR suggests its conservation as a heterodimeric partner for EcR. The recovery of EcR, USP, and orphans such as E75 inspired a more exhaustive search for nuclear receptors in *D. melanogaster*, and the annotated genome now lists 21 nuclear receptors. Among them, only EcR unambiguously interacts physically with an activating ligand, though there is evidence that USP binds to juvenile hormone (Jones and Sharp, 1997; Jones *et al.*, 2001).

The recovery and characterization of EcR, USP, other orphan receptors, and various ecdysteroid-inducible targets in other insects has proceeded at a quickening pace in recent years, and, along with it, a proliferation of DNA probes and antibodies have been developed to examine these players. Several insect researchers have actively identified orthologs for EcR and USP and its puff gene targets so that a rapidly expanding body of comparative information is appearing in the literature. The abundance of information accumulated in the *Drosophila* system has heavily influenced mechanistic interpretations of ecdysteroid action, though it is apparent from the comparative studies done so far that ecdysteroid action at the developmental level varies among insect species, consistent with their wide range of adaptations to the environment. Complete EcR and USP sequences have so far been reported for a few dozen species among four of the insect orders (Diptera, Lepidoptera, Orthoptera, and Coleoptera) and two other arthropod classes. If the variety of results obtained from these studies provide any indication, then only a fraction of the potential information concerning the ecdysteroid receptor has so far been retrieved and assimilated.

7.1.3. Chapter Organization

The straightforward thesis of the ecdysteroid hierarchy based on the chromosomal puff response seems to contrast with the complexity found in many

in vivo experiments. It is apparent that ecdysteroid responsiveness varies widely by tissue or cell type, species, incubation conditions, developmental time, activating ligand, ligand concentration, and treatment duration, presumably because the functional capacity of the receptor itself varies in these regimes. Nevertheless, several common and conserved themes have emerged from this work that are valuable not only for insect biologists, but also for other geneticists, cell biologists, and endocrinologists.

The organization of this chapter is based largely on the criteria by which the EcR of *D. melanogaster* was originally defined: (1) the deduced EcR amino acid sequence includes the zinc fingers and ligand-binding domain helices that typify nuclear receptors, (2) extracts of cells expressing EcR bind to ^{125}I -iodoponasterone and this binding disappears with the addition of anti-EcR antibodies, (3) insect cellular extracts carry a protein that binds specifically to an *hsp27* EcRE and this binding disappears in the presence of anti-EcR antibodies, (4) ecdysteroid-inducible transcription is restored to an ecdysone-insensitive *Drosophila* cell line by transfecting the cells with EcR cDNA, and (5) the pattern of spatial and temporal expression of EcR during pre-metamorphic development is consistent with its role as a mediator of the ecdysteroid response (Koelle *et al.*, 1991).

This chapter will examine the information gathered about the ecdysteroid receptor at three levels, and provide brief descriptions and references for the tools used to undertake those experiments. First, the receptor will be viewed as a structural entity, with particular emphasis on the sequence characteristics of EcR and USP as well as their biophysical and biochemical properties. These studies build upon the first three properties noted for EcR in its original characterization. Second, and as in the original report, the transcriptional function of the ecdysteroid receptor will be examined, with particular regard to its interactions with ligand, other protein factors, and promoter elements. This section will also describe a few of the ecdysteroid-inducible systems which have been developed for various applications. Third, the ecdysteroid receptor will be viewed from a cellular and developmental perspective *in vivo*, with special emphasis on the spatial and temporal diversity of ecdysteroid-mediated action, along with the approaches used to address these questions. Finally, the chapter will offer a prognosis concerning important and unresolved problems surrounding ecdysteroid action via its receptors, along with possible experimental technologies and strategies that might clarify them.

7.2. Ecdysteroid Receptor Structure, Biophysics, and Biochemistry

7.2.1. Domain Organization and Amino Acid Alignment

Both EcR and USP belong to the superfamily of nuclear receptors first described for several steroid hormones and vitamins among the vertebrates. Like their evolutionary counterparts, EcR and USP are structurally modular, that is, they are composed of distinct domains responsible for specific molecular functions (Figure 2). Individual domains are at least partially autonomous in their function, since domains of different nuclear receptors can be swapped to create structural and functional chimeras. Chimeras derived from the EcR and USP of different insect species have been used to compare and differentiate their functional capabilities (e.g., Suhr *et al.*, 1998; Wang *et al.*, 2000; Henrich *et al.*, 2000).

The basic organization of nuclear receptors has been described extensively in other reviews, and will be examined here exclusively in terms of the functional features found in insects and associated with each of the domains: (1) the N-terminal (A/B) domain through which nuclear receptors interact with other transcriptional factors, (2) the DNA binding domain (DBD) or C domain which is responsible for the receptor's recognition of specific DNA response elements in the genome and which is comprised of two cysteine–cysteine zinc fingers that define proteins as members of the nuclear receptor superfamily, (3) the hinge region (D domain), which has been implicated in ligand-dependent heterodimerization along with the E domain, nuclear localization, and DNA recognition, (4) the E- or ligand-binding domain (LBD), which is usually comprised of 12 alpha-helices that form a ligand-binding pocket, and (5) the F domain, which is found as a nonconserved sequence in all of the insect EcRs but not in any known USP or RXR sequence.

7.2.1.1. The A/B domain This domain is sometimes referred to as the *trans*-activation domain because this portion of the nuclear receptor interacts with the cell's transcriptional machinery and is responsible for a ligand-independent transcriptional activation function (AF1). The A/B domain tends to be variable among nuclear receptors, though there is modest similarity in this portion of EcR and USP among insect species. In several species, multiple isoforms of EcR and/or USP with different A/B domains arise through the activity of alternative promoters and/or alternative pre-mRNA splicing. The occurrence of multiple isoforms for EcR and

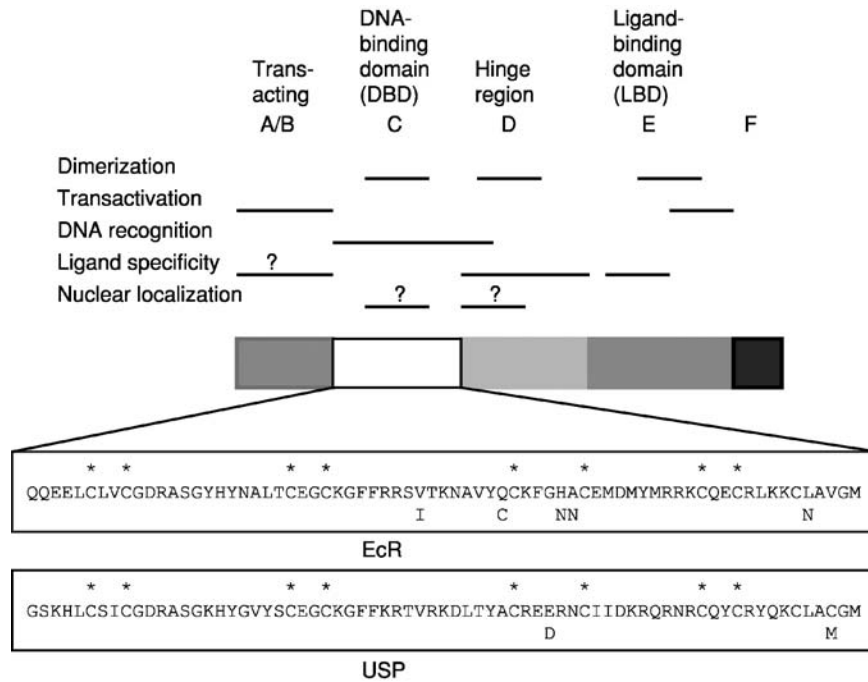


Figure 2 Domain organization of nuclear receptor superfamily members. Bars denote regions of the receptor associated with specific subfunctions or suspected subfunctions (as indicated with a ?). Boxed regions indicate consensus amino acid sequences for the DNA-binding domain of insect EcR and USP based on Bonneton *et al.* (2003). Asterisks (*) indicate positions of two sets of four cysteines which ligand to zinc ion to form each of the two zinc fingers.

USP does not follow any apparent pattern and therefore does not seem to be tied to an obvious evolutionary mechanism. For instance, so far only the *DmEcR* gene among insects encodes three isoforms (A, B1, and B2; Talbot *et al.*, 1993). Among cycloraphous flies the Mediterranean fruit fly (*Ceratitidis capitata*, *Cc*) EcR gene specifies both the A and B1 isoforms (Verras *et al.*, 2002), whereas only the B1 isoform has been found so far in the sheep blowfly (*Lucilia cuprina*, *Lc*) genome (Hannan and Hill, 1997).

Among other Diptera, the mosquito (*Aedes aegypti*, *Aa*) EcR encodes the A and B1 isoforms (Wang *et al.*, 2002), but the midge (*Chironomus tentans*, *Ct*) encodes only the B1-like isoform (Imhof *et al.*, 1993). The same variable pattern is found among the lepidopteran species. The corn earworm (*Heliothis virescens*, *Hv*; Martinez *et al.*, 1999a) has only EcR-B1, whereas the spruce budworm (*Choristoneura fumiferana*, *Cf*; Perrera *et al.*, 1999a), the rice stem borer (*Chilo suppressalis*, *Cs*), the tobacco hornworm (*Manduca sexta*, *Ms*; Jindra *et al.*, 1996), and the silkworm (*Bombyx mori*, *Bm*; Swevers *et al.*, 1995; Kamimura *et al.*, 1997) express both an A and B1 isoform. An A and B1 isoform of EcR has also been recovered from the mealworm (*Tenebrio molitor*, *Tm*) genome (Mouillet *et al.*, 1997) and a single EcR isoform has been recovered from the migratory

locust (*Locusta migratoria*, *Lm*; Saleh *et al.*, 1998). The most complex EcR gene organization is found in a noninsect species, *A. americanum*, wherein at least three A/B isoforms have been identified, each of which resembles the *D. melanogaster* A isoform sequence (Guo *et al.*, 1997). The functional properties of the insect EcR isoforms and their distinct developmental roles will be discussed later in the chapter, though it is noteworthy that the B2 isoform uniquely associated with *D. melanogaster* is also the most efficient for rescuing larval development in EcR homozygous mutants that would otherwise die during embryonic development (Li and Bender, 2000). This observation simultaneously highlights the possible uniqueness of the fruit fly EcR and the potential significance of the A/B domain for comparison among the insects.

In one species, *C. tentans*, multiple USP isoforms differing in their A/B domain occur along with only a single EcR isoform (Vogtli *et al.*, 1999). The migratory locust (*Locusta migratoria*; *Lm*) also expresses multiple USP isoforms along with the single EcRB1 isoform found so far, though the USP variants do not arise from differential splicing in the A/B domain (Hayward *et al.*, 1999, 2003). Along with two EcR isoforms, two USP isoforms exist in *A. aegypti* (Kapitskaya *et al.*, 1996), *M. sexta* (Jindra *et al.*, 1997), and *B. mori* (Tzertzinis

et al., 1994). Two RXR/USP isoforms also exist in *A. americanum*, along with its three EcR isoforms (Guo *et al.*, 1998). Other complete USP sequences have been reported for *L. cuprina* (Hannan and Hill, 2001) and *C. fumiferana* (Perera *et al.*, 1998).

7.2.1.2. The C domain or DNA-binding domain (DBD) The members of the nuclear receptor superfamily are defined by a 66–68 amino acid region known as the cysteine–cysteine zinc finger (Figures 2 and 3). The EcR DBD closely resembles the vertebrate FXR, and both EcR/USP and FXR/RXR recognize a palindromic inverted repeat sequence separated by a single nucleotide (IR1); the *hsp27* EcRE follows this arrangement. A consensus sequence based on the investigation of several functional IR1 elements is: 5'-PuG(G/T)T(C/G)A(N)TG(C/A(C/A(C/t)Py (Antoniewski *et al.*, 1993). As will be noted later, the EcR/USP complex is also capable of recognizing direct repeat elements

(Antoniewski *et al.*, 1996; Vogtli *et al.*, 1998). The DBD amino acid sequence is highly conserved among the Diptera and Lepidoptera, but specific residues are substituted in the EcR DBD of other insects (Henrich and Brown, 1995; Bonneton *et al.*, 2003).

7.2.1.3. The D domain or hinge region The D domain includes the amino acids which lie between the zinc fingers and the ligand binding domain (LBD). Portions of this region are highly conserved among all the insect EcRs, including a T-box motif that plays a role in DNA recognition (Devarakonda *et al.*, 2003; Figure 3) and an A-box, a helical structure that is essential for high affinity recognition of a DNA response element (Niedziela-Majka *et al.*, 2000). The D region of EcR is also essential for ligand dependent heterodimerization with USP (Suhr *et al.*, 1998; Perera *et al.*, 1999b). The USP hinge region includes a highly conserved

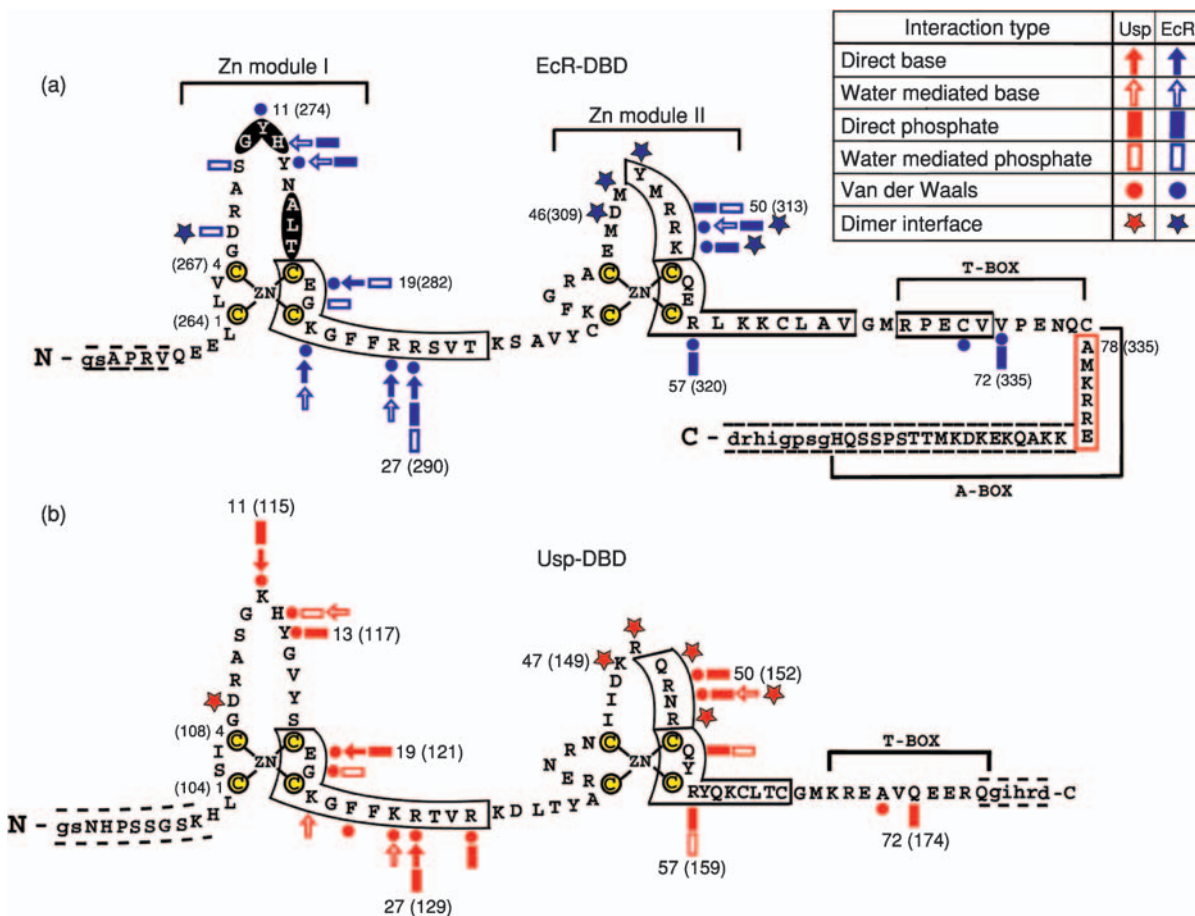


Figure 3 The protein constructs used for a crystallization study, designating amino acid contact sites with noted DNA interactions. (a) Sequence of *Drosophila melanogaster* EcR DBD with liganded zinc fingers, T-box, and A box. (b) Sequence of *D. melanogaster* USP DBD with liganded zinc fingers and T-box. Lower case letters designate artifacts of cloning in the constructs used for the study. (Reproduced with permission from Devarakonda, S., Harp, J.M., Kim, Y., Ozyhar, A., Rastinejad, F., 2003. Structure of the heterodimeric ecdysone receptor DNA-binding complex. *EMBO J.* 22, 5827–5840.)

T-box motif, which plays a role in site recognition of DNA response elements but is not essential for heterodimerization (Figure 3; Niedziela-Majka *et al.*, 2000; Devarakonda *et al.*, 2003).

7.2.1.4. The E domain or ligand binding domain (LBD) The domain of the nuclear receptor which is responsible for interacting with the receptor's cognate ligand is the E domain or LBD. The EcR and USP LBD fall within the canonical organization observed for almost all other members of the nuclear receptor superfamily. The region is comprised of 12 α -helices that form a ligand-binding pocket which holds the cognate ligand. The sequence of the EcR LBD is highly conserved among the insects, consistent with the widespread occurrence of the molting hormone, 20E, among the insect orders (see Figure 4). Nevertheless, the EcR LBD sequences from some insect orders have not been reported, and possibly important divergences among them remain unknown. For EcR, a ligand-dependent transcriptional activation function (AF2) is localized in the most carboxy-terminal helix 12, which folds over the pocket to hold the ligand molecule inside. This folding thereby creates an interactive surface with other proteins that ultimately modulates the receptor's transcriptional activity. For nuclear receptors generally, a dimerization interface lies along helices 9 and 10 (Perlmann *et al.*, 1996) and ligand-independent transcriptional functions (AF1) have also been associated with various portions of the EcR LBD (Hu *et al.*, 2003). Later portions of the chapter will explore not only the functional features of the LBD based on biochemical, cellular, and *in vivo* experiments, but will also discuss the effects of LBD modification and species-based sequence differences upon EcR and USP capabilities.

As noted elsewhere, the USP LBD has diverged considerably over evolutionary time. Whereas the USP LBD of some insect orders bears a fairly close resemblance to the vertebrate RXR, the dipteran and lepidopteran USP LBDs include a loop between helices 1 and 3 (Figure 5; Billas *et al.*, 2001; Clayton *et al.*, 2001). The diversity is further elaborated in the fly sequences, which include several additional, glycine-rich stretches that are not found in other insects. The aforementioned *Lm* USP includes short and long variants in the LBD which arise by an unidentified molecular process (Hayward *et al.*, 2003).

Among the 21 nuclear receptors in *D. melanogaster*, all but two of these proteins (Knirps, Nauber *et al.*, 1988; and Knirps-related, Oro *et al.*, 1988) carry the 12 alpha-helices that typify the LBD.

Except for EcR and possibly USP, none of these 19 superfamily members are known to interact physically with a cognate ligand. Several show patterns of expression that suggest a role in mediating ecdysteroid-inducible responses (Sullivan and Thummel, 2003) and one, DHR38, is activated by ecdysteroids via a novel mechanism (Baker *et al.*, 2003).

7.2.1.5. F domain Only a few members of the nuclear receptor superfamily possess the F domain, but the EcR family members carry up to 226 amino acids (in *D. melanogaster*) beyond the helix 12 region, though these are not conserved among the insect EcR sequences. Apparently, the F-domain of *DmEcR* is totally dispensable, since its cleavage produces a receptor that has the same transcriptional capabilities as the full-length EcR (Hu *et al.*, 2003). Recently, a variant of the *Aedes albopictus* EcR has been discovered which lacks seven amino acids in this carboxy-terminal region. While the functional significance, if any, of this deviation is not known, only one of the forms normally predominates in a mosquito cell line (Jayachandran and Fallon, 2001).

7.2.2. Strategies for Identifying Insect Receptors

The identity of EcR described earlier by using a DNA probe derived from the E75 "early puff" gene quickly led to similar strategies for identifying EcR orthologs in other insects. These attempts have sometimes employed conventional probes, typically derived from the DBD sequence of *DmEcR*, to screen cDNA libraries (e.g., Mouillet *et al.*, 1997; Guo *et al.*, 1997). In many instances a combination of approaches utilizing cDNA library screening along with polymerase chain reaction (PCR) using primers from conserved EcR regions have been utilized to obtain full-length EcR cDNA clones (e.g., Swevers *et al.*, 1995). Similarly, the use of PCR to amplify a small portion of the EcR DBD sequence, followed by cDNA library searches has also resulted in the successful recovery of EcR cDNA clones (e.g., Hannan and Hill, 1997). Because many insects encode multiple EcR isoforms, 5' and 3' rapid amplification of cDNA ends (RACE) has been used with primers derived from regions of the EcR cDNA shared among all the isoforms. These extensions from a common region within the EcR sequence such as the DBD or LBD permit the rapid recovery of novel transcripts with isoform-specific coding regions (e.g., Minakuchi *et al.*, 2002).

The recovery of the *D. melanogaster ultraspiracle* (*usp*) gene resulted from three independent screens using vertebrate RXR as a probe (Oro *et al.*, 1990),

EcR

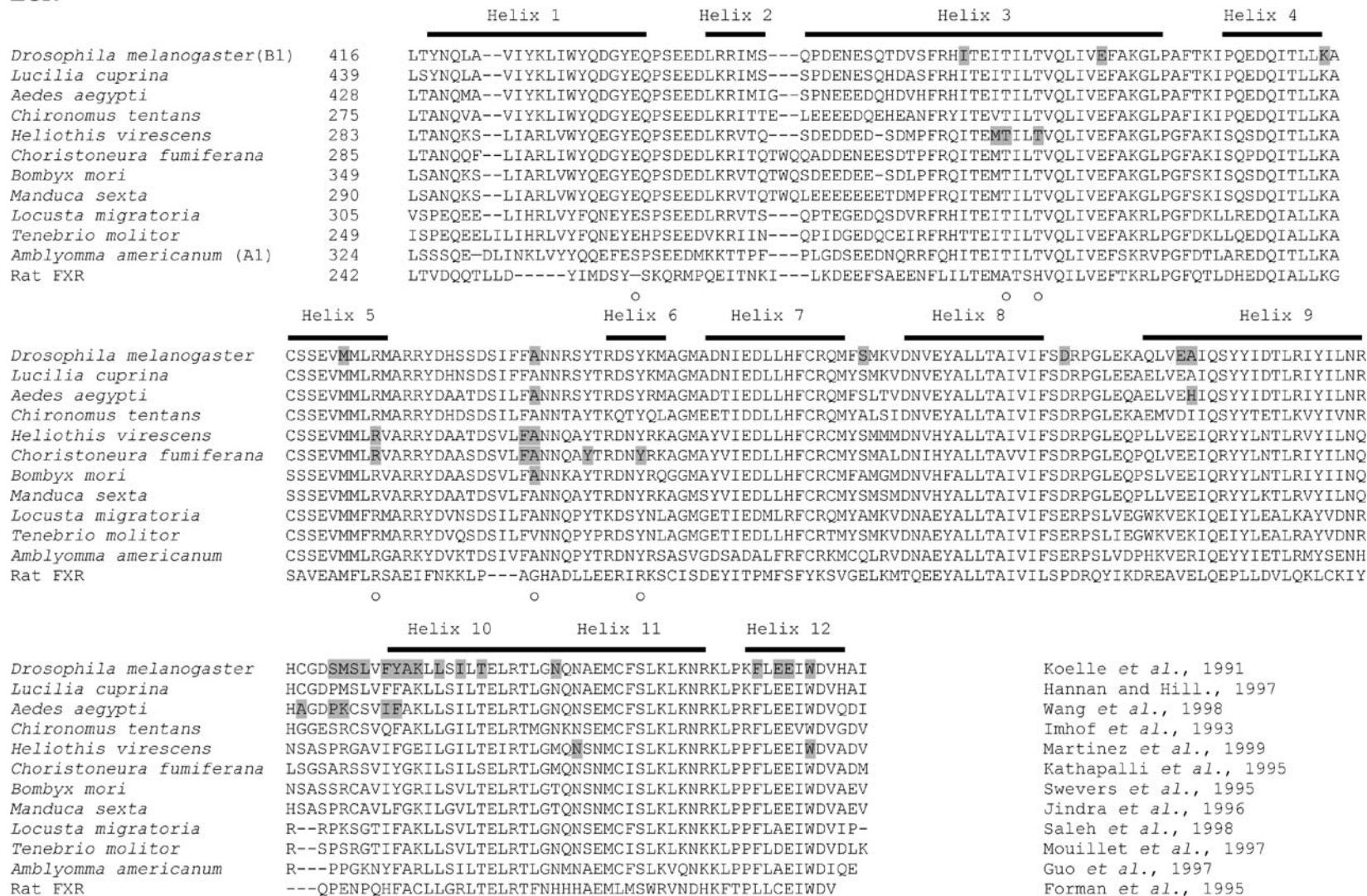


Figure 4 Amino acid alignment of representative insect EcR sequences (order Diptera: *D. melanogaster*, *Lucilia cuprina*, *Aedes aegypti*, *C. tentans*; order Lepidoptera: *Heliothis virescens*, *Bombyx mori*, *Manduca sexta*, *Choristoneura fumiferana*; order Orthoptera: *Locusta migratoria*; order Coleoptera: *Tenebrio molitor*). Also included: ixodid tick (*Amblyomma americanum*), and rat FXR. Amino acids designate number of first residue in alignment. References indicate original report. Bars designate each of the 12 helices, and shaded residues indicate those which have been mutated and reported (references given in the text). Binding sites of *HvEcR* with 20E indicated with circle (o).

USP

		<u>Helix 1</u>	<u>Helix 2</u>	<u>Helix 3</u>
<i>Drosophila melanogaster</i>	233	SVSR---DFSIERIIEAEQRAETQCGDRALTPLRVG--PYSTVQPDYKGAVS--ALCQVVKQLFQMVYARMPHFAQVP		
<i>Lucilia cuprina</i>	203	SSLR---DLTIERIIEAEQKAEISLGGDNVLPFLRVG--NNSMVQHDYKGAVS--HLCQMVKQYQMVYARRTPHFTHLQ		
<i>Aedes aegypti</i> USP-A	229	SSVR---DVTIERIIEAEQLEQSEQKSGDNAIPYLRVG--SNSMIPPEYKGAVS--HLCQMVKQYQILDFARRVPHFTINLP		
<i>Chironomus tentans</i>	295	GPGR---DITVERLMEADQMSBARGCKSIQYLRVAASNTMIPPEYRAPVS--AICAMVVKQVQHMDFCRRLLPHFTKLP		
<i>Heliothis virescens</i>	201	SSVQV--ELSIERLLEMESLVADPSEE--FQFLRVG--PDSNVPPKFRAPVS--SLCQIGNKQIAALVVWARDIPHFSQLE		
<i>Choristoneura fumiferana</i>	210	SSVQVDELSIERLLEMESLVADPSEE--FQFLRVG--PDSNVPPRYRAPVS--SLCQIGNKQIAALVVWARDIPHFGQLE		
<i>Bombyx mori</i>	202	SSVQ---ELSIERLLELEALVADSAAE--LQILRVG--PESGVPAPYRAPVS--SLCQIGNKQIAALVVWARDIPHFGQLE		
<i>Manduca sexta</i>	201	SSVQ---ELSIERLLEIESLVADPPEE--FQFLRVG--PESGVPAPYRAPVS--SLCQIGNKQIAALVVWARDIPHFGQLE		
<i>Locusta migratoria</i>	177	SSLH---TDMFVERILEAEKRVECKAEN-----QVEY-----ESTMNNICQAANICQATNKQLFQLVWAKHIPHFTSLP		
<i>Tenebrio molitor</i>	176	SNMQ--AEMPLDRIIEAEKRIECPAG-----GSGG-----VGEQHDGVN--NICQATNKQLFQLVQWAKLIPHFTSLP		
<i>Amblyomma americanum</i>	158	TSGGAPPEMPLERILEAELRVES-----QTGLTSESAQQDD-----PVS--SICQAADRQLHLVQWAKHIPHFEELP		
<i>Homo sapiens</i> RXRA	224	SSAN--EDMPVERILEAEELAVEPKTET-----YVEANMGLNPSSPNDPVT--NICQAADKQLFTLVEWAKRIPHFEELP		

		<u>Helix 4</u>	<u>Helix 5</u>	<u>Helix 6</u>
<i>Drosophila melanogaster</i>		IDDQVILLKAAWIELLIANVAWCSIVSLDDGGAGGGGGLGHGDSFERRSPGLQPQQLFLNQSFYHHRNSAIKAGVSAI		
<i>Lucilia cuprina</i>		REDQILLKAGWNELLIANVAWCSIESLDAEYASPG---TVHDGSGFRRSPVRQPQQLFLNQNFYHHRNSAIKANVVSII		
<i>Aedes aegypti</i> USP		RDDQVMLLRCCGNWELIIAAVAWRSMYEIETER-----SSDGSRIIV---RQPQLMCLGPNFTLHRNSAQAGVDTL		
<i>Chironomus tentans</i>		LNDQMYLLKQSNELIILNIAVMSIQYVEPDRR-----NADGSLERR--QISQMCLSRNYTLGRNMAVQAGVVQI		
<i>Heliothis virescens</i>		MEDQILLIKGSWNELLLFAIAWRSMEFLETERD-----GVDGTGNRT--TSPQMLMCLMPGMTLHRNSALQAGVGQI		
<i>Choristoneura fumiferana</i>		LDQVVLKASWNELLLFAIAWRSMEYLEDERE-----NGD--GTRS--TTQPQLMCLMPGMTLHRNSAQAGVGAI		
<i>Bombyx mori</i>		IDDQILLIKGSWNELLLFAIAWRSMEFFLDERE-----NVD--SRN--TAPPQLICLMPGMTLHRNSALQAGVGQI		
<i>Manduca sexta</i>		LEDQILLIKSNWELLLFAIAWRSMEYLTDERE-----NVD--SRS--TAPPQLMCLMPGMTLHRNSALQAGVGQI		
<i>Locusta migratoria</i>		LEDQVLLLRAGWNELIIAASFHRSVDVKDG-----IVLATGLTVHHRNSAHQAGVGVTI		
<i>Tenebrio molitor</i>		MSDQVLLLRAGWNELIIAASFHRSIQAQDA-----IVLATGLTVNKTSAHAHVGVGNI		
<i>Amblyomma americanum</i>		LEDRMVLKAGWNELIIAASFHRSVDVDRD-----IVLATGLVVQRHSAHGAGVGAI		
<i>Homo sapiens</i> RXRA		LDDQVILLRAGWNELIIASFHRSIAVKDG-----ILLATGLVHHRNSAHSAGVGAI		

		<u>Helix 7</u>	<u>Helix 8</u>	<u>Helix 9</u>	<u>Helix 10</u>
<i>Drosophila melanogaster</i>		FDRILSELSVKMKRLLNDRRELSCLKAIIILNPNDIRGKISRAEIECREKVYACLDEHCRLEHPGDDGRFAQLLLRLPALR			
<i>Lucilia cuprina</i>		FDRILSELSIKMKRLNIDRSELSCLKAIIILFNPNIRGLKCRADVEVCREKIYACLDEHCRTEHPGDDGRFAQLLLRLPALR			
<i>Aedes aegypti</i> USP		FDRILCELGIKMKRLLDVTRAEIVLKAIIILFNPNIRGLKQKEIDGMREKIYACLDEHCKQHPSEDGRFAQLLLRLPALR			
<i>Chironomus tentans</i>		FDRILSELSVKMKRLLDLDATLCLKSIVVFNPNVVRTLDDRKSIDLRSRIYASLDEYCRQKHPNEDGRFAQLLLRLPALR			
<i>Heliothis virescens</i>		FDRVLSELSLKMRTLVRDQAEYVALKAIILNPNVKGKLNKQEVVLEKMFCLDEYCRRSRSEEGRFAALLRLPALR			
<i>Choristoneura fumiferana</i>		FDRVLSELSLKMRTLVRDQAEYVALKAIILNPNVKGKLNKQEVVLEKMFCLDDYCRRSRSEEGRFAALLRLPALR			
<i>Bombyx mori</i>		FDRVLSELSLKMRTLVRDQAEYVALKAIILNPNVKGKLNKQEVVLEKMFCLDEYCRRSRSGEEGRFAALLRLPALR			
<i>Manduca sexta</i>		FDRVLSELSLKMRTLVRDQAEYVALKAIILNPNVKGKLNKPEVVVLEKMFCLDEYVRRSRCAEEGRFAALLRLPALR			
<i>Locusta migratoria</i>		FDRVLTTELVAKMRKMDKTELGLRSVILFNPEVRLKSAQEVLEKRYAALEEYTRTHPDEPGRFAKLLRLPSLR			
<i>Tenebrio molitor</i>		YDRVLSSELVNMKEMKMDKTELGLRAIILYNPTCRGKISVQEVLEKRYVLEEYTRTHPNEPGRFAKLLRLPALR			
<i>Amblyomma americanum</i>		FDRVLTTELVAKMRKMDKTELGLRAVILFNPEAKGLRTPSGGPEGESVASALEEHCRRQYDPQGRFAKLLRLPALR			
<i>Homo sapiens</i> RXRA		FDRVLTTELVSKMRMDKMDKTELGLRAVILFNPNDSKGLSNPAEVEALREKVYASLEAYCKHKYEPQGRFAKLLRLPALR			

		<u>Helix 11</u>	<u>Helix 12</u>	
<i>Drosophila melanogaster</i>		SISLKCQDHLFLFRITSDRPLEELFLEQLEAPP		Oro et al. (1990), Shea et al. (1990), Henrich et al. (1990)
<i>Lucilia cuprina</i>		SISLKCCLDHLFFRRLIGERALEELIAEQLEAPI		Hannan and Hill (2001)
<i>Aedes aegypti</i> USP		SISLKCCLDHLNFRILSDRHLDSFTIVEMLDMPI		Kapitskaya et al. (1996)
<i>Chironomus tentans</i>		SISLKCCLDHLFYQLIDDKNVNSVIEEFHKLN		Vogtli et al. (1999)
<i>Heliothis virescens</i>		SISLKSFEHLFFHVLVADTSIAGYIRDALRNHA		unpublished
<i>Choristoneura fumiferana</i>		SISLKSFEHLYFFHLVAEGSISGYIREALRNHA		Perera et al. (1998)
<i>Bombyx mori</i>		SISLKSFEHLYLFFHLVAEGSVSISYIRDALCNHA		Tzertzinis et al. (1994)
<i>Manduca sexta</i>		SISLKCFEHLYFFHVLVADTSIASYIHDAALRNHA		Jindra et al. (1997)
<i>Locusta migratoria</i>		SIGLKCLEHLFFRRLIGDVPIDTFMEMLESPS		Hayward et al. (1999)
<i>Tenebrio molitor</i>		SIGLKCSEHLFFFKLIGDVPIDTFMEMLESAPS		de laChambre (unpublished)
<i>Amblyomma americanum</i>		SIGLKCLEHLFFFKLIGDTPIDNFLMSLEAPS		Guo et al. (1998)
<i>Homo sapiens</i> RXRA		SIGLKCLEHLFFFKLIGDTPIDTFMEMLEAPH		Mangelsdorf et al. (1990)

Figure 5 Continued

employing an oligonucleotide derived from a highly conserved portion of the DBD to screen an embryonic cDNA library (Henrich *et al.*, 1990), and by using a promoter element in the *s15* chorion gene to screen an expression library (Shea *et al.*, 1990). A genomic clone residing near the tip of the *D. melanogaster* X chromosome rescues larvae homozygous for the early larval lethal mutation, *ultraspiracle* (*usp*; Oro *et al.*, 1990), so named because mutant larvae fail to shed their first instar cuticle and appear to develop an extra row of spiracles (Perrimon *et al.*, 1985). The same combination of PCR-based and library screening approaches used for finding EcR have led to the recovery of complete USP sequences from other insects.

The discovery that E75 was not only a target of ecdysteroid action, but a nuclear receptor itself, proved to be the first of several serendipitous discoveries of other nuclear receptors in the *Drosophila* genome that are associated directly with the salivary gland puffing response mediated by EcR and USP. Another “early late” puff gene product, E78, is a member of the nuclear receptor superfamily (Stone and Thummel, 1993) as is DHR3, which had been recovered in the same library screen that yielded EcR (Koelle *et al.*, 1992); DHR3 is also a heterodimeric partner of EcR (White *et al.*, 1997). Still another nuclear receptor, β FTZ-F1, a transcriptional regulator of the embryonic segmentation gene *fushi tarazu* (Lavorgna *et al.*, 1991) is the product of the stage-specific 75CD chromosomal puff that occurs during the mid-prepupal ecdysteroid peak (Woodard *et al.*, 1994).

More systematic screens have yielded other *Drosophila* hormone receptors (DHRs), including DHR4 (Sullivan and Thummel, 2003), DHR39 (Horner *et al.*, 1995), and DHR78 (Fisk and Thummel, 1998), which are orphans and ecdysteroid-regulated. Based on USP's possible homology to RXR, a yeast two-hybrid assay employing USP as bait led to the isolation of an interacting orphan receptor, DHR38, that is orthologous to the vertebrate nerve growth factor 1B (NGF1B; Sutherland *et al.*, 1995). The *seven-up* (*svp*) gene, originally defined by a mutation that disrupts photoreceptor function, encodes the *Drosophila* ortholog of another vertebrate orphan, the chicken ovalbumin upstream promoter-transcription factor (COUP-TF; Mlodzik *et al.*,

1990). COUP-TF heterodimerizes with RXR, and analogously, SVP forms a functional heterodimer with USP as shown by its ability to compete for dimerization with EcR in both *D. melanogaster* (Zelhof *et al.*, 1995a) and *A. aegypti* (Zhu *et al.*, 2003a).

Therefore, the orphan nuclear receptors not only resemble EcR and USP structurally, it is increasingly apparent that they also play a role in the orchestration of ecdysteroid response, both as targets of EcR and USP-mediated transcriptional activity and as heterodimeric partners for the two functional components of the ecdysteroid receptor. Each of these orphans is sufficiently unique in sequence that a combination of library screening and PCR approaches have led to the successful recovery of orphan orthologs. A listing of the orphans implicated in some aspect of ecdysteroid regulation from other insect species is given in Table 1. Later in the chapter, the regulation and developmental roles of these orphans in *D. melanogaster* and other insects will be explored and compared further. The transcript levels of all 21 nuclear receptors found in the *D. melanogaster* genome have been analyzed preliminarily to reveal their potential connection with ecdysteroid-regulated developmental events. Some of these have not been functionally associated with ecdysteroid action and play a role in other developmental processes (Sullivan and Thummel, 2003, and references therein); these will not be considered further in this chapter.

7.2.3. Homology Models and Crystal Structure for Ecdysteroid Receptor Components

The need to understand the interaction of EcR with its natural ligand and other ecdysteroid agonists has prompted efforts to elucidate the crystal structure of the EcR LBD. Homology models based on a comparison of the EcR LBD with the known crystal structures of the vertebrate retinoic acid receptor (RAR) and vitamin D receptor (VDR) have been employed to formulate predictions about the three-dimensional structure of the EcR LBD (Wurtz *et al.*, 2000). The docking model resulting from this comparison with known crystal structures suggests that the ligand-binding pocket of EcR consists of a shallow tube that is just large enough to accommodate 20E and a bulky envelope. The shape and size of the

Figure 5 Amino acid alignment of representative insect USP sequences (order Diptera: *D. melanogaster*, *L. cuprina*, *A. aegypti*, *C. tentans*; order Lepidoptera: *H. virescens*, *B. mori*, *M. sexta*, *C. fumiferana*; order Orthoptera: *L. migratoria*; order Coleoptera: *T. molitor*). Also included: ixodid tick (*A. americanum*) and human RXR- α . Amino acids designate number of first residue in alignment. References indicate original report. Bars designate each of the 12 helices, and shaded residues indicate those which have been mutated and reported (references given in the text).

Table 1 Nuclear receptors experimentally associated with ecdysteroid action in *Drosophila melanogaster*. Relevant references given in text

<i>Drosophila</i> receptor	Noninsect ortholog	Insect orthologs	Mutational effect in <i>Drosophila</i>
EcR	vert. FXR	see Figure 4	Embryonic/larval lethal
USP	vert. RXR	see Figure 5	Larval lethal
DHR4		<i>Manduca</i>	Not reported
DHR38	vert. NGF1B	<i>Aedes</i>	Disrupted cuticle development
DHR39			No discernible effect
E75	<i>Metapanaeus</i> (shrimp) Vert. PPAR γ	<i>Aedes, Bombyx, Choristoneura, Galleria, Manduca</i>	Larval/adult lethal
E78	<i>Dirofilaria</i> (filarial parasite)		Disrupted chromosome puffing
DHR3	<i>C. elegans</i> CHR3, ROR	<i>Aedes, Choristoneura Galleria, Manduca, Bombyx, Tenebrio</i>	Embryonic lethal
DHR78		<i>Bombyx, Tenebrio</i>	Larval lethal
FTZF1	vert. Steroidogenic factor-1 (SF1)	<i>Aedes, Bombyx, Manduca, Tenebrio</i>	Embryonic/larval lethal
SVP	vert. COUP-TF	<i>Aedes, Tenebrio</i>	Disrupted eye development

pocket predicted by this model also indicate that the more compact bisacylhydrazines that behave as nonsteroidal agonists of EcR fill up only a portion of the pocket. The crystal structure of *HvEcR* has shown that the EcR LBD is highly flexible, and that the shape of its ligand-binding pocket adapts to the ligand that occupies it, whether it be 20E, or a nonsteroidal agonist (**Figure 6**; Billas *et al.*, 2003). Just as the crystal structure predicts, substitution of an amino acid that normally contacts 20E (A398P in *HvEcR*; see **Figure 4** for its position in other EcR sequences) destroys *HvEcR*'s ability to mediate an inducible transcriptional response to 20E, but the same mutation does not affect the inducibility caused by a nonsteroidal agonist. The crystal structure further predicts that a valine residue residing in helix 5 of Lepidoptera EcR sequences (V384 in *HvEcR*) is responsible for the high affinity of a lepidopteran-specific agonist, BY106830. Most insect EcRs from other orders encode a methionine at this position in helix 5 (see **Figure 4**). The flexibility of the EcR LBD which allows it to bind structurally disparate ligands, presumably is stabilized by heterodimerization with USP.

When USP is purified for crystal structure analysis, a phospholipid is copurified that is partially embedded in the relatively large ligand-binding pocket of USP. As a consequence, the USP ligand-binding domain is held in an antagonistic, apo-conformation in which the carboxy-terminal region including the last alpha-helix (helix 12) is held out (**Figure 7**; Billas *et al.*, 2001; Clayton *et al.*, 2001). Another modeling study has shown that the USP LBD possesses the theoretical capability to reconfigure itself into an agonist position and interact with protein cofactors, although the residues involved in

these interactions would be different from those exposed on the RXR- α homolog when it assumes an agonist conformation (Sasorith *et al.*, 2002). Modelling has also been employed to determine the capability for the three major juvenile hormones (JH1, JH2, and JH3) to bind to the ligand-binding pocket of USP. At least two ligand-binding configurations were identified, one associated with USP forms that carry an arginine residue at a critical position in helix 5 (see **Figure 5**). This residue prevails among the non-dipteran species for which the model predicted that JH acids could form a stable binding configuration.

For the dipteran species, which typically carry a nonpolar amino acid at this position in helix 5, JH esters showed better affinity than JH acids, consistent with the results of biochemical binding studies performed on the *Drosophila* USP in which a purified USP fusion protein shows half-maximal binding in the range of 1–5 μ M using a tryptophan fluorescence assay (Jones *et al.*, 2001). While the modeling demonstrates the theoretical capability for various JH forms to bind to either lepidopteran or dipteran forms of USP, the occupancy rate of the ligand-binding pocket for them is relatively low for nuclear receptors. Nevertheless, other studies indicate that biological relevance does not always depend upon a high ligand binding affinity (Forman *et al.*, 1995; Kitareewan *et al.*, 1996; Staudinger *et al.*, 2001). For instance, the affinity of the vertebrate peroxisome proliferator activating receptors (PPARs) involves ligand interactions of low specificity and affinity that approximates the levels predicted for USP and JH forms (Schmidt *et al.*, 1992). Viewed from this perspective, the low affinity of a receptor for its activating ligand actually suggests a

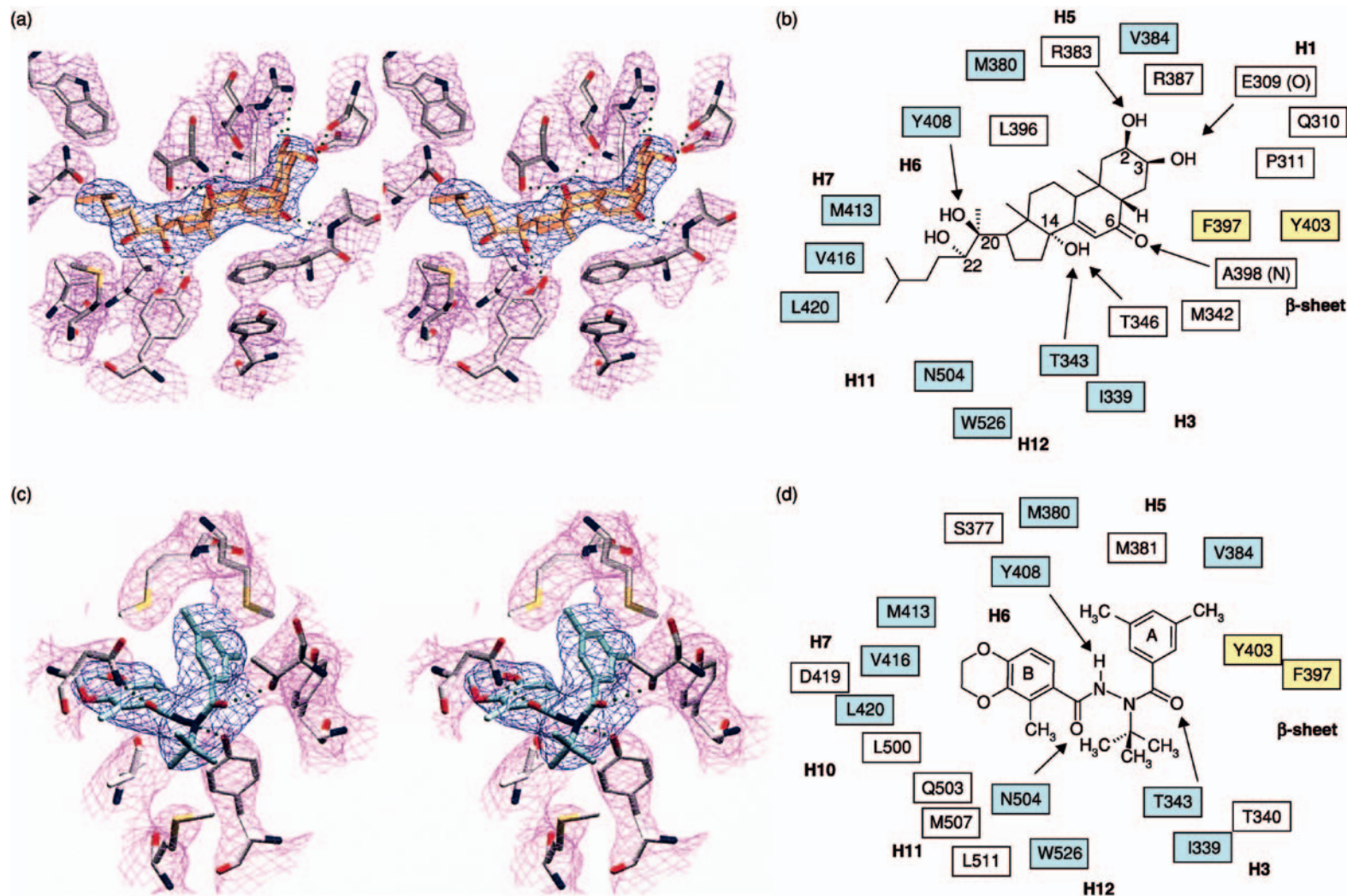


Figure 6 Two ligand binding modes for *HvEcR* LBD as described in Billas *et al.* (2003). (a) Two weighted omit stereoview maps of the electron density for ponasterone A-bound *HvEcR*-LBD at 2.9 Å resolution. Ligand shown in blue and selected amino acid residues shown in magenta. Interactions between residues and ligand indicated as green dotted lines. (b) Schematic representation of the interactions between ligand-binding residues and ponasterone A. Arrows correspond to hydrogen bonds between ligand and amino acid residues. Residues in blue are common to both stereoview structures in (a), residues in white are those depicted only in the first stereoview map, and those in yellow are depicted only in the second (righthand) map. (c) two weighted stereoview maps, as above, with the nonsteroidal agonist, BY106830. (d) schematic representation of the interactions between ligand-binding residues and BY106830. (Adapted with permission from Billas, I.M., Iwema, T., Garnier, J-M., Mitschier, A., Rochel, N., *et al.*, 2003. Structural adaptability in the ligand-binding pocket of the ecdysone hormone receptor. *Nature* 426, 91–96.)

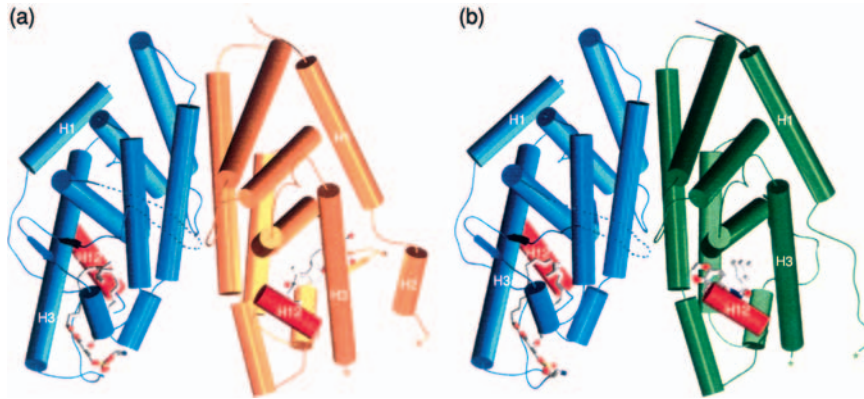


Figure 7 The dimerization structures of *HvEcR* LBD and *HvUSP* LBD. (a) EcR/USP LBD heterodimer with bound ponasterone A. (b) EcR/USP LBD heterodimer with nonsteroidal agonist, BY106830. Helix 12 is shown in red, USP shown in blue. The missing loop that connects USP Helix 5 to the β -sheet is shown as a dashed blue line that projects closely to EcR helix 9–10. (Adapted with permission from Billas, I.M., Iwema, T., Garnier, J.-M., Mitschier, A., Rochel, N., *et al.*, 2003. Structural adaptability in the ligand-binding pocket of the ecdysone hormone receptor. *Nature* 426, 91–96.)

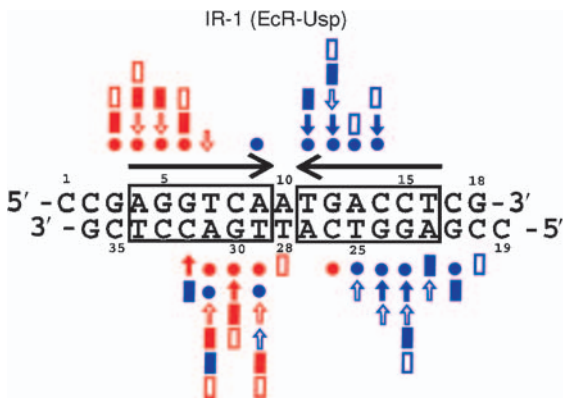


Figure 8 An idealized palindromic repeat element with a single nucleotide spacer used in the EcR-USP complex. Symbols as in **Figure 3**. Red indicates USP interaction sites, blue indicates EcR interaction sites. Solid arrows indicate a direct base interaction and hollow arrows indicate a water-mediated base interaction. Solid bars indicate a direct phosphate interaction, and hollow bars indicate a water-mediated phosphate interaction. Circles indicate van der Waals interaction. (Reproduced with permission from Devarakonda, S., Harp, J.M., Kim, Y., Ozyhar, A., Rastinejad, F., 2003. Structure of the heterodimeric ecdysone receptor DNA-binding complex. *EMBO J.* 22, 5827–5840.)

mechanism by which fairly tight regulation is achieved when ligand titers reach relatively high levels in the cell. A discussion of functional experiments concerning the effects of JH on the ecdysteroid receptor components, EcR and USP, is presented later.

The crystal structure of the EcR and USP DBD at a canonical EcRE DNA-binding site reveals the nature and location of DNA-binding residues for the two DBD regions, as well as their points of protein-protein interaction (**Figure 8**). While RXR forms a functional dimer with EcR (Christopherson *et al.*, 1992; Yao *et al.*, 1993), the physical interaction

between USP/RXR and EcR is different than it is for RXR with several of its natural partners (**Figure 9**; Devarakonda *et al.*, 2003).

The ability of the DHR38/USP heterodimer to induce transcription in response to ecdysteroids, even though the complex does not physically interact with its ligand, has prompted a crystal structure analysis of the DHR38 LBD. DHR38 lacks a true ligand-binding pocket – the space is filled with four phenylalanine side chains. The dimerization interface apparently lies along helix 10, but DHR38 lacks helix 12, and its AF2 function may reside in a unique sequence lying between helix 9 and 10 in an activated (agonistic) conformation (Baker *et al.*, 2003).

7.2.4. Biochemical Analysis of EcR and USP

Numerous biochemical experiments have been performed to assess the properties associated with EcR and USP function in insects, including: (1) the affinity of EcR for ecdysteroids and nonsteroidal agonists, (2) the ability of EcR and USP to dimerize and interact with DNA target sequences, (3) the physical interaction of EcR and USP with other orphan receptors and proteins, and (4) the detection and demonstration of posttranslational and other modified forms of EcR and USP.

The necessity for heterodimerization between EcR and USP to produce a functional ecdysteroid receptor was demonstrated by showing that the *in vitro* translated EcR and USP products bind to a radiolabelled *hsp27* EcRE, but that neither product alone is capable of binding to the same element, based on the results of an electrophoretic mobility shift assay (EMSA). Moreover, this effect is enhanced by the simultaneous presence of muristerone A or

20E, indicating that the hormone stabilizes the heterodimer at an EcRE site. Further, the affinity of radiolabelled ^{125}I -iodoponasterone, an ecdysteroid with high specific activity and affinity for EcR (Cherbas *et al.*, 1988), is substantially higher for EcR/USP dimers than for EcR alone ($\sim K_d = 1 \text{ nM}$; Yao *et al.*, 1993). A similar level of ligand affinity is obtained with extracts taken from cultured *Drosophila* Kc and S2 cells, which contain endogenously expressed USP (Koelle *et al.*, 1991). EcR also heterodimerizes with the vertebrate RXR and this heterologous dimer is responsive to muristerone A, but not 20E, indicating that USP plays some role in determining the ligand specificity of the complex (Christopherson *et al.*, 1992; Yao *et al.*, 1993). The ability of EcR and USP candidates to heterodimerize on an *hsp27* EcRE has become a standard for verification (e.g., Swevers *et al.*, 1996; Hannan and Hill, 2001; Durica *et al.*, 2002; Minakuchi *et al.*, 2002).

The EcR/USP heterodimers from other insects show an affinity for ^{125}I -iodoponasterone and [^3H]-ponasterone A that approximates the *DmEcR*/USP heterodimer (e.g., $K_d = 1.1 \text{ nM}$; Swevers *et al.*, 1996). For *AaEcR* and *AaUSP*, the detection of EcR/USP heterodimers in a mammalian cell culture extract increases approximately 25-fold with the addition of $5 \mu\text{M}$ 20E. This accompanies a decrease in the quantity of *AaUSP*/*AaSVP* heterodimers, thus illustrating the competitive dynamics of these two protein-protein interactions involving USP (Zhu *et al.*, 2003a).

The ability of nonsteroidal agonists, such as the diacylhydrazines, RH5849 (1,2-dibenzoyl-1-*tert*-butylhydrazine) and RH5992 (tebufenozide), to displace radiolabelled ponasterone has been used to assess EcR specificity for these compounds. When extracts from a lepidopteran (Sf9) and dipteran insect cell line (Kc) are compared for the ability of the diacylhydrazines to displace [^3H]-ponasterone A, the affinity of all RH compounds is considerably lower in the Kc cells (Nakagawa *et al.*, 2002). Also, none of several RH compounds fail to displace [^3H]-ponasterone A in extracts containing the migratory locust's EcR and USP, indicating that the orthopteran EcR, like those of other ancient insect orders, possesses substantially different binding properties than those of the Lepidoptera (Hayward *et al.*, 2003). Ligand binding requires the entire LBD, and deletion of a portion of helix 12 at the carboxy-terminal end of EcR is sufficient to eliminate ponasterone A binding (Perera *et al.*, 1999b; Hu *et al.*, 2003; Grebe *et al.*, 2003).

Fusion proteins expressing wild-type and mutated versions of *DmEcR* and *DmUSP* LBDs in yeast cells have also been subjected to tests of their affinity

for ponasterone A in cell extracts (Grebe *et al.*, 2003). The association rate of a *DmEcR* LBD fusion protein is modestly elevated by the presence of USP, and the dissociation rate is reduced by about 20-fold, so that the dissociation constant is reduced from about 40 nM in EcR alone to about 0.5 nM in the presence of USP. At least one mutation of the *Drosophila* EcR LBD (N626K) alters the rate of dissociation without affecting association rate, suggesting that an ecdysteroid enters and exits the EcR ligand-binding pocket by different routes, though this effect has not been tested in the intact EcR or *in vivo*. Purified *DmUSP* has the potential to form homodimers and other oligomers, even in the absence of a DNA-binding site, though this capability is reduced by removal of the A/B domain (Rymarczyk *et al.*, 2003).

Along with its ligand-binding properties, the ability to recognize a DNA element, usually the consensus *hsp27* EcRE by an EcR/USP or EcR/RXR dimer, is a standard for identifying a functional ecdysteroid receptor. The *hsp27* EcRE was originally localized by DNase I footprinting and its ability to confer ecdysteroid-inducibility on an otherwise noninducible promoter in S1 *Drosophila* cultured cells. The sequence is 23 bp long and arranged in an inverted palindrome separated by a single nucleotide (Riddihough and Pelham, 1987). The EcR and USP zinc finger interactions with an idealized palindromic EcRE and with each other are described diagrammatically (Figure 3; Ozyhar and Pongs, 1993; Devarakonda *et al.*, 2003) and shown graphically (Figure 8). A diagrammatic depiction of the nature of individual nucleotide interactions between the element and the zinc fingers is also shown in Figure 9 (Devarakonda *et al.*, 2003). A closely related response element taken from an ecdysteroid-inducible gene in Kc cells, *Eip28/29*, has also been used experimentally (Cherbas *et al.*, 1991). The EcR/USP heterodimer also recognizes half sites arranged into direct repeats (DR) separated by 0–5 nucleotide spacers (DR0–DR5) with the DR4 element showing the highest affinity. Various direct repeat elements were tested with the ecdysteroid-inducible and fat body-specific *fbp1* promoter. When these direct repeat elements are connected to the minimal *fbp1* promoter, they are unable to induce higher transcription in the presence of ecdysteroids. However, when the DR0 and DR3 elements are substituted for the natural *fbp1* EcRE in its normal promoter context, both elements are ecdysteroid-inducible and fat body specific (Antoniewski *et al.*, 1996).

Context is also important for the intermolt, *sgs4* gene promoter, which requires that an EcRE be surrounded by serum element binding protein

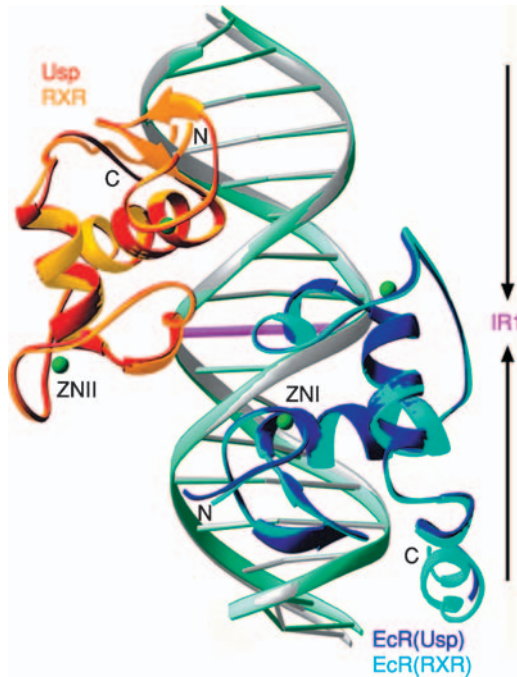


Figure 9 A superposition of the *DmEcR/DmUSP/IR1* DNA complex and the *DmEcR/HsRXR/IR1* DNA complex. USP is shown in red, RXR is shown in gold. EcR with USP is shown in purple, and EcR with RXR is shown in light blue. Zinc ions are shown in green and the single base pair spacer nucleotide is shown as magenta. (Reproduced with permission from Devarakonda, S., Harp, J.M., Kim, Y., Ozyhar, A., Rastinejad, F., 2003. Structure of the heterodimeric ecdysone receptor DNA-binding complex. *EMBO J.* 22, 5827–5840.)

(SEBP) binding sites in order to mediate a receptor-inducible response (Lehman and Korge, 1995).

A perfect palindrome carrying the half-site sequence, GAGGTCA separated by a single A/T nucleotide (PAL1) shows the highest affinity for the *DmEcR/DmUSP* complex in extracts taken from *Drosophila* S2 cells. By testing the ability of other elements to compete for this element, the order of affinity is been determined: PAL1 > DR4 > DR5 > PAL0 > DR2 > DR1 > *hsp27*, DR3 > DR0. In all cases, affinity is elevated by the presence of muristerone A. In cell culture experiments testing the inducibility of these elements when attached to a minimal promoter, inducibility correlates with affinity, except that the *hsp27* EcRE is a stronger inducing element than DR1, DR2, or PAL0. When DR4 is placed in a reverse orientation relative to the promoter, it maintains its ability to mediate a transcriptional response (Vogtli *et al.*, 1998). A similar correlation between DNA affinity and inducibility has been noted for the *AaEcR/AaUSP* complex and its response elements (Wang *et al.*, 1998).

A natural direct repeat EcRE has been identified in the ecdysteroid-regulated intermolt 3C puff of

D. melanogaster that is separated by 12 nucleotides (DR12) which is capable of conferring ecdysteroid-responsiveness to a heterologous promoter in cell cultures (D'Avino *et al.*, 1995). The affinity of the EcR/USP complex for the DR12 element is offset by the presence of other orphan receptors (DHR38, DHR39, β FTZF1), and a weak inductive response to 20E is also offset by the presence of these orphans (Crispi *et al.*, 1998). A similar competition scenario exists between *DmEcR* and *DmSVP* as heterodimeric partners with *DmUSP*. The SVP/USP dimer preferentially binds to a DR1 element, whereas the EcR/USP dimer displays a heightened affinity for an *Eip28/29* EcRE (Zelhof *et al.*, 1995a).

The recognition of these canonical elements also depends upon specific features of the ecdysteroid receptor itself. In at least one instance, DNA recognition depends upon isoform-specific EcR and USP combinations. The EcR heterodimers formed with each of the two USP/RXR isoforms in the ixodid tick, *A. americanum*, display substantially different affinities for DNA response element sequences, with an RXR1/EcR dimer showing strong affinity for both palindromic and direct repeat sequences, whereas an RXR2/EcR dimer shows only weak affinity for a PAL1 element (Palmer *et al.*, 2002).

Ligand-binding specificity also plays a role in determining the affinity of EcR/USP for a response element. *AaEcR/DmUSP* recognition of an *hsp27* EcRE on EMSAs is normally enhanced by the presence of ecdysone, but only 20E enhances affinity of the *DmEcR* in this assay. Domain swapping between the *AaEcR* and the *DmEcR* localizes a region within the LBD that is responsible for the differential effect of ecdysone on the two EcRs. Within the swapped region, a single amino acid conversion in the *DmEcR* (Y611F) to the corresponding *AaEcR* sequence results in a mutated receptor that shows the ability to dimerize in the presence of both 20E and ecdysone (Wang *et al.*, 2000). Interestingly, all insect EcR sequences encode either a tyrosine or phenylalanine at this position in helix 10, which lies along a dimerization interface. The subtle yet significant difference in sequence, when considered in the framework of the numerous residue differences that exist among the insect EcR LBDs, illustrates the dimensions of potential functional diversity among them.

Generally, the interpretation of EMSA results must be handled circumspectively because purified EcR/USP proteins do not always exhibit the same DNA binding properties as cell extracts which include the receptor components (e.g., Lan *et al.*, 1999; Palmer *et al.*, 2002). This is exemplified in the extreme by the observation that affinity column

purified *DmEcR* and *DmUSP*, when mixed together, fail to bind to the *hsp27* EcRE unless other chaperone proteins are added. While no single chaperone is essential for DNA binding, the elimination of any one of them proportionally reduces EcRE recognition. The formation of this chaperone/receptor complex also requires ATP, although none of these chaperones is necessary for ligand binding. Only two of the six chaperones described, Hsp90 and Hsc70, are *Drosophila* proteins; the others used in the study were human chaperone proteins (Arbeitman and Hogness, 2000).

By expressing GST fusion proteins in *E. coli* and using affinity chromatography to purify them, preparations of *Chironomus* EcR and USP have been recovered which retain essential DNA-binding and ligand-binding characteristics as long as detergents are absent from the purification process. Scatchard plots of bacterially expressed *CtEcR* reveal two high-affinity ecdysteroid binding sites, as do extracts from a *Chironomus* epithelial cell line (Grebe *et al.*, 2000). Bacterial GST-fusion proteins expressing the *Drosophila* EcR and USP DNA-binding domains have also been isolated for the purpose of testing their affinity to DNA elements, although the GST motif alters the DNA-binding properties of the fusion protein (Niedziela-Majka *et al.*, 1998; Grebe and Spindler-Barth, 2002). Similarly, the recovery of a fusion protein encoding the *DmEcR* LBD is enhanced by cleaving the C-terminal F-domain, which is considerably longer in *D. melanogaster* than other species (Halling *et al.*, 1999); this cleavage exerts no discernible effect on transcriptional activity (Hu *et al.*, 2003).

Since EcR and USP function as part of a protein complex, orphan receptors and cofactors described throughout the chapter typically require the discovery and/or demonstration of a physical interaction. These demonstrations include the use of yeast two-hybrid assays (e.g., Sutherland *et al.*, 1995; Beckstead *et al.*, 2001), the interaction of *in vitro* translated EcR and USP with a protein or protein domain (e.g., Bai *et al.*, 2000), and the identification of interacting proteins that coprecipitate with EcR and/or USP (e.g., Sedkov *et al.*, 2003). A member of another class of proteins, the immunophilins, which are known to interact with vertebrate steroid receptor complexes, coprecipitates as part of an EcR/USP complex taken from *M. sexta* prothoracic (ecdysteroidogenic) glands. Ligand affinity of the complex falls within an expected range, though it is unknown whether the interaction between the immunophilin, FKBP46, and EcR/USP is direct and also unknown whether this interaction affects transcriptional activity (Song *et al.*, 1997).

The colocalization of proteins such as RNA polymerase II and EcR on *Chironomus* polytene chromosomes by immunostaining illustrates a possible relationship between the complex and the cell's transcriptional machinery (see Figure 1; Yao *et al.*, 1993; Wegmann *et al.*, 1995). A zinc finger protein that preferentially localizes at active puffs along *Drosophila* polytene chromosomes, known as peptide on ecdysone puffs (PEP), has been characterized by anti-PEP antibody decoration of polytene chromosomes, but has not yet been functionally associated with ecdysteroid action (Amero *et al.*, 1991).

The effect of posttranslational modifications upon ecdysteroid activity has also not been explored extensively, though a phosphorylated form of *DmUSP* in fly larvae has been identified by showing that phosphatase activity eliminates a high molecular weight band in larval extracts. The phosphorylated form of USP in larval salivary glands is elevated by incubation with 20E (Song *et al.*, 2003). Similarly, the presence of a phosphorylated form of both *CtEcR* and *CtUSP* extracted from *C. tentans* larvae has been demonstrated on Western immunoblots (Rauch *et al.*, 1998). The relative abundance of phosphorylated forms of the two USP isoforms in the *M. sexta* prothoracic gland is altered by the presence of 20E and has been associated with changes in ecdysteroidogenic activity, suggesting a feedback mechanism by which ecdysteroid synthesis is downregulated in prothoracic glands (Song and Gilbert, 1998).

While several groups have employed DNA cloning methods to recover receptor genes from which protein sequences are deduced, other efforts have demonstrated receptor purification through measurements of their activity. Partial purification of an ecdysteroid-binding protein from *Drosophila* embryos produces two peptides that are radiolabelled by a 20E derivative, one of 150 kDa and another of 90 kDa (Strangmann-Diekmann *et al.*, 1990), the latter being the molecular weight predicted from the *Drosophila* EcR cDNA sequence (Koelle *et al.*, 1991). Purification of the protein through its affinity to a magnetic *hsp27* EcRE failed to resolve the disparity between the measured size of the purified product and the predicted size of the protein (Ozyhar *et al.*, 1992). Purification of the 20E receptor yielded a protein that binds to the appropriate response element sequence of the *hsp27* promoter, as well as an *hsp23* promoter element, and elevates transcription rates by 100-fold (Luo *et al.*, 1991). The presence of ecdysteroids increases the affinity of the receptor protein as measured by EMSA.

7.3. Functional Characterization of the Ecdysteroid Receptor

7.3.1. Cell Culture Studies: Rationale

The study of EcR and USP *in vivo* is complicated because ecdysteroid responses vary both spatially and temporally. In fact, the effects of ecdysteroids can be obscured by the heterogeneity of response at the promoter of a single gene (Andres and Cherbas, 1992; Andres and Cherbas, 1994). Therefore, it is often difficult to unravel *in vivo* transcriptional and cellular responses mechanistically, even in a single cell type, since the response to ecdysteroids typically triggers ongoing changes in the target cell, that in turn, affect later ecdysteroid responses.

Cell cultures provide the benefit of working with a stable, relatively homogeneous cell type that does not require the rigorous staging and rearing conditions necessary for meaningful studies *in vivo*. Thus, cultured cells can provide a variety of important and useful insights about EcR and USP by establishing a foundation for subsequent *in vivo* hypothesis testing. Because they are easy to grow, it is also relatively easy to recover cellular extracts for biochemical testing. Nevertheless, stably cultured cells probably do not duplicate any actual cell exactly, so that observations reflect what a cell can do, but not necessarily what a cell does. Therefore, subsequent *in vivo* verification is essential for insights garnered through cell culture experiments.

Numerous experiments involving cultured cell lines have been conducted over the years to test the ability of the ecdysteroid receptor to regulate transcriptional activity induced by ecdysteroid treatment. Early experiments focused on endogenous changes in cell morphology and gene expression in insect cells challenged with ecdysteroids such as 20E, which express EcR and USP endogenously. As transfection technology has developed, increasingly sophisticated cell systems have been developed to analyze and dissect receptor functions. In recent years these systems have been developed to screen for novel agonists and devise “new and improved” ecdysteroid induction systems for agricultural, biomedical, and commercial application.

7.3.2. Cell Cultures: Characterization

The most prominent cell line for early studies of ecdysteroid action was the *D. melanogaster* Kc cell line, and derivatives of it are still employed today (Hu *et al.*, 2003). In response to 20E at 10^{-7} to 10^{-8} M, Kc cells start to develop extensions within hours, produce acetylcholinesterase, and undergo a

proliferative arrest a few days later. These cells are even more responsive to the phytoecdysteroids, ponasterone A and muristerone A, than to 20E (Cherbas *et al.*, 1980 and references therein). With prolonged exposure to ecdysteroids, Kc cells become insensitive to 20E and EcR levels become depressed (Koelle *et al.*, 1991). The nonsteroidal agonist RH5849 not only mimics the effects of 20E when tested on sensitive Kc cells, it also fails to evoke a response from insensitive Kc cells, thus arguing that the RH5849 acts via a common mechanism with ecdysteroids (Wing, 1988).

Other cell lines, Schneider 2 and 3 (S2 and S3), also become insensitive to ecdysteroid action by reducing their titer of EcR, and have been used because they are relatively easy to transfect transiently with fusion and reporter genes. As noted earlier, the identity of the *DmEcR* gene was partly demonstrated by introducing the *DmEcR* cDNA into ecdysteroid-insensitive S2 cells and thus restoring their responsiveness to 20E (Koelle *et al.*, 1991). More recently, Kc cell lines have been improved by developing protocols for stable integration of transgenes by P-element transposition into the genome (Segal *et al.*, 1996) and by using parahomologous gene targeting to “knock out” an endogenous gene target (Cherbas and Cherbas, 1997). In this way, a cell line, L57-3-11 containing no endogenous EcR has been produced which allows the introduction of modified versions of EcR for subsequent experimentation, without the complications normally posed by the presence of endogenous EcR (Hu *et al.*, 2003).

The Kc cell line has been used to identify ecdysteroid-regulated transcriptional targets. By comparing hormone-treated and control Kc cells, Savakis *et al.* (1980) isolated and identified three ecdysteroid-inducible peptides (EIPs) named by their molecular mass in kiloDaltons, EIP28, EIP29, and EIP40, whose synthesis is elevated 10-fold at 10^{-8} M 20E. JH supplementation of the cell culture medium inhibits acetylcholinesterase induction but not EIP induction (Cherbas *et al.*, 1989).

The use of ecdysteroid-responsive lines for other insects is expanding continuously, largely in order to understand the causes of insecticidal resistance to ecdysteroid agonists and to identify novel insecticidal candidates. A *C. tentans* epithelial cell line was used to isolate several stable and ecdysteroid-resistant clones that metabolizes 20E relatively quickly (Spindler-Barth and Spindler, 1998). Clones from the same line were later selected for their resistance to the inductive effects of RH5992 and differential metabolism of RH5992 was ruled out as a cause for the resistance. While the profile of EcR in these cells is approximately normal, some

lines shows a modest decline in the abundance of phosphorylated USP forms that is not reversible by addition of 20E. A variety of differences in EcR ligand-binding characteristics were noted among the cell lines tested that presumably reflect the role of unknown factors underlying the resistance (Grebe *et al.*, 2000).

Another widely used lepidopteran cell line is Sf9, derived from ovarian cells of the fall armyworm, *Spodoptera frugiperda*. The affinity of ponasterone A for Sf9 and Kc cells is very similar, but the nonsteroidal agonist ANS-118 (chromafenozide) displays a much higher affinity for extracts derived from Sf9 cells than from Kc cells (Toya *et al.*, 2002). Similarly, cell lines derived from *C. fumiferana* show a much greater responsiveness to RH5992 than *Drosophila* cultured cells, which do not respond to the compound and excrete it at an elevated rate via an ABC transporter system (Retnakaran *et al.*, 2001). *Choristoneura* cell lines, with prolonged exposure to RH5992 become irreversibly insensitive to ecdysteroids and to the agonist, though the mechanism is unknown (Hu *et al.*, 2004). A more comprehensive discussion of ecdysteroid agonist properties is provided elsewhere in this series, though it is clear that the differences among species are derived primarily from structural diversifications in EcR and possibly, USP.

Other lines are particularly useful for studying ecdysteroid action in economically important insect species even when a complete EcR and USP have not been yet been isolated. These lines include the European corn borer (*Ostrinia nubilalis*; Trisyono *et al.*, 2000), the tent caterpillar (*Malacosoma disstria*; Palli *et al.*, 1995), the fall armyworm (*Spodoptera frugiperda*; Chen *et al.*, 2002a), and the cotton boll weevil (*Anthonomus grandis*; Dhadialla and Tzertzinis, 1997). The ecdysteroid responsiveness has been established for at least some of these lines by showing that orthologs for ecdysteroid responsive *D. melanogaster* genes, notably the genes encoding E75 (early puff) and DHR3 (early-late puff), are activated transcriptionally when the cells are challenged with 20E or an agonist.

A major benefit of using insect cell lines is that they provide the cofactors and machinery that are necessary for transcription to occur. On the other hand, other factors may modify or obscure an aspect of ecdysteroid response in a given experimental regime as silent and unidentified participants. As is evident from *in vivo* studies, a given experiment will generate substantially different cellular responses depending upon the milieu of proteins that contribute to ecdysteroid responsiveness in a given cell type. For this reason, mammalian cell lines containing no endogenous ecdysteroid responsiveness have been

used to reconstruct an ecdysteroid responsive transcriptional system by introducing the genes encoding EcR, USP, cofactors, and reporter genes in order to analyze their individual effects on ecdysteroid action (e.g., Christopherson *et al.*, 1992; Yao *et al.*, 1993; Palli *et al.*, 2003; Henrich *et al.*, 2003). Heterologous cells and cell lines also pose special difficulties, since it is conceivable that endogenous proteins provide novel functions or act as surrogates for similar insect proteins. Conversely, these cells might render false negative results in some experiments because they are missing one or more crucial cofactors.

7.3.3. Cell Cultures: Functional Receptor Studies

A variety of studies have examined the capability of EcR and USP to function in insect and other cell lines. Using the same approach, several orphan receptors from *D. melanogaster* have also been tested for their ability to respond to candidate ligands. These studies have generally focused on: (1) the responsiveness and activity of target DNA sequences in promoters, (2) the effect of various ecdysteroids, agonists, and antagonists upon receptor activity, (3) the properties of specific domains on receptor function and the effect of structural modifications upon transcriptional activity, and (4) the effect of heterodimerization and other cofactors, including orphan receptors, upon transcriptional activity.

7.3.3.1. Promoter studies The *hsp27* EcRE connected to a weak constitutive promoter is capable of inducing the transcription of a reporter gene in a *Drosophila* cell line by over 100-fold when challenged with 20E (Riddihough and Pelham, 1987). When *hsp27* EcREs are organized in a tandem repeat and attached to a weak promoter, the gene is not only responsive to 20E, but also is repressed in the absence of hormone, suggesting a repressive role for the ecdysteroid receptor via this element (Dobens *et al.*, 1991). A plethora of cell culture transcriptional tests and accompanying EMSAs have been reported which followed from this early work, and this is an important criterion for determining the functionality of an EcR/USP heterodimer, as it was in the original report (Koelle *et al.*, 1991).

The most fundamental task concerning promoter activity is to define the functional promoter elements to which the ecdysteroid receptor complex binds and exerts its effects on transcription. This was accomplished for the genes encoding the aforementioned EIP28 and 29, which are translated from

alternatively spliced mRNAs of the same gene (Schulz *et al.*, 1986). Three specific ecdysone response elements are ecdysteroid-responsive *in vivo* (Andres *et al.*, 1992; Andres and Cherbas, 1994). One of these Eip28/29 EcREs lies in the promoter region though it is not required for ecdysteroid-inducibility in Kc cells, possibly because this element is not used in the embryonic hemocytes from which the cells are derived. The other two elements lie downstream from the polyadenylation site, that is, on the 3' side of the gene (Cherbas *et al.*, 1991). The EIP40 gene is also ecdysteroid-inducible *in vivo* (Andres and Cherbas, 1992) and the genomic region that includes the EIP40 gene has been analyzed (Rebers, 1999). Just as cell culture experiments have been used to identify EcREs *in vitro* for subsequent *in vivo* analysis, the reverse relationship has also been undertaken successfully – the ecdysteroid-responsive promoter elements in the β 3 tubulin gene, which is ecdysteroid-responsive *in vivo* (Sobrier *et al.*, 1989), were identified by testing their inducibility by 20E in Kc cells (Bruhat *et al.*, 1993).

Cell culture experiments have been used not only to dissect the functional ecdysteroid response elements within a promoter region, but also to examine the possibility that different factors compete for these sites to modulate transcriptional activity. The relationship of *M. sexta* EcRB1 and two MsUSP isoforms has been explored in *M. sexta* GV1 cell cultures by observing the ecdysteroid-inducible MHR3 promoter (Lan *et al.*, 1997). Both EcR/USP complexes display about the same level of ligand affinity, but the EcRB1/USP1 complex induces much higher levels of expression in response to 20E from an intact promoter and also represses basal expression in the absence of 20E. Further, the EcRB1/USP1 complex binds to a canonical EcRE in the promoter and activates transcription, but the addition of USP2 prevents transcription and blocks binding to the EcRE. *In vitro* translated EcRB1/USP2 can bind to this same EcRE, indicating that other cellular cofactors are responsible for the differential action of the two MsUSP isoforms (Lan *et al.*, 1999).

The two isoforms of CtUSP show somewhat different ligand-binding capabilities, and one of the forms, when purified, recognizes a DR1 element that is not recognized by the other. However, both show about the same ability to induce transcription with DmEcR in human HeLa cells. The CtEcR does not possess the ability to induce transcription in cell cultures (Vogtli *et al.*, 1999).

7.3.3.2. Transactivation studies The appearance of multiple EcR isoforms in *D. melanogaster* has

prompted comparisons of their transcriptional responsiveness in cell cultures and in yeast. The B1 and B2 N-terminal domains from DmEcR, when combined with a GAL4 DBD and transfected into yeast, are capable of mediating transcription via a GAL4-responsive universal activation site (UAS) in the promoter. This AF1 (ligand-independent) transcriptional function is further elevated by removing an inhibitory region within the B1 domain (Mouillet *et al.*, 2001). In human HeLa cells, DmEcRB1 and DmEcRB2 showed about the same level of AF1 function and both also possessed AF2 (ligand-dependent) transcriptional activity. By contrast, EcRA reduced basal transcriptional activity below the level obtained from an EcR with no A/B domain at all.

Similar results have been obtained in a mammalian Chinese hamster ovary (CHO) line, except that the B1 isoform displays a highly elevated AF1 function compared to the A and B2 isoforms (Henrich *et al.*, 2003). This activity difference measured for B1 in the two cell lines suggests that the HeLa cell line carries a repressor that interacts with the B1 domain which does not exist in CHO cells. It also illustrates the diversity of function that is possible among different cell types owing to differences in the cellular milieu. In the EcR-deficient L57-3-11 line, all three EcR isoforms are responsive to ecdysteroids, though the A domain contributes no AF1 function, nor does the DmUSP A/B domain. The B1 domain contains several regions that when deleted impose a modest impact on AF1 functions. By contrast, specific point mutations in the B2 domain dramatically reduce AF1 functions (Hu *et al.*, 2003), suggesting that a localized interaction with other transcriptional factors is essential for activity.

Lezzi *et al.* (2002) devised a yeast two-hybrid assay which fused the EcR and USP LBD to the yeast GAL4 activation domain and DNA-binding domain, respectively. A variety of site-directed mutations of the LBDs were used to test their effects on functional dimer formation and both AF1 and AF2 (ligand-dependent) transcriptional activity as measured by UAS-mediated lacZ activity. The wild-type LBDs dimerize and induce activity at a low rate even in the absence of muristerone A, and this rate increases more than tenfold in the presence of muristerone A (but not other ecdysteroids such as 20E). Mutations of critical residues in EcR's helix 10 all but eliminate both dimerization and transcriptional activity, as expected. Deletion or mutation of helix 12, which normally folds over the ligand-filled pocket, eliminates EcR's AF2 function, as do mutations that affect ligand binding, but AF1 function is still detected in these mutant forms of

EcR. Substitution at a consensus cofactor-interacting residue, K497E, results in a strong elevation of basal transcriptional activity, though this mutant EcR has a reduced capacity for ligand-dependent induction and low ligand affinity (Bergman *et al.*, 2004; Grebe *et al.*, 2003). Virtually all point mutations in the *Dm*USP LBD eliminate transcriptional activity, though many retain the capability to dimerize with EcR, suggesting that USP is required for normal AF1 activity. Deletion of the carboxy-terminal region of *Dm*USP, including helix 12, does not disrupt homodimerization detected by gel filtration, but eliminates ligand-binding. Substitutions of specific residues in helix 12 modestly reduce basal transcriptional activity in yeast. However, while ligand-binding for the mutant USP is only slightly reduced, the ligand-dependent transcription of the EcR/USP complex is virtually eliminated (Przibilla *et al.*, 2004). This result is counterintuitive since the crystallized *Dm*USP is locked into an antagonistic conformation which implies that AF2 activity is impossible. It also contrasts with the normal activity seen in several helix 12 mutations of USP that were tested in the *Drosophila* L57-3-11 cell line, which may express enough *Dm*USP to mask the mutational effects (Hu *et al.*, 2003).

7.3.3.3. Ligand effects At least three issues pertaining to ligand responsiveness of the ecdysteroid receptor have been addressed through the use of cell cultures. First, one has focused on the responsiveness of a given receptor to ecdysteroids and agonists, particularly novel compounds. Second, modified receptors have been tested for their capacity to respond to a given ligand or to assess the structural basis of ligand specificity. Finally, recent studies have focused on the possibility that other orphan receptors are responsive to ecdysteroids and/or other ligands.

By testing ecdysteroid-induced transcriptional activity in cell culture, new nonsteroidal agonists, including both artificial compounds (Mikitani, 1996) and a variety of phytochemicals which act as agonists, including 8-*O*-acetylharpagide (Elbrecht *et al.*, 1996) or antagonists, including several flavones (Oberdorster *et al.*, 2001). Environmental chemicals including polyaromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) induce ecdysteroid-responsive genes, suggesting an EcR-mediated basis for molting defects commonly found among aquatic species residing in polluted water (Oberdorster *et al.*, 1999). The use of cell culture assays has also been utilized to analyze the effects of juvenile hormone and its analogs upon transcriptional activity. The topic of juvenile hormone and its effects upon EcR and USP will be addressed later in the chapter.

Based on lines of evidence gleaned from experimental studies over the years, the possibility that ecdysteroids other than 20E display biological activity has been speculated about and even suspected for years (Somme-Martin *et al.*, 1990; Grau and Lafont, 1994; Henrich and Brown, 1995). In S2 cells, the 20-hydroxylated derivatives of the major and natural ecdysteroid products of the *D. melanogaster* larval ring gland, 20E and makisterone A are the most efficacious in activating a reporter gene whose promoter carried three tandem copies of the *hsp27* EcRE. By contrast, precursor and metabolite ecdysteroids, as well as nonsteroidal agonists, evoke considerably less activity in the cell culture assay. As noted in other studies, ponasterone A displays the highest potency, and two other phytoecdysteroids, muristerone A and cyasterone, also are more inducible than 20E in these cells (Baker *et al.*, 2000).

Receptor modifications have also been utilized to delineate the basis for differences in ligand specificity, as noted earlier for the effects of the A398P mutation to test predictions based on the *Hv*EcR crystal structure (Billas *et al.*, 2003). Similarly, a substitution in the *Cf*EcR (A393P) eliminates both its ligand-binding affinity for ponasterone A and its transcriptional inducibility in cell cultures, possibly by impairing the receptor's interaction with a coactivator (GRIP1), but the mutation exerts no debilitating effect on responsiveness to a nonsteroidal agonist (Kumar *et al.*, 2002). A chimeric *Drosophila/Bombyx* EcR carrying only the *Bombyx* D-domain is responsive to RH5992 in mammalian CV-1 cells. The effect is largely attributable to the relatively high level of RH5992-dependent dimerization between the D-domain of the chimera and endogenous RXR or cotransfected USP (Suhr *et al.*, 1998). Based on a deletion analysis of EcR, both its D and E domain are required for ligand-dependent dimerization with USP (Perera *et al.*, 1999b).

As already noted, insect cells may provide endogenous components that influence ecdysteroid activity. EcR itself masks a second ecdysteroid responsive activity in *Drosophila* S2 cells. A fusion protein carrying the DHR38 LBD was tested for its ability to dimerize with *Dm*USP in response to several ecdysteroids using S2 cells from which endogenous EcR activity was eliminated by RNA interference. A response to 20E occurs that is further induced by adding an RXR activator, indicating that the DHR38/RXR heterodimer is responsible for the effect. Similarly, cotransfection with a chimeric and constitutively active form of USP also confers the ability of DHR38 to respond to 20E (Baker *et al.*, 2003). The DHR38/USP dimer also responds to at least six other ecdysteroids, including some, such as

3-dehydro-20E and 3-dehydromakisterone A, which are abundant during late larval development in *Drosophila* and display unusually high inducibility in fat body (Somme-Martin *et al.*, 1990). The relevance of this pathway for ecdysteroid-driven events represents an important avenue for investigation in *Drosophila* and other insects.

Cell culture experiments have also been used to demonstrate the effect of orphan receptors and receptor cofactors upon ecdysteroid response, including AHR38 (Zhu *et al.*, 2000), AaSVP (Zhu *et al.*, 2003a), DmSVP (Zelhof *et al.*, 1995a), and DHR78 (Zelhof *et al.*, 1995b). These will be discussed in connection with their biological relevance for modulating ecdysteroid receptor-mediated activities.

7.3.4. Ecdysteroid-Inducible Cell Systems

The use of ecdysteroid-inducible cellular systems has emerged rapidly in recent years since ecdysteroids and nonsteroidal agonists are inexpensive and harmless to humans. Yeast assays have been developed to screen for ecdysteroid agonists of potential commercial value. When EcR by itself is transformed into yeast cells, it is capable of inducing a high level of transcription that is ligand-independent and which is not appreciably increased by the presence of USP or RXR (De la Cruz and Mak, 1997). Through modifications of the A/B domain, an inducible system was successfully produced (De la Cruz *et al.*, 2000).

A second ecdysteroid-inducible yeast system was produced by making at least two modifications. First, by removing the AaEcR A/B domain, the receptor's AF1 function is reduced when transformed into yeast. Second, the addition of the mammalian receptor coactivator GRIP1 confers yeast cells with the ability to be induced more strongly by a range of ecdysteroid agonists. Conversely, the addition of the receptor corepressor SMRT represses transcription (Tran *et al.*, 2001a). A yeast inducible assay utilizing the CfEcR and CfUSP also requires the removal of the A/B domain from both EcR and USP, along with the addition of the GRIP1 coactivator (Tran *et al.*, 2001b). In fact, the USP A/B domain often displays repressive properties or is inactive in heterologous cells, thus necessitating its deletion and/or replacement with a constitutively active N-terminal domain, such as VP16 (Tran *et al.*, 2001b; Hu *et al.*, 2003; Henrich *et al.*, 2003).

The field use of the bisacylhydrazines as insecticides has also inspired the introduction of ecdysteroid-inducible systems into plants which could be used to promote the expression of beneficial genes. Nonsteroidal agonist-inducible expression has been obtained from *Zea mays* (corn) protoplasts

(Martinez *et al.*, 1999b), tobacco, and *Arabidopsis* (Padidam *et al.*, 2003) via EcR fusion proteins. This induction in *Arabidopsis* has been successfully linked to the expression of a coat protein gene from the tobacco mosaic virus (TMV), which confers resistance to TMV infection (Koo *et al.*, 2004) and also induces the expression of a factor in maize that restores fertility to a male-sterile strain (Unger *et al.*, 2002).

As noted, ecdysteroids evoke no discernible responses upon mammalian cells, and therefore, attempts to implant an ecdysteroid-inducible gene expression system into cells for therapeutical purposes has been undertaken. EcR dimerizes with endogenously expressed RXR to form a functional heterodimer that responds to biologically inert ecdysteroids or nonsteroidal agonists (Christopherson *et al.*, 1992; Palli *et al.*, 2003). By producing and testing a variety of chimeras, a heterologous receptor system has been developed in mammalian cells that evokes induction rates of almost 9000-fold, with very rapid reduction when the ecdysteroid agonist is removed (Palli *et al.*, 2003). A mouse strain carrying a transgenically introduced *Drosophila* EcR gene has been produced in which tissues are capable of responding to ecdysteroids without any other discernible phenotypes other than the expression of an *hsp27* EcRE regulated reporter gene (No *et al.*, 1996). The ecdysone switch has been shown to be responsive to several ecdysteroids and ecdysteroid agonists, and the maximal response can be further elevated by the addition of RXR activators. RXR superinduction requires that EcR be previously bound by its cognate ligand since RXR activators by themselves exert no effect upon EcR/RXR activity (Saez *et al.*, 2000). Ecdysteroid-inducible expression of medically important genes has been accomplished in human cell lines (Choi *et al.*, 2000), as well as the introduction of an ecdysteroid-inducible gene into rats via an adenovirus vector used for somatic gene therapy (Hoppe *et al.*, 2000).

7.4. Cellular, Developmental, and Genetic Analysis

7.4.1. The Salivary Gland Hierarchy: A Model for Steroid Hormone Action

Many of the insights concerning steroid hormone regulation of transcriptional activity were first recognized in the larval salivary gland of several insect species. By observing the location, timing, and size of these chromosomal puffs, the activity of an ecdysteroid-inducible gene can be inferred. The progression of events, and their relation to

developmental changes in the salivary gland and the whole animal are described here for *D. melanogaster* (see Thummel, 2002 and references therein). During the period of the third instar that precedes the wandering stage, “intermolt puffs” appear at several specific chromosomal sites. In response to the late larval ecdysteroid peak, these puffs regress and another set of “early” ecdysteroid-responsive puffs appear. The timing and duration of these “early” puffs varies (some are referred to as “early-late” puffs) and these eventually regress as another set of “late puffs” appear at other chromosomal sites. The puffing pattern accompanies the production and secretion of glue protein from the salivary gland, which is extruded as the larva becomes immobile and tanning of the larval cuticle begins (puparium formation).

Several hours later, a second smaller pulse of ecdysteroid induces a second wave of “early” puff activity, which is followed by a second wave of “late” puff activity. The two peaks evoke similar, but not identical, transcriptional changes, and these subtle differences underlie important developmental consequences. The second wave culminates in the expression of β FTZ-F1, a specific isoform of an orphan receptor encoded by the stage-specific (that is, the prepupal specific) gene puff located at the chromosomal interval, 75C. β FTZ-F1, in turn, sets off a cascade that ultimately includes the expression of another stage-specific puff, E93, involved in the histolysis of the gland (Broadus *et al.*, 1999; Lee *et al.*, 2002).

As expected, 20E shows the greatest potency in terms of puff induction, followed by 3-dehydro-20E, which is an endogenously produced ecdysteroid in the *Drosophila* third instar (Somme-Martin *et al.*, 1988). Significantly, however, while the potency of individual ecdysteroids varies, no individual ecdysteroid evokes a unique puffing site, or alternatively, fails to evoke puffing at a site induced by other ecdysteroids. In other words, there is no evidence for multiple ecdysteroid signaling pathways in the *Drosophila* salivary gland (Richards, 1976).

7.4.2. Molecular and Genetic Characterization of the Puff Hierarchy

Numerous genetic and molecular studies have verified the basic tenets of the Ashburner model. Ashburner proposed that the ecdysteroid receptor directly induces the early puffs while simultaneously repressing late puff genes, and that the early puff gene products not only increase late puff transcription, but also feed back to downregulate their own expression. The use of chromosomal deletions and duplications of the region that contains E74 and

E75 provides a way to alter the intracellular dosage of early puff gene products and thereby test the model. Late puffs appear earlier and are larger when the early puff genes are duplicated, consistent with the accumulation of an activating factor. On the other hand, late puff appearance is delayed in chromosomes with early puff deletions (Walker and Ashburner, 1981).

Further evidence that late puffing events are dependent upon early puffing events was shown ingeniously by testing permeabilized salivary glands 20E in combination with cellular extracts (Myohara and Okada, 1987). When ecdysteroid and a cellular extract from unstimulated salivary glands are mixed together, no response is registered from the late puff at 78C, but this puff appears when 20E, along with a cellular extract from stimulated salivary glands, is tested on permeabilized salivary glands.

Another line of inquiry has focused on demonstrating that EcR and USP interact directly with puff site regions. Immunostaining has shown specific instances in which EcR and USP colocalize to early puff sites (Yao *et al.*, 1993), and that EcR and RNA polymerase II antibodies colocalize on *C. tentans* polytene chromosomes (see Figure 1). EcR has also been associated with late puff sites (Talbot *et al.*, 1993), inferring that it directly influences the regulation of late puff genes. Further, the products of the early puff gene, E75, have been associated by immunostaining with early and late puff sites, as predicted by the Ashburner model (Hill *et al.*, 1993).

The puff hierarchy actually begins before the onset of pupariation in *Drosophila* with the appearance of several intermolt puffs (see Lehman, 1995 and references therein). The *sgs4* gene encodes a salivary glue protein and is regulated by the synergistic interplay of an ecdysone receptor element, and a secretion enhancer binding protein (SEBP3) site. A second SEBP binding site has been associated with products of the early puff product, Broad, indicating that the proper regulatory interplay between the ecdysone receptor and other transcriptional factors depends upon the cellular milieu (von Kalm *et al.*, 1994; Lehman and Korge, 1995). As ecdysteroid titers increase, the intermolt puffs regress.

Three early puff genes have been the subject of intensive ongoing study in *D. melanogaster* and other insects. All three genes encode transcription factors, consistent with a role in regulating late puff expression: the Broad-Complex (Br-C) encodes a zinc finger protein (DiBello *et al.*, 1991), E74 is an *ets* protooncogene (Burtis *et al.*, 1990), and E75 encodes an orphan receptor (Segraves and Hogness, 1990). All three possess a level of organizational

complexity that is not evident from the cytological studies alone. Br-C encodes several different isoforms derived from alternative splicing (DiBello *et al.*, 1991). Broad proteins carry a common core domain and a subset of several different variable domains (see Bayer *et al.*, 1997), and the expression of each isoform varies both spatially and temporally (e.g., Huet *et al.*, 1993; Mugat *et al.*, 2000; Brennan *et al.*, 2001; Riddiford *et al.*, 2003). The switchover of expression among combinations of Br-C isoforms has been associated with the modulatory regulation of many ecdysteroid-inducible genes including the transcription factor gene *hedgehog* (*hb*; Brennan *et al.*, 1998), micro-RNA (Sempere *et al.*, 2003), and *fbp1* (Mugat *et al.*, 2000). The E74 puff encodes two isoforms, one of which is transcribed at low ecdysteroid levels (E74B; Karim and Thummel, 1991) and disappears as titers become elevated while the transcript which specifies the E74A isoform becomes more abundant. Finally, three isoforms exist for the nuclear receptor at the E75 puff (A, B, and C; Segraves and Hogness, 1990) resulting from alternative splicing. One of these, E75B, carries only the second zinc finger within its DBD. Several other ecdysteroid responsive puff genes have also been identified which play cellular roles (see Thummel 2002, and references therein).

7.4.3. Developmental Regulation of Ecdysteroid Response in *D. melanogaster*

Transcript levels of EcR, the intermolt genes, and the individual isoforms of the early puff genes (BR-C, E74, E75) vary temporally among individual tissues during the late larval/prepupal period in *D. melanogaster* (Huet *et al.*, 1993). It is apparent that the relative abundance of the early puff isoforms changes continuously as ecdysteroid titers surge and decline, that the profile of “early puff” expression varies among tissues at any given time, and that the pattern changes are specific for a given tissue. For instance, EcR levels are very low throughout the larval/prepupal period in imaginal discs, whereas they remain elevated in the gut throughout this period. The relative abundance of EcR and its early puff products plays some role in the timing of subsequent gene expression, as exemplified by the *Ddc* (dopa decarboxylase) gene. It behaves as an “early late” gene and is induced by the combined action of EcR and Br-C, which is offset, in turn, by a repressive promoter element in epidermis and imaginal discs (Chen *et al.*, 2002b). A similar mechanism has been described for the suppression of *Ddc* in *M. sexta* (Hiruma *et al.*, 1995).

The complexity of *in vivo* response to ecdysteroids is also revealed by comparing the puffing

response seen along the polytene chromosomes of the larval fat body during the same time that the salivary gland puff response occurs (Richards, 1982). While many aspects of the fat body response resemble those seen in the salivary gland, individual fat body puff sites are responsive to both ecdysone and 20E in ranges below 10^{-6} M, whereas salivary gland puffing is much more responsive to 20E. While one obvious explanation lies with differences in metabolism, the fat body response to ecdysone does not lag the response to 20E in the fat body, as would be expected if conversion to 20E preceded receptor-mediated response. Fat body chromosomes also evoke different puffing patterns than salivary gland chromosomes, and these presumably reflect functional differences between the two compared cell types.

Diversity of response has been seen for other ecdysteroid-inducible genes. For instance, the fat body protein-1 (*fbp1*) gene is ecdysteroid-inducible and expressed only in the fat body during the onset of metamorphosis (Maschat *et al.*, 1991). The aforementioned *Eip28/29* gene is ecdysteroid-responsive but displays several different patterns of expression in individual tissues at the onset of metamorphosis (Andres and Cherbas, 1992, 1994). The complexity of *in vivo* regulation is also exemplified by the *E93* gene, which falls at an endpoint in the salivary gland hierarchy because the *E93* gene product plays a central role in salivary gland histolysis (Lee *et al.*, 2002). Nevertheless, its expression in other tissues is not responsive to 20E (Baehrecke and Thummel, 1995).

The *D. melanogaster* imaginal discs and ring gland are notable in that they apparently fail to respond to ecdysteroids during the larval/prepupal period, as measured with a GAL4-EcR/GAL4-USP *in vivo* system in which lacZ staining reports the level of ligand-dependent heterodimerization between the two fusion proteins (Kozlova and Thummel, 2002). The lack of responsiveness may reflect the effect of cofactors (or their absence) and/or mechanisms by which 20E is either removed from the cells via a transporter mechanisms (Hock *et al.*, 2000) or metabolized.

The same GAL4 *in vivo* reporter system indicates that EcR and USP heterodimerization occurs in response to the ecdysteroid peak during *D. melanogaster* mid-embryogenesis and that the receptor plays a role in germ band retraction and head involution (Kozlova and Thummel, 2003a). Moreover, the transcriptional relationship among ecdysteroid-regulated genes is generally maintained during the course of individual ecdysteroid peaks throughout premetamorphic development (Sullivan and

Thummel, 2003). Mutations of the early-late gene, DHR3, cause embryonic lethality, further implicating not only EcR but the ecdysteroid hierarchy itself (Carney *et al.*, 1997).

Of course, the existence of functionally distinct EcR isoforms provides an important basis for differential ecdysteroid responses. During the late larval stage, the EcRA isoform of *D. melanogaster* is generally predominant in imaginal discs, whereas the B isoforms are associated primarily with larval tissues that undergo histolysis during metamorphosis (Talbot *et al.*, 1993). Issues surrounding the diversity of isoform function will be discussed further in Section 7.4.5.

7.4.4. Conservation of Ecdysteroid Response Among Insects

The isolation of EcR and USP from other insects and the assembly of their developmental profiles now allows direct analysis of ecdysteroid action in other organisms. So far, these studies show general consistency with those obtained in *D. melanogaster*, specifically, EcR expression varies over time and peak levels of expression coincide with ecdysteroid peaks, and different isoforms predominate among different tissues. For instance, the expression patterns of the two EcR isoforms in *M. sexta* vary among cell types (Jindra *et al.*, 1996) and expression levels of the two USP isoforms switch in conjunction with molts (Jindra *et al.*, 1997), providing a regulatory framework for the early-late response of MHR3 discussed earlier (Lan *et al.*, 1999).

The recovery of ecdysteroid-inducible target genes such as E74, E75, FTZF1, and HR3 orthologs also allows a comparison of the ecdysteroid-inducible cascade in other organisms, and the resulting proteins play biological roles in a variety of regulatory processes that is too extensive to discuss here. The comparative analysis over preadult development is most complete in *M. sexta*, and the essential features of the ecdysteroid-inducible hierarchy are maintained (Riddiford *et al.*, 2003, and references therein). Similarly, the players and timing of ecdysteroid-responsive genes during *B. mori* choriogenesis are conserved (Swevers and Iatrou, 2003), as they are in *A. aegypti* vitellogenesis (Zhu *et al.*, 2003a). Interestingly, however, USP's other partners, DHR38 and SVP, have not been associated with whole body responses in any organism. In flies, SVP is involved in eye differentiation (Mlodzik *et al.*, 1990) and DHR38 is involved in cuticle formation (Fisk and Thummel, 1998) at the onset of metamorphosis; both players are involved in mosquito vitellogenesis (Zhu *et al.*, 2003a).

At least two points emerge from these considerations. First, while the early response at the core of the ecdysteroid hierarchy appears to be highly conserved among insects at the global level, local ecdysteroid responses likely recruit specific players to mediate specific developmental events. Second, combinations of the EcR/SVP/DHR38/USP orphan receptor group might be incorporated into various ecdysteroid-regulated processes for the purpose of regulating a linear series of events such as mosquito vitellogenesis and fly eye differentiation. One intriguing aspect of this orphan group is that the broad spectrum response of DHR38/USP might be important for regulating responses via the ecdysone precursor to 20E and via 20E metabolites occurring after 20E exerts its effects, with SVP providing activation of an alternative pathway by competing with the 20E-inducible response itself. The context of EcR action will be interesting to explore in other insect processes, such as the color pattern formation of butterfly wings (Koch *et al.*, 2003) and insect diapause.

Historically, numerous studies of puffing on polytene chromosomes preceded and have continued along with the work on *D. melanogaster*. The salivary gland response of *Sciara coprophila* (fungal fly) provides an interesting comparison to *D. melanogaster*, and illustrates the importance of investigating the features of each system without pretense. The *S. coprophila* II/9A puff appearing in response to ecdysteroid treatment reflects the initiation of both bi-directional DNA replication (Liang *et al.*, 1993) and the elevated transcription of two similar mRNA species which bear no resemblance to ecdysteroid-responsive genes found in *Drosophila* (DiBartolomeis and Gerbi, 1989). When the promoter region of the *S. coprophila* II/9-1 gene is transgenically introduced into *D. melanogaster*, it is transcriptionally responsive to ecdysteroids in the salivary gland, indicating that the ecdysteroid-inducible transcriptional machinery is conserved evolutionarily between the two species (Bienz-Tadmor *et al.*, 1991). However, there is no evidence that the DNA amplification is induced by 20E in flies or mediated by the ecdysteroid receptor.

In *Trichosia pubescens*, Amabis and Amabis (1984) found that ecdysteroid treatment of salivary glands generates both DNA and RNA "early" puffs, whose subsequent regression is accompanied by the appearance of other "late" DNA amplification and RNA puffs. Cycloheximide treatments after early puff appearance block late puff expansion. In this species, large puffs appear after considerable delay, and the appearance of early puffs is not obvious, suggestive of a single broad ecdysteroid peak, rather

than the two peaks that occur in *D. melanogaster* over the same developmental timespan. In this respect, the response pattern is comparable to *C. tentans*, which in turn relies upon an interplay of ecdysone and 20E to achieve ecdysteroid-induced regulation (Stocker and Pavan, 1977).

In yet another sciarid fly, *Bradysia hygida*, 20E induces DNA amplification and transcription of a temporally “late” puff gene (BhC4-1). When this gene is transgenically introduced into *D. melanogaster*, it retains its “late” puff characteristics in terms of salivary gland timing, but transcription of the gene is not blocked by repression of protein translation with cycloheximide, suggesting that the late induction is actually a form of derepression in the *D. melanogaster* salivary gland (Basso *et al.*, 2002). This interpretation is also based on the observation that 20E exerts both repressive and inductive effects upon the protein composition of the *B. hygida* salivary gland during the fourth and final larval stage (de Carvalho *et al.*, 2000).

As evidenced by the cases presented here, beyond the superficial similarity, there are numerous unsolved mysteries about the integration of ecdysteroid response into biological processes, especially when one considers that the most complete hierarchical comparisons so far involve only lepidopteran and dipteran species. Several ecdysteroid-inducible genes have been identified for the coleopteran, *T. molitor* (Mouillet *et al.*, 1999), at least indicating that several of the orphan receptors involved in mediating ecdysteroid responses exist in more primitive insects. Further, the existence of an E78 ortholog in the filarial parasite, *D. immitis*, implies that at least portions of the hierarchy are conserved in other invertebrates (Crossgrove *et al.*, 2002).

7.4.5. *In Vivo* Molecular and Genetic Analysis of EcR and USP

The pattern of EcR gene transcription during pre-metamorphic development fluctuates dramatically, with high levels accompanying each of the embryonic and larval molts, followed by peaks of expression during the prepupal and pupal-adult stages of metamorphosis (Koelle *et al.*, 1991). Moreover, the EcR gene encodes three different isoforms with the A isoform’s transcription governed by a promoter and two other isoforms (B1 and B2) generated by alternative splicing via a second promoter (Talbot *et al.*, 1993). All three isoforms share a common sequence that includes a small portion of the A/B domain and the other domains, but the amino-terminal side of the A/B region for each isoform is unique. The A isoform predominates in fly tissues which undergo morphogenetic changes during

metamorphosis, including the imaginal discs, the ring gland, the imaginal rings of the foregut, hindgut, and salivary gland (Talbot *et al.*, 1993), and in a heterogeneous subset of neurons that degenerate after adult emergence (Truman *et al.*, 1994) (see **Chapter 4**), whereas the B1 isoform is found in larval tissues including the salivary gland, the fat body, larval muscle (Talbot *et al.*, 1993), and proliferative neurons (Truman *et al.*, 1994).

Functional studies of EcR based on the isolation of mutations within the *D. melanogaster* gene have led to further insights about EcR’s developmental role, and essentially confirmed its ecdysteroid-mediating function. As might be expected, null mutations of a common region in EcR, and thereby disrupt all three isoforms and result in embryonic lethality, whereas mutations that retain a reduced ability to mediate ecdysteroid responses allow mutant survival through some or all of the larval stages. Some of these weaker mutations affect nonconserved residues, but at least one of these prepupal lethal mutations involves a highly conserved phenylalanine in the DBD; conversely, there are nonconserved residues that cause embryonic lethality when substituted. One of the lethal mutations (A483T) replaces the amino acid residue that interacts with the SMRTER corepressor and is a conditional third instar lethal mutation. Presumably, the lability of the mutant protein disrupts the normal interaction between EcR and SMRTER at higher temperatures (29 °C). The A483T mutation (along with other EcR mutations; **Table 2**) also disrupts adult female fecundity at its restrictive temperature, indicating that the corepressor interaction is essential for at least late larval development and oogenesis (Bender *et al.*, 1997; Carney and Bender, 2000). B-specific mutations cause early larval lethality (Schubiger *et al.*, 1998); an A-specific EcR mutation reduces but does not eliminate mutant survival to the adult stage and disrupts the normal expression of EcRB1 (D’Avino and Thummel, 2000; Schubiger *et al.*, 2003) (see **Chapter 4**). Other A-specific mutations cause pupal lethality (Carney *et al.*, 2004). A gain-of-function mutation of *DmEcR* (K497A) that dimerizes in the absence of hormone, possibly because of disrupted corepressor binding, has also been identified in cell cultures, though its potential *in vivo* effect is unknown (Bergman *et al.*, 2004).

Over developmental time, the B2 isoform, when expressed under the control of a heat shock promoter, rescues larval development in EcR-null mutants, though heat shocks are required in each instar to accomplish it. The A and B1 isoforms rescue development through the first instar, but fail thereafter, suggesting that a common function is required

Table 2 A selected list of EcR mutations and their functional consequences. Mutations correspond to shaded residues in **Figure 4**

Mutation	Species	Effect
A393P	<i>C. fumiferana</i>	Destroys 20E response, but not nonsteroidal agonist response (Kumar <i>et al.</i> , 2002; Billas <i>et al.</i> , 2003). Impaired coactivator (GRIP1) interaction (<i>CfEcR</i> only; Kumar <i>et al.</i> , 2002)
A398P	<i>H. virescens</i>	
A522P	<i>D. melanogaster</i>	
A534P	<i>A. aegypti</i>	
A559P	<i>B. mori</i>	
Y403A	<i>H. virescens</i>	Reduces nonsteroidal agonist response, but not 20E response (Billas <i>et al.</i> , 2003)
A483T	<i>D. melanogaster</i>	Conditional late larval lethal mutation; disrupts oogenesis (Li and Bender, 2000); binding site for SMRTER corepressor (Tsai <i>et al.</i> , 1999)
K497A, E	<i>D. melanogaster</i>	Constitutive transcriptional activity in cell culture (Bergman <i>et al.</i> , 2004)
Y611F	<i>D. melanogaster</i>	Elevated binding to <i>hsp27</i> EcRE in presence of ecdysone (Wang <i>et al.</i> , 2000)
F645A	<i>D. melanogaster</i>	Dimerizes normally but is not 20E inducible; dominant negative lethal mutation <i>in vivo</i> (Cherbas <i>et al.</i> , 2003)
W650A	<i>D. melanogaster</i>	No dimerization (Cherbas <i>et al.</i> , 2003)

during the first instar and more differentiated EcR functions are essential later. The rescued B2 transformants become sluggish during the wandering stage of the late third instar, then immobile, and eventually die (Li and Bender, 2000).

Isoform-specific mutations in conjunction with transformation rescue have further delineated EcR-based functions in fly development. As expected, an EcRB1-specific mutation disrupts developmental activities in those cells where it is expressed. For instance, the salivary gland's ecdysteroid-induced puffing is disrupted in B1 mutants (but not eliminated), and only transformation with EcRB1 restores normal puffing of various early and early-late genes, though B2 exerts a partial rescue. Similarly, B1-expressing abdominal histoblasts and midgut cells develop abnormally (Bender *et al.*, 1997). Neuronal remodeling during metamorphosis is disrupted in genetic mosaics that do not express either of the two B isoforms in proliferating neurons (Schubiger *et al.*, 1998) (see **Chapter 4**), but remodeling is rescued by the expression of either B isoform within these cells (Schubiger *et al.*, 2003). Remodeling is not disrupted in mutations of the early puff genes, indicating that this aspect of the cascade is not specifically tied to the failure of B isoform mutant effects (Lee *et al.*, 2000).

A related strategy for discriminating EcR functions involves introducing an isoform or fusion protein transgenically into flies, expressing the transgene ectopically, and then observing the dominant negative phenotypic consequences of such expression. At least three variations have been reported: (1) expression of a wild-type isoform ectopically under the control of an UAS promoter regulated by the yeast GAL4 transcription factor (Schubiger *et al.*, 2003), (2) the expression of a GAL4-EcR LBD fusion protein that forms an

inactive dimer with cellular USP in a nonisoform specific manner (Kozlova and Thummel, 2002, 2003a), and (3) UAS-controlled expression of a dominant negative EcRB1 isoform (F645A in *D. melanogaster*) that forms an inactive dimer with intracellular USP, and is then tested for rescuability by the concomitant expression of a specific isoform (Cherbas *et al.*, 2003). In the case of wild-type overexpression, each isoform generates a unique pattern of phenotypes. Overexpression of EcRA suppresses posterior puparial tanning, and affects ecdysteroid-inducible gene expression in posterior compartments of the wing disc but does not affect viability. By contrast, overexpression of EcRB1 and B2 during puparial formation greatly reduces viability (Schubiger *et al.*, 2003) (see **Chapter 4**). Ectopic expression of a GAL4-EcR during the late third instar causes a failure of puparial contraction and cuticular tanning that resembles the traits displayed by mutant EcR larvae (Kozlova and Thummel, 2002). The isoform-specific rescue of the EcR^{F645A} mutants, however, reveals a poor correlation between tissue-specific effects and intracellular titers of each isoform, though the accumulation of disrupted responses in several individual tissues leads to a stage-specific developmental arrest in the third instar (Cherbas *et al.*, 2003).

Further insights about the functional differences associated with the *D. melanogaster* EcR isoforms will likely emerge by understanding the basis for isoform-specific mRNA transcription. Two promoters, one associated with the A isoform, and the other associated with the two B isoforms, regulate the appearance of these transcripts. Promoter segments responsible for high levels of EcRA expression during metamorphosis have been identified (Sung and Robinow, 2000). While the level of EcRA detected is homogeneous among those neurons which express

EcRA during metamorphosis, the underlying promoter regulation is surprisingly heterogeneous among them, indicating that the observed pattern of expression obscures an underlying regulatory complexity. A TGF- β /activin signaling pathway has been implicated in the regulation of EcRB1 transcription. In activin mutants, EcRB1 transcription is reduced and neurons in the mushroom bodies of the brain fail to undergo remodeling during metamorphosis. The expression of EcRB1 rescues the remodeling defects, indicating that EcRB1 levels are regulated through the activin signaling pathway (Zheng *et al.*, 2003).

By comparison with EcR's complexity, the regulation of the *usp* gene in *D. melanogaster* is relatively simple, with no introns and no alternative splicing forms, though the profile is more complex in species with multiple USP isoforms (Jindra *et al.*, 1997; Vogtli *et al.*, 1999). In flies, the expression of USP through development is relatively stable, though it is unclear whether EcR or USP is the rate-limiting partner in developing tissues (Henrich *et al.*, 1994). The *usp* gene is defined by several recessive early larval lethal mutations: three missense substitutions (*usp*³, *usp*⁴, and *usp*⁵) that mutate amino acid residues in the USP DBD and directly contact phosphate residues in the DNA backbone, along with a nonsense mutation, *usp*², that truncates the DBD (Oro *et al.*, 1990; Henrich *et al.*, 1994; Lee *et al.*, 2000). The null-*usp*² allele evokes a different effect on gene expression than the other *usp* alleles. Whereas all the mutations disrupt the normal repression of the BrC-Z1 isoform, only USP2 is incapable of activating BrC-Z1 transcription. USP3 and USP4 are also able to mediate ecdysteroid-induced gene transcription through an *hsp27* EcRE-regulated promoter (Ghbeish *et al.*, 2001).

This dual capability has been analyzed *in vivo* during ommatidial assembly and differentiation in the eye disc. Briefly, the differentiation of the eight retinula cells in each of the 700 or so ommatidia that become the compound eye occurs through the recruitment of undifferentiated cells as a wave moves from the posterior to anterior end of the eye disc. Cells along this progressing wave, the morphogenetic furrow, undergo a host of transcriptional changes and some aspects of the process are ecdysteroid-dependent *in vitro*. Furrow advancement accelerates in mutant *usp* patches on the eye disc (Zelhof *et al.*, 1997), whereas an ecdysteroid deficit retards its advancement (Brennan *et al.*, 1998). The apparently paradoxical action results from the failure of a repressive *usp* function as evidenced by the abnormal appearance of Br-C Z1 expression in mutant patches lying along the front of the moving furrow.

The *usp*² allele causes an absence of Z1 behind the furrow, where it is normally present, further demonstrating the dual roles. USP has been similarly implicated in both wing margin bristle differentiation (Ghbeish and McKeown, 2002), and the repression of premature neuronal differentiation in the wing imaginal discs (Schubiger and Truman, 2000) (see **Chapter 4**). The maintenance of USP3 and USP4 activation functions likely explains the normal appearance of mutant *usp* imaginal clones (Oro *et al.*, 1992).

Maternal contribution of normal *usp* transcript is essential for the completion of embryogenesis (Oro *et al.*, 1992). Mutant *usp* larvae are rescued through the third instar with a USP connected to a heat shock promoter. The lethal phenotype of these partially rescued larvae is reminiscent of the effects seen with larval EcR mutations and internal morphology is similar at the time of larval arrest. Only the *usp* mutants, however, develop a supernumerary larval cuticle and fail to wander off the food (Hall and Thummel, 1998; Li and Bender, 2000). DHR38 mutants also undergo abnormal cuticle apolysis (Kozlova *et al.*, 1998), suggesting that the DHR38/USP dimer may be essential for this aspect of premetamorphic development, rather than the EcR/USP dimer.

It is beyond the realm of this chapter to explore the effects of ecdysteroid-inducible genes in depth and the reader is directed to other reviews (Henrich *et al.*, 1999; Riddiford *et al.*, 2000; Thummel, 2002). Isoform-specific mutational analysis of ecdysteroid-responsive genes has generally confirmed essential roles at metamorphosis with the two isoforms of E74 (Fletcher *et al.*, 1995), two of the E75 isoforms (Bialecki *et al.*, 2002), DHR3 (Lam *et al.*, 1999), and the β FTZF1 isoform (Broadus *et al.*, 1999). The complex genetic complementation of several Br-C mutations (Kiss *et al.*, 1988) has been associated with each of four isoforms (Z1–Z4; Bayer *et al.*, 1997). The *npr1* (nonpupariation) third instar lethal mutations of Br-C fail to complement all other alleles by impairing a common and essential premetamorphic function. Some Br-C mutations affect the gene's own expression (Gonzy *et al.*, 2002), consistent with predictions of the Ashburner model. As noted elsewhere, Br-C isoforms play a variety of inductive and repressive roles, often through switchover of isoform expression, in a large spectrum of premetamorphic processes.

7.4.6. Ecdysteroid Receptor Cofactors

As noted earlier, the ecdysteroid receptor is part of a complex of proteins which affect both its inductive

and repressive transcriptional functions. In the case of Hsc70, a chaperone that physically interacts with *DmEcR*, this functional importance is demonstrated by the genetic interaction between EcR and Hsc70 mutations (*hsc4* gene) in *D. melanogaster*. Trans-heterozygotes for these two mutations develop severely blistered wings and bent legs typically associated with the mutational impairment of EcR function (Bender *et al.*, 1997; Arbeitman and Hogness, 2000).

Based on findings from experiments with vertebrate nuclear receptors, transcriptional cofactors and their roles have begun to be recognized and explored; the process of eye ommatidial differentiation during the third instar of *D. melanogaster* that was described earlier has become the focus of efforts to understand the role of cofactors in development. The dominant negative EcR^{F645A} mutation severely disrupts eye development (Cherbas *et al.*, 2003; Sedkov *et al.*, 2003), and USP function is also required for normal furrow progression (Zelhof *et al.*, 1997).

One cofactor implicated in the process is the product of the trithorax-related (*trr*) gene, a histone methyltransferase that conceivably plays a role in remodeling the chromatin in promoter regions, just as vertebrate factors do to facilitate receptor-mediated transcription (Stallcup, 2001). The role of TRR as an ecdysone-dependent coactivator is evidenced by the fact that it is recovered as part of a complex that includes EcR and a trimethylated form of the histone-3 protein (presumably modified by TRR) from the ecdysteroid inducible promoter of the *Drosophila hedgehog* (*hh*) gene in extracts derived from 20E challenged S2 cells. The methylation is reduced in complexes taken from *trr* mutant embryos. Further, the trans-heterozygotic combination of a *trr* mutation and the EcR^{F645A} mutation causes almost complete lethality, revealing an essential *in vivo* interaction. The aforementioned Hedgehog (Hh) protein regulates the progression of eye cell differentiation, and *trr*-mutant somatic cell clones express Hh at reduced levels. It follows that TRR is normally a coactivator associated with elevated ecdysteroid-inducible activity leading to higher Hh levels.

EcR retains its repressive capabilities in the absence of its inducible activity, and another cofactor, the corepressor SMRTER, has also been implicated in the regulation of Hh expression and eye differentiation. The lethal and mutant phenotypic effects of the dominant negative EcR^{F645A} mutation are reversed by a hypomorphic mutation of the *Smr* corepressor (i.e., a partial loss of repression), just as EcR mutant effects are overcome by increasing TRR coactivator activity (Sedkov *et al.*, 2003).

Both TRR and SMRTER carry the LXXLL amino acid motifs (L refers to leucine and X refers to any amino acid) associated with nuclear receptor interactions, and physical interaction sites with EcR have been mapped in both cases (Tsai *et al.*, 1999; Sedkov *et al.*, 2003). Moreover, the SMRTER interactive site in *DmEcR* has been mapped to an amino acid residue in helix 5 that when mutated (A483T) results in conditional larval lethality (Bender *et al.*, 1997; Tsai *et al.*, 1999). Another *Drosophila* coactivator, Taiman (TAI), was first identified in a screen for mutations that disrupt oogenesis. TAI resembles a human ortholog (AIB1) belonging to the p160 steroid receptor coactivator family (SRC), whose members are typified by a basic helix-loop-helix (bHLH) domain, a PAS domain of unknown function, several LXXLL motifs associated with nuclear receptor interaction, and several glutamine-rich stretches. These proteins form a bridge between hormone receptors, chromatin modifying enzymes such as the histone acetyl transferases, and the transcriptional machinery. TAI expression colocalizes with EcR and USP in the border cells of the ovary and colocalizes with USP on the polytene chromosomes of *Drosophila* larval salivary glands. TAI also elevates ecdysteroid-inducible transcription in a cell culture and coprecipitates with EcR, but not USP (Bai *et al.*, 2000). Its role in premetamorphic processes has not yet been elucidated.

Still another EcR-interacting protein containing the LXXLL motif, *rigor mortis* (*rig*), is required for ecdysteroid signaling during larval development. Mutant *rig* larvae fail to survive beyond the advent of metamorphosis, while displaying phenotypes resembling other mutations defective in ecdysteroid synthesis or response. *Rig* is required as a coactivator for induction of the E74A isoform which normally appears as ecdysteroid titers increase, but is not required for E75A, EcR, or USP transcription; the effect on transcription is likely to be indirect since *Rig* contains no DNA-binding motifs. The protein interacts physically with EcR and USP, and also with the orphan receptors DHR3, β FTZ-F1, and SVP. Even when helix 12 is deleted from β FTZ-F1, its interaction with *Rig* is detectable, suggesting that the relationship between *Rig* and nuclear receptors is ligand-independent (Gates *et al.*, 2004). A *D. melanogaster* corepressor, Alien, that is highly conserved phylogenetically, also interacts with several receptors, including EcR, SVP, and β FTZ-F1 (but not DHR3, DHR38, DHR78, or DHR96; Dressel *et al.*, 1999). No mutations of Alien have been reported so far.

The pattern of receptor interactions seen with TAI, *Rig*, and Alien suggests that a level of regulation

remains to be elucidated in connection with the ecdysteroid hierarchy. This is further highlighted by the effects of another cofactor, Bonus (Bon), which belongs to a class of proteins (TIF1) that do not bind to DNA directly, but which repress transcriptional activity. Homozygous *bon* mutants display many of the defective β FTZ-F1 phenotypes noted earlier (Broadus *et al.*, 1999), and Bon physically interacts with helix 12 of β FTZ-F1 via an LXXLL motif. In some *bon* mutants, transcript levels of EcR B1, E74A and B, and BR-C are reduced, but DHR3 transcript levels are elevated (Beckstead *et al.*, 2001). Non-DNA binding cofactors, MBF1 and MBF2, associated with *Bombyx* β FTZ-F1 activity have also been identified which interact with the TATA binding protein to induce transcription (Li *et al.*, 1994).

Finally, it is notable that the methoprene-resistant (Met) mutation in *D. melanogaster* defines a gene which specifies another bHLH-PAS transcription factor (Ashok *et al.*, 1998) (see Chapter 8). Cellular extracts from flies homozygous for Met mutations show little binding to the juvenile hormone analog methoprene (Shemshidini and Wilson, 1990). The implications of this identity and its significance for explaining the modulatory effects of JH on ecdysteroid-inducible transcriptional activity will be addressed later.

7.4.7. Orphan Receptor Interactions with EcR and USP

In addition to those players which have been tied directly to the transcriptional response elicited by the EcR/USP heterodimer (DHR3, E75, E78, β FTZF1), several other nuclear receptors also modulate receptor activity by either dimerizing directly with USP, or by competing for EcR/USP promoter sites.

The DHR38/USP interaction is particularly interesting because *in vitro* results suggest that it is this “receptor” complex that responds to a broad spectrum of ecdysteroids, including many known to exist in *D. melanogaster* larvae. Further, DHR38 mutations disrupt cuticle formation, as do *usp* mutations, but EcR mutations do not impair this aspect of development, leaving open the possibility that this aspect of ecdysteroid regulation does not involve EcR at all (Hall and Thummel, 1998; Kozlova *et al.*, 1998). DHR38 is broadly expressed but transcript levels appear to be in low abundance (Sullivan and Thummel, 2003), though the DHR38/USP heterodimer is more responsive to 20E than EcR/USP (Baker *et al.*, 2003). In any case, the mechanism of action is novel, since the transcriptional

activation via DHR38/USP is not associated with a physical ligand interaction.

A similar dimer between AHR38 and *Aa*USP has been noted prior to vitellogenesis in *A. aegypti*. The nonresponsive heterodimer is displaced by the EcR/USP heterodimer in response to a 20E titer peak following a bloodmeal (Zhu *et al.*, 2000). As noted earlier, USP also dimerizes with SevenUp (SVP), the aforementioned ortholog of COUP-TF. As the 20E peak that stimulates *A. aegypti* vitellogenin expression ensues, a similar competition between EcR and SVP for USP as a heterodimeric partner leads to a downregulation of *A. aegypti* vitellogenin gene transcription after egg-laying (Zhu *et al.*, 2003a), illustrating a mechanism by which an ecdysteroid response can be terminated.

Ectopic expression of the *svp*⁺ gene in flies causes lethality, but this effect can be rescued by simultaneous ectopic expression of *usp*⁺ (Zelhof *et al.*, 1995a). This interaction apparently is a relevant aspect of photoreceptor differentiation, since both SVP and USP function are essential for this process to occur normally (Mlodzik *et al.*, 1990; Zelhof *et al.*, 1997). The potential relevance of the SVP/USP interaction has also been established by demonstrating that SVP competes with EcR for USP’s partnership, and thereby reduces ecdysteroid-induced transcription in cell cultures. Further, the functional SVP/USP dimer preferentially interacts with DR1 DNA elements, whereas the EcR/USP dimer interacts with the Eip28/29 element.

Still another orphan receptor that is essential for normal metamorphosis in flies is DHR78. Mutations of this receptor cause late larval lethality, disruptions of the tracheal system, and the impairment of EcR, E74B, and BR-C transcription. DHR78 binds to numerous ecdysteroid inducible sites in salivary glands (Fisk and Thummel, 1998; Astle *et al.*, 2003). DHR78 inhibits 20E-dependent induction of transcription in cell culture assays (Zelhof *et al.*, 1995b) and it has been proposed that DHR78 activity may interact with an “upstream” ligand in the period that precedes the late third instar ecdysteroid peak to prime a later ecdysteroid response (Fisk and Thummel, 1998). A *Bombyx* ortholog, BHR78, has been shown to dimerize directly with BmUSP (Hirai *et al.*, 2002) suggesting that DHR78 may block 20E-dependent transcription by competing for USP with EcR.

7.4.8. Ecdysteroid Action and Other Developmental Processes

The ecdysteroid response is highly heterogeneous among cell types, owing to differences in the

quantitative levels of EcR and USP isoforms, rates of 20E conversion from ecdysone, 20E metabolism and cellular exclusion, and yet it is evident that the circulation of ecdysteroids in the insect hemolymph provides the organism with a means to coordinate its individual developmental programs. Therefore, it is expected that ecdysteroids set off general responses and also specific responses that involve either EcR and USP or targets that are regulated by them.

For organismal investigations, the dramatic changes associated with the late larval ecdysteroid peak in *D. melanogaster* evoke substantial changes in transcript levels which can be detected using genomic approaches. As already noted, a combination of standard technology and a genomic outlook has motivated the assembly of a detailed profile of orphan receptor transcript levels through pre-adult development (Sullivan and Thummel, 2003). A limitation of the whole body approach is evident in the case of DHR38, which is widely expressed but at such low levels that the transcript is barely evident on Northern blots. An earlier study using subtractive hybridization led to the characterization of several ecdysteroid-inducible genes, most of which were unknown and not associated with ecdysteroid-inducible events directly (Hurban and Thummel, 1993). Screens of hematopoietic Kc cells (Savakis *et al.*, 1980) and imaginal discs (Apple and Fristrom, 1991) have yielded several ecdysteroid-dependent genes which do not overlap with those recovered from other screens, and these transcripts are largely associated with tissue-specific responses. However, while the timing of early ecdysteroid gene expression is delayed in the discs (Huet *et al.*, 1993), at least one ecdysteroid-responsive pupal cuticle gene, EDG84A, is regulated by FTZ-F1, which as noted, also regulates metamorphic processes in the salivary gland (Murata *et al.*, 1996).

Microarray analysis has been used more recently to investigate the changes in transcription on a genome-wide basis, using the onset of the white prepupal (pupal) stage as a reference point. The early puff genes change in a predictable, ecdysteroid-regulated manner, and genes involved in processes such as myogenesis, apoptosis, and imaginal disc differentiation are upregulated at appropriate times during the period. As ecdysteroid levels peak, transcription of most genes encoding glycolytic enzymes are substantially reduced, as are genes whose enzymatic products regulate the citric acid and fatty acid cycles, oxidative phosphorylation, and amino acid metabolism (White *et al.*, 1999). Adult flies heterozygous for a lethal EcR mutation survive longer than wild-type flies and show greater resistance to oxidative stress, heat, and dry

conditions, though activity levels are normal and a possible connection to metabolic function has not been made (Simon *et al.*, 2003).

Important mechanistic activities at the cellular level, however, will continue to require careful functional analysis. In the case of eye differentiation in flies, morphogenetic furrow movement during the late third instar is dependent on 20E, but not on normal EcR gene activity (Brennan *et al.*, 2001). The repressive action of USP described earlier for this process (Ghbeish and McKeown, 2002) is offset by the positive regulation of Hedgehog (Hh) transcription that involves EcR (Sedkov *et al.*, 2003). Hh, in turn, facilitates a switchover from the Z2 to the Z1 isoform at the ecdysteroid-responsive BR-C gene locus. The temporal coordination of hormonal signaling with the regulatory activities along the furrow, therefore, provide a balance of signals that both stimulates morphogenetic furrow movement (in the form of EcR-mediated Hh expression) and regulates its rate of progression (in the form of a repressive USP function). The mechanism of furrow movement is not fully elucidated, and it is conceivable that a second hormonal signaling pathway is involved in the process.

The interaction between JH and ecdysteroid action remains elusive, despite the clear recognition that there must be cross-talk between these pathways. The JH analog methoprene induces several disruptions of the central nervous system that resemble those caused by mutations of the BR-C gene. These defects are not seen in flies homozygous for the methoprene-resistant (MET) mutation (Restifo and Wilson, 1998).

7.4.9. Juvenile Hormone Effects upon Ecdysteroid Action

A central tenet of insect biology is that ecdysteroids mediate larval-larval molts in the presence of the sesquiterpenoid, JH (see **Chapter 8**). In the absence of JH, by contrast, ecdysteroid induces a larval-pupal transition. Implicit in this view is the recognition that JH via its receptor competes with the EcR/USP dimer at an EcRE (Berger *et al.*, 1992) or modifies components of the ecdysteroid receptor itself (Jones *et al.*, 2001; Henrich *et al.*, 2003). Nevertheless, no uniform explanation for the action of JH, alone or in conjunction with ecdysteroids, has been forthcoming.

The structural similarity of 9-*cis* retinoic acid, the cognate ligand of vertebrate RXR, with JH inspired experiments to test the possibility that the insect RXR ortholog, USP, is the JH receptor, and like RXR, switches between a homodimeric and heterodimeric state to process incoming hormonal

signals. This enticing possibility is further bolstered by the observation that RXR is inducible by binding to the acid metabolite of the JH analog, methoprene (Harmon *et al.*, 1995). Jones and Sharp (1997) reported that a *Drosophila* USP fusion protein binds to JH forms found in flies, as measured by the dose-dependent suppression by JH3 and JH3-bis epoxide on the fluorescence of tryptophan residues in the ligand binding domain of USP. Later studies have demonstrated that purified USP fusion proteins exist in homodimeric and homotetrameric forms which show a saturable ability to bind to JH3, with about 50% binding at 4 μ M, while the closely related farnesol exerts no effect. Using nuclear extracts from the lepidopteran Sf9 cell line, it was further shown that USP binds specifically as a homodimer to a direct repeat response element separated by 12 nucleotides (DR12). Further, JH3 strongly induces a reporter gene in which the DR12 element is attached to the core promoter of the gene encoding the *Trichoplusia ni* JH esterase (Jones *et al.*, 2001). A DR4 element in the *C. fumiferana* JH esterase gene promoter is induced by JH1 and suppressed by 20E, suggesting that an interaction between these hormones occurs at the DNA level (Kethidi *et al.*, 2004). The biochemical examination of these promoters, along with those of JH-inducible genes found recently in cell cultures through microarray analysis, will hopefully bring a convergence of views on JH action (Dubrovsky *et al.*, 2000).

Along a different line of reasoning, experiments have been undertaken based on the sequence similarity between the EcR LBD and the vertebrate FXR LBD, which surprisingly, is responsive to JH3 in the 10–50 μ M range with an RXR dimer partner in mammalian cell cultures (Forman *et al.*, 1995). When tested in this regime, JH3 potentiates the ability of the EcR/USP complex to induce a weak transcriptional induction via an *hsp27* EcRE in the presence of submaximal muristerone A levels. The potentiation apparently requires prior binding of EcR to ecdysteroids and there is no evidence yet that JH3 is binding with USP in this assay, and no response is noted with EcR/USP to bile acid, the most potent FXR activator. When tested with 20E, only EcRB2 among the three *Drosophila* isoforms is additionally potentiated with JH3 (Henrich *et al.*, 2003), and, as noted, the B2 isoform is the only one capable of rescuing larval development in EcR mutants (Li and Bender, 2000). The potentiation is not analogous to the responsiveness of an EcR/RXR heterodimer described earlier (Saez *et al.*, 2000), and the low ecdysteroid levels which accompany JH titers during larval development suggests its possible biological relevance. In both *Drosophila*

and *Manduca*, one of the isoforms of Broad (Z1) is ectopically induced in cuticle by the application of JH analogs, further supporting the possibility that JH acts directly upon the ecdysteroid receptor's transcriptional capability (Zhou and Riddiford, 2002), since the BR-C gene is a direct target of the receptor in both *Drosophila* and *Manduca*.

In reality, differences such as the promoter and receptor constructs among the experimental regimes leave open the possibility that USP acts by multiple mechanisms, particularly if one considers the possibility that posttranslational modifications such as phosphorylation also can modify receptor activity. Possibly distinct roles of USP during larval and metamorphic development are evidenced *in vivo* by the fact that a chimeric USP transgene (in which the *Drosophila* USP LBD is replaced by the equivalent domain from *C. tentans*) completely rescues larval development in *usp* mutants that otherwise die in the first instar. Such genetically rescued larvae, however, suddenly die as the onset of the prepupal stage approaches (Henrich *et al.*, 2000). Another potentially important factor is the protein defined by the Methoprene-tolerant (Met) mutation that belongs to the bHLH-PAS family of transcription factors, and includes several cofactors known to interact with nuclear receptors (Ashok and Wilson, 1998). The effects of Met upon the ecdysteroid receptor await further experimentation in *Drosophila* and other insects.

JH3 plays an essential role for the translation of β FTZ-F1 from existing transcripts in the fat body of newly emerged *A. aegypti* females. In turn, FTZF1 activity is required for the subsequent transcription of the mosquito "early puff" genes regulating vitellogenesis (Zhu *et al.*, 2003b). While this may be seen as another mode of action, the effect on β FTZF1 translation may be indirect, that is, JH3 may regulate the transcription of genes whose products play a role in mRNA stability and/or protein translation.

In summary, the diverse functional effects of JH upon ecdysteroid-inducible gene expression have elicited several possible mechanisms from investigators over the years. As it becomes increasingly apparent that EcR and USP are the two most recognizable components of a protein complex of diverse functional capability, it seems highly plausible that there is not a single JH receptor, but rather, a JH receptive protein complex (or complexes).

7.5. Prognosis

An explosion of information about the ecdysteroid receptor has occurred recently which will

undoubtedly influence the field far into the future. There are three levels of progress that will be central to the development of the field: (1) continued progress on understanding the biological action of ecdysteroids using functional genomics and genetics in *D. melanogaster*, (2) the adaptation of transgenic and genomic methods into receptor studies of other insects, and (3) continued modeling of insect receptor complexes along with tests of these models through both *in vitro* and *in vivo* experimentation.

The emergence of clever new methodologies, notably the *in vivo* lacZ reporter system reported by Kozlova and Thummel (2003b), provides *in vivo* tools that overcome the limitations of biochemical isolation and testing from flies. Microarray analysis has already been employed to investigate changes in gene transcription at the onset of metamorphosis (White *et al.*, 1999) and to compare expression in Kc167 and SL2 cell lines (Neal *et al.*, 2003). Microarray analysis has also been employed in *D. melanogaster* to follow expression events throughout larval and metamorphic development. The results suggest that while the larval stage is relatively quiescent, there are unique patterns of expression associated with this early developmental time which in some cases, may prove to be repressive functions (Arbeitman *et al.*, 2002). In this respect, microarrays also may prove important for gaining a better grasp of JH's mode of action (Dubrovsky *et al.*, 2000) during larval stages. Inevitably, the genomic approach will unravel entire gene networks that interact with and operate within the ecdysteroid-regulated hierarchy (Arbeitman *et al.*, 2002; Stathopoulos and Levine, 2002). In any event, investigating the entire genome is important for identifying temporal connections at a gene expression level which potentially coincide with important functional relationships, as the connection between the glycolytic pathway and the onset of metamorphosis has already exemplified (White *et al.*, 1999). Sooner rather than later, it is likely that genome-wide approaches will be applied to identify EcR and/or USP binding sites (Ren *et al.*, 2000).

Another feature of ecdysteroid action which has received virtually no attention is the role that nutritional and cellular states play in determining and modifying ecdysteroid action (Zinke *et al.*, 2002). As the case with glycolysis shows, it seems inevitable that a relationship exists here, given that the most important problem for premetamorphic insects stems from their need to process nutrients as they grow and undergo molting. The role of orphan receptors and their ligands seems an obvious place to explore this possibility, since most of the identified orphans among vertebrates have proven to be

responsive to a variety of dietary compounds and intracellular metabolites, including EcR's vertebrate relatives, FXR and LXR.

The regulation of EcR and USP expression has begun to be undertaken and should also lead to important insights about endocrine regulation generally, and the interaction of ecdysteroid regulation with other biological processes (Sung and Robinow, 2000; Zheng *et al.*, 2003). EcR undergoes fairly dramatic fluctuations in transcription, though little is known about the relative titers of EcR and USP intracellularly (Koelle *et al.*, 1991). In other words, it is not clear which one of the two is rate-limiting in any given cell type, and thus, a quantitative question remains to be fully considered. In fact, there has been a tendency to ascribe regulation to the roles of individual players, without regard to the fact that cellular protein titers, ligand titers, rate of metabolism, and the relative affinity of promoter elements all play a role in determining the level of transcriptional response at any given gene at any given time. When ecdysteroid action is viewed as a challenge of cellular equilibrium, it is seen as a continuous and interactive process rather than a directed and responsive one. Interestingly, there are still unanswered questions about ecdysteroid regulation within the *Drosophila* salivary gland hierarchy which will be particularly useful for examining ecdysteroid response and processing, particularly with the judicious use of transgenic constructs.

A second important cornerstone for future progress will depend upon a more complete depiction of ecdysteroid receptor function among the insect orders. A survey of known EcR and USP sequences shows that only Diptera and Lepidoptera are extensively represented at this time. Based upon the variations in agonist responsiveness already discovered among the insect receptors in these two orders alone, the need for obtaining and testing other insect receptors is plainly obvious.

The ability to assess the effects of EcR, USP, and genes targeted by the ecdysteroid receptor in other insects will be greatly enhanced by continued development of transformation procedures. The ability to produce "null" mutations via RNA interference with transgenic constructs will be particularly important for assessing functional processes in other insects. Uhlirova *et al.* (2003) illustrated this possibility by showing that Sindbis virus-induced transformation with an RNAi eliminates Br-C expression in *Bombyx mori*. The interference exacerbates the same developmental defects in *Bombyx* as previously noted for the effects of Br-C null mutations in flies.

Related to the discovery and characterization of ecdysteroid responses in insect processes will be the

continued examination of the insect receptors themselves. The use of chimeras and mutational analysis, in conjunction with the predictive powers provided by improved modeling and crystal structures, will lead to further insights concerning the capabilities of receptor function and its response to ligands, which may include a broad spectrum based on the flexibility of its ligand-binding pocket (Billas *et al.*, 2003). Many mutations affect receptor function nonspecifically (Bender *et al.*, 1997; Bergman *et al.*, 2004), and site-directed mutagenesis provides a particularly useful approach for finding mutations that specifically disrupt EcR and USP subfunctions essential for interpreting receptor function in cellular and *in vivo* systems.

In summary, the information reported here represents only a fraction of the progress on ecdysteroid receptor action that has taken place over the last 20 years. The convergence of research interests mentioned at the outset has created a synergy among approaches that will continue to yield important new insights about endocrine action and its effects on development, new possibilities for insecticidal discovery, and new applications for the use of ecdysteroid-regulated transcriptional cell systems.

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References

- Amabis, D.C., Amabis, J.M., 1984. Effects of ecdysterone in polytene chromosomes of *Trichosia pubescens*. *Devel. Biol.* 102, 1–9.
- Amero, S.A., Elgin, S.C.R., Beyer, A.L., 1991. A unique zinc finger protein is associated preferentially with active ecdysone-responsive loci in *Drosophila*. *Genes Devel.* 5, 188–200.
- Andres, A.J., Cherbas, P., 1992. Tissue-specific ecdysone responses: Regulation of the *Drosophila* genes Eip28/29 and Eip40 during larval development. *Development* 116, 865–876.
- Andres, A.J., Fletcher, J.C., Karim, F.D., Thummel, C.S., 1993. Molecular analysis of the initiation of insect metamorphosis: a comparative study of *Drosophila* ecdysteroid-regulated transcription. *Devel. Biol.* 160, 388–404.
- Andres, A.J., Cherbas, P., 1994. Tissue-specific regulation by ecdysone: distinct patterns of Eip28/29 expression are controlled by different ecdysone response elements. *Devel. Genet.* 15, 320–331.
- Antoniewski, C., Laval, M., Lepesant, J.A., 1993. Structural features critical to the activity of an ecdysone receptor binding site. *Insect Biochem. Mol. Biol.* 23, 105–114.
- Antoniewski, C., Mugat, B., Delbac, F., Lepesant, J.-A., 1996. Direct repeats bind the EcR/Usp receptor and mediate ecdysteroid responses in *Drosophila melanogaster*. *Mol. Cell. Biol.* 16, 2977–2986.
- Apple, R.T., Fristrom, J.W., 1991. 20-hydroxyecdysone is required for, and negatively regulates, transcription of *Drosophila* pupal cuticle protein genes. *Devel. Biol.* 146, 569–582.
- Arbeitman, M.N., Hogness, D.S., 2000. Molecular chaperones activate the *Drosophila* ecdysone receptor, an RXR heterodimer. *Cell* 101, 67–77.
- Arbeitman, M.N., Furlong, E.E., Imam, F., Johnson, E., Null, B.H., *et al.*, 2002. Gene expression during the life cycle of *Drosophila melanogaster*. *Science* 297, 2270–2275.
- Asahina, M., Jindra, M., Riddiford, L.M., 1997. Developmental expression of Ultraspiracle proteins in the epidermis of the tobacco hornworm, *Manduca sexta*, during larval life and the onset of metamorphosis. *Devel. Genes Evol.* 207, 381–388.
- Ashburner, M., Chihara, C., Meltzer, P., Richards, G., 1974. Temporal control of puffing activity in polytene chromosomes. *Cold Spring Harbor Symp. Quant. Biol.* 38, 655–662.
- Ashok, M., Turner, C., Wilson, T.G., 1998. Insect juvenile hormone resistance gene homology with the bHLH-PAS family of transcriptional regulators. *Proc. Natl Acad. Sci. USA* 95, 2761–2766.
- Astle, J., Kozlova, T., Thummel, C.S., 2003. Essential roles for the Dhr78 orphan nuclear receptor during molting of the *Drosophila* tracheal system. *Insect Biochem. Mol. Biol.* 33, 1201–1209.
- Baehrecke, E.H., Thummel, C.S., 1995. The *Drosophila* E93 gene from the 93F early puff displays stage- and tissue-specific regulation by 20-hydroxyecdysone. *Devel. Biol.* 17, 85–97.
- Bai, J., Uehara, Y., Montell, D.J., 2000. Regulation of invasive cell behavior by Taiman, a *Drosophila* protein related to AIB1, a steroid receptor coactivator amplified in breast cancer. *Cell* 103, 1047–1058.
- Baker, K.D., Warren, J.T., Thummel, C.S., Gilbert, L.I., Mangelsdorf, D.J., 2000. Transcriptional activation of the *Drosophila* ecdysone receptor by insect and

- plant ecdysteroids. *Insect Biochem. Mol. Biol.* 30, 1037–1043.
- Baker, K.D., Shewchuk, L.M., Kozlova, T., Makishima, M., Hassell, A., *et al.*, 2003. The *Drosophila* orphan nuclear receptor DHR38 mediates an atypical ecdysteroid signaling pathway. *Cell* 113, 731–742.
- Bashirullah, A., Pasquinelli, A.E., Kiger, A.A., Perrimon, N., Ruvkun, G., *et al.*, 2003. Coordinate regulation of small temporal RNAs at the onset of *Drosophila* metamorphosis. *Devel. Biol.* 259, 1–8.
- Basso, L.R., Vasconcelos, C., Fontes, A.M., Hartfelder, K., Silva, J.A., *et al.*, 2002. The induction of DNA puff BhC4-1 is a late response to the increase in 20-hydroxyecdysone titers in last instar dipteran larvae. *Mech. Devel.* 110, 15–26.
- Bayer, C.A., von Kalm, L., Fristrom, J.W., 1997. Relationships between protein isoforms and genetic functions demonstrate functional redundancy at the Broad-Complex during *Drosophila* metamorphosis. *Devel. Biol.* 191, 311–312.
- Becker, H.J., 1959. Die puffs der speicheldrüsenchromosomen von *Drosophila melanogaster*. *Chromosoma* 10, 654–678.
- Beckstead, R., Ortiz, J.A., Sanchez, C., Prokopenko, S.N., Chambon, P., *et al.*, 2001. Bonus, a *Drosophila* homolog of TIF1 proteins, interacts with nuclear receptors and can inhibit β FTZ-F1-dependent transcription. *Mol. Cell.* 7, 753–765.
- Bender, M., Imam, F.B., Talbot, W.S., Ganetzky, B., Hogness, D.S., 1997. *Drosophila* ecdysone receptor mutations reveal functional differences among receptor isoforms. *Cell* 91, 777–788.
- Berger, E.M., Goudie, K., Klieger, L., Berger, M., DeCato, R., 1992. The juvenile hormone analog, methoprene, inhibits ecdysterone induction of small heat shock protein gene expression. *Devel. Biol.* 151, 410–418.
- Bergman, T., Henrich, V.C., Schlattner, U., Lezzi, M., 2004. Ligand control of interaction *in vivo* between ecdysteroid receptor and ultraspiracle ligand-binding domain. *Biochem. J.* 378, 779–784.
- Bialecki, M., Shilton, A., Fichtenberg, C., Segraves, W.A., Thummel, C.S., 2002. Loss of the ecdysteroid-inducible E75A orphan nuclear receptor uncouples molting from metamorphosis in *Drosophila*. *Devel. Cell* 3, 209–220.
- Bienz-Tadmor, B., Smith, H.S., Gerbi, S.A., 1991. The promoter of DNA puff gene II/9–1 *Sciara coprophila* is inducible by ecdysone in late prepupal salivary glands of *Drosophila melanogaster*. *Cell Regul.* 2, 875–888.
- Billas, I.M., Moulinier, I., Rochel, N., Moras, D., 2001. Crystal structure of the ligand-binding domain of the ultraspiracle protein Usp, the ortholog of retinoid X receptors in insects. *J. Biol. Chem.* 276, 7465–7474.
- Billas, I.M., Iwema, T., Garnier, J-M., Mitschier, A., Rochel, N., *et al.*, 2003. Structural adaptability in the ligand-binding pocket of the ecdysone hormone receptor. *Nature* 426, 91–96.
- Blumberg, B., Evans, R.M., 1998. Orphan nuclear receptors – new ligands and new possibilities. *Genes Devel.* 12, 3149–3155.
- Bonneton, F., Zelus, D., Iwema, T., Robinson-Rechavi, M., Laudet, V., 2003. Rapid divergence of the ecdysone receptor in Diptera and Lepidoptera suggests coevolution between EcR and Usp-RXR. *Mol. Biol. Evol.* 20, 541–555.
- Brennan, C.A., Ashburner, M., Moses, K., 1998. Ecdysone pathway is required for furrow progression in the developing *Drosophila* eye. *Development* 125, 2653–2664.
- Brennan, C.A., Ashburner, M., Moses, K., 2001. Broad-Complex, but not ecdysone receptor, is required for progression of the morphogenetic furrow in the *Drosophila* eye. *Development* 128, 1–11.
- Broadus, J., McCabe, J.R., Endrizzi, B., Thummel, C.S., Woodard, C.T., 1999. The *Drosophila* β FTZ-F1 orphan nuclear receptor provides competence for stage-specific responses to the steroid hormone ecdysone. *Mol. Cell* 3, 143–149.
- Bruhath, A., Dreau, D., Drake, M.E., Tourmente, S., Chapel, S., *et al.*, 1993. Intronic and 5' flanking sequences of the *Drosophila* beta 3 tubulin gene are essential to confer ecdysone responsiveness. *Mol. Cell. Endocrinol.* 94, 61–71.
- Burtis, K.C., Thummel, C.S., Jones, C.W., Karim, F.D., Hogness, D.S., 1990. The *Drosophila* 74EF early puff contains E74, a complex ecdysone-inducible gene that encodes two ets-related proteins. *Cell* 61, 85–99.
- Carney, G.E., Bender, M., 2000. The *Drosophila* ecdysone receptor (EcR) gene is required maternally for normal oogenesis. *Genetics* 154, 1203–1211.
- Carney, G.E., Robertson, A., Davis, M.B., Bender, M., 2004. Creation of EcR isoform-specific mutations in *Drosophila melanogaster* via local P element transposition, imprecise P element excision, and male recombination. *Mol. Genet. Genomics* 271, 282–290.
- Carney, G.E., Wade, A.A., Sapra, R., Goldstein, E.S., Bender, M., 1997. DHR3, an ecdysone-inducible early-late gene encoding a *Drosophila* nuclear receptor, is required for embryogenesis. *Proc. Natl Acad. Sci. USA* 94, 12024–12029.
- Chan, S.M., 1998. Cloning of a shrimp (*Metapanaeus ensis*) cDNA encoding a nuclear receptor superfamily member: an insect homologue of E75 gene. *FEBS Lett.* 436, 395–400.
- Chen, J.H., Turner, P.C., Rees, H.H., 2002a. Molecular cloning and induction of nuclear receptors from insect cell lines. *Insect Biochem. Mol. Biol.* 32, 657–667.
- Chen, L., O'Keefe, S.L., Hodgetts, R.B., 2002b. Control of Dopa decarboxylase gene expression by the Broad-Complex during metamorphosis in *Drosophila*. *Mech. Devel.* 119, 145–156.
- Cherbas, L., Cherbas, P., 1997. “Parahomologous” gene targeting in *Drosophila* cells: an efficient, homology-dependent pathway of illegitimate recombination near a target site. *Genetics* 145, 349–358.

- Cherbas, P., Cherbas, L., Lee, S.-S., Nakanishi, K., 1988. 26-[¹²⁵I]Iodoponasterone A is a potent ecdysone and a sensitive radioligand for ecdysone receptors. *Proc. Natl Acad. Sci. USA* 85, 2096–2100.
- Cherbas, L., Hu, X., Zhimulev, I., Belyaeva, E., Cherbas, P., 2003. EcR isoforms in *Drosophila*: testing tissue-specific requirements by targeted blockade and rescue. *Development* 130, 271–284.
- Cherbas, L., Koehler, M.M.D., Cherbas, P., 1989. Effects of juvenile hormone on the ecdysone response in *Drosophila* Kc cells. *Devel. Genet.* 10, 177–188.
- Cherbas, L., Lee, K., Cherbas, P., 1991. Identification of ecdysone response elements by analysis of the *Drosophila* Eip28/29 gene. *Genes Devel.* 5, 120–131.
- Cherbas, L., Yonge, C.D., Cherbas, P., Williams, C.M., 1980. The morphological response of Kc-H cells to ecdysteroids: hormonal specificity. *Wilhelm Roux's Archives* 189, 1–15.
- Choi, D.S., Wang, D., Tolbert, L., Sadee, W., 2000. Basal signaling activity of human dopamine D2L receptor demonstrated with an ecdysone-inducible mammalian expression system. *J. Neurosci. Methods* 94, 217–225.
- Christopherson, K.S., Mark, M.R., Bajaj, V., Godowski, P.J., 1992. Ecdysteroid-dependent regulation of genes in mammalian cells by a *Drosophila* ecdysone receptor and chimeric transactivators. *Proc. Natl Acad. Sci. USA* 89, 6314–6318.
- Chung, A.C.-K., Durica, D.S., Clifton, S.W., Roe, B.A., Hopkins, P.M., 1998. Cloning of crustacean EcR and RXR homologs and elevation of RXR mRNA by retinoic acid. *Mol. Cell. Endocrinol.* 139, 209–237.
- Clayton, G.M., Peak-Chew, S.Y., Evans, R.M., Schwabe, J.W.R., 2001. The structure of the ultraspiracle ligand-binding domain reveals a nuclear receptor locked in an inactive conformation. *Proc. Natl Acad. Sci. USA* 98, 1549–1554.
- Crispi, S., Giordano, E., D'Avino, P.P., Furia, M., 1998. Cross-talking among *Drosophila* nuclear receptors at the promiscuous response element of the ng-1 and ng-2 intermolt genes. *J. Mol. Biol.* 275, 561–574.
- Crossgrove, K., Laudet, V., Maina, C.V., 2002. *Dirofilaria immitis* encodes Di-nhr-7, a putative ortholog of the *Drosophila* ecdysone regulated E78 gene. *Mol. Biochem. Parasitol.* 119, 169–177.
- D'Avino, P.P., Thummel, C.S., 2000. The ecdysone regulatory pathway controls wing morphogenesis and integrin expression during *Drosophila* metamorphosis. *Devel. Biol.* 220, 211–224.
- D'Avino, P.P., Drispi, S., Cherbas, L., Cherbas, P., Furia, M., 1995. The moulting hormone ecdysone is able to recognize target elements composed of direct repeats. *Mol. Cell. Endocrinol.* 113, 1–9.
- de Carvalho, D.P., Coelho, P.S.R., de Almeida, J.C., 2000. A dual role of 20-hydroxyecdysone in the control of protein synthesis related to DNA puff activity in the anterior region of *Bradysia hygida* (Diptera, Sciaridae) salivary gland. *Insect Biochem. Mol. Biol.* 30, 541–548.
- De la Cruz, F.E., Kirsch, D.R., Heinrich, J.N., 2000. Transcriptional activity of *Drosophila melanogaster* ecdysone receptor isoforms and ultraspiracle in *Saccharomyces cerevisiae*. *J. Mol. Endocrinol.* 24, 183–191.
- De la Cruz, F., Mak, P., 1997. *Drosophila* ecdysone receptor functions as a constitutive activator in yeast. *J. Steroid Biochem. Mol. Biol.* 62, 353–369.
- Devarakonda, S., Harp, J.M., Kim, Y., Ozyhar, A., Rastinejad, F., 2003. Structure of the heterodimeric ecdysone receptor DNA-binding complex. *EMBO J.* 22, 5827–5840.
- Dhadialla, T., Carlson, G.R., Le, D.P., 1998. New insecticides with ecdysteroidal and juvenile hormone activity. *Annu. Rev. Entomol.* 43, 545–569.
- Dhadialla, T.S., Tzertzinis, G., 1997. Characterization and partial cloning of ecdysteroid receptor from a cotton boll weevil embryonic cell line. *Arch. Insect Biochem. Physiol.* 35, 45–57.
- DiBartolomeis, S.M., Gerbi, S.A., 1989. Molecular characterization of DNA puff II/9A genes in *Sciara coprophila*. *J. Mol. Biol.* 210, 531–540.
- DiBello, P.R., Withers, D.A., Bayer, C.A., Fristrom, J.W., Guild, G.M., 1991. The *Drosophila* Broad-Complex encodes a family of related proteins containing zinc fingers. *Genetics* 129, 385–397.
- Dobens, L., Rudolph, K., Berger, E.M., 1991. Ecdysterone regulatory elements function as both transcriptional activators and repressors. *Mol. Cell. Biol.* 11, 1846–1853.
- Dressel, U., Thormeyer, D., Altincicek, B., Paululat, A., Eggert, M., et al., 1999. Alien, a highly conserved protein with characteristics of a corepressor for members of the nuclear hormone receptor superfamily. *Mol. Cell. Biol.* 19, 3383–3394.
- Dubrovsky, E.B., Dubrovskaya, V.A., Bilderback, A.L., Berger, E.M., 2000. The isolation of two juvenile hormone-inducible genes in *Drosophila melanogaster*. *Devel. Biol.* 224, 486–495.
- Durica, D.S., Wu, X., Anilkumar, G., Hopkins, P.M., Chung, A.C.-K., 2002. Characterization of crab EcR and RXR homologs and expression during limb regeneration and oocyte maturation. *Mol. Cell. Endocrinol.* 189, 59–76.
- Elbrecht, A., Chen, Y., Jurgens, T., Hensens, O.D., Zink, D.L., et al., 1996. 8-O-acetylharpagide is a nonsteroidal ecdysteroid agonist. *Insect Biochem. Mol. Biol.* 26, 519–523.
- Elke, C., Vogtli, M., Rauch, P., Spindler-Barth, M., Lezzi, M., 1997. Expression of EcR and Usp in *Escherichia coli*; purification and functional studies. *Arch. Insect Biochem. Physiol.* 35, 59–69.
- Fisk, G.J., Thummel, C.S., 1995. Isolation, regulation, and DNA-binding properties of three *Drosophila* nuclear hormone receptor superfamily members. *Proc. Natl Acad. Sci. USA* 92, 10604–10608.
- Fisk, G.J., Thummel, C.S., 1998. The DHR78 nuclear receptor is required for ecdysteroid signaling during the onset of *Drosophila* metamorphosis. *Cell* 93, 543–555.
- Fletcher, J.C., Burtis, K.C., Hogness, D.S., Thummel, C.S., 1995. The *Drosophila* E74 gene is required for

- metamorphosis and plays a role in the polytene chromosome puffing response to ecdysone. *Development* 121, 1455–1465.
- Forman, B.M., Goode, E., Chen, J., Oro, A.E., Bradley, D.J., *et al.*, 1995. Identification of a nuclear receptor that is activated by farnesol metabolites. *Cell* 81, 687–693.
- Gates, J., Lam, G., Ortiz, J.A., Losson, R., Thummel, C.S., 2004. rigor mortis encodes a novel nuclear receptor interacting protein required for ecdysone signaling during *Drosophila* larval development. *Development* 131, 25–36.
- Ghbeish, N., Tsai, C.-C., Schubiger, M., Zhou, J.Y., Evans, R.M., *et al.*, 2001. The dual role of ultraspiracle, the *Drosophila* retinoid X receptor, in the ecdysone response. *Proc. Natl Acad. Sci. USA* 98, 3867–3872.
- Ghbeish, N., McKeown, M., 2002. Analyzing the repressive function of ultraspiracle, the *Drosophila* RXR, in *Drosophila* eye development. *Mech. Devel.* 111, 89–98.
- Gonzy, G., Pokholkova, G.V., Perronet, F., Mugat, B., Demakova, O.V., *et al.*, 2002. Isolation and characterization of novel mutations of the Broad-Complex, a key regulatory gene of ecdysone induction in *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* 32, 121–132.
- Grau, V., Lafont, R., 1994. Metabolism of ecdysone and 20-hydroxyecdysone in adult *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* 24, 49–58.
- Grebe, M., Przibilla, S., Henrich, V.C., Spindler-Barth, M., 2003. Characterization of the ligand-binding domain of the ecdysteroid receptor from *Drosophila melanogaster*. *Biol. Chem.* 384, 93–104.
- Grebe, M., Rauch, P., Spindler-Barth, M., 2000. Characterization of subclones of the epithelial cell line from *Chironomus tentans* resistant to the insecticide RH5992, a nonsteroidal moulting hormone agonist. *Insect Biochem. Mol. Biol.* 30, 591–600.
- Grebe, M., Spindler-Barth, M., 2002. Expression of ecdysteroid receptor and ultraspiracle from *Chironomus tentans* (Insecta, Diptera) in *E. coli* and purification in a functional state. *Insect Biochem. Mol. Biol.* 32, 167–174.
- Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J.-M., *et al.*, 1986. Human oestrogen receptor cDNA: sequence, expression, and homology to v-erb-A. *Nature* 320, 134–139.
- Guo, X., Harmon, M.A., Laudet, V., Mangelsdorf, D.J., Palmer, M.J., 1997. Isolation of a functional ecdysteroid receptor homologue from the ixodid tick *Amblyomma americanum* (L.). *Insect. Biochem. Mol. Biol.* 27, 945–962.
- Guo, X., Xu, Q., Harmon, M., Jin, X., Laudet, V., *et al.*, 1998. Isolation of two functional retinoid X receptor subtypes from the ixodid tick *Amblyomma americanum* (L.). *Insect Biochem. Mol. Biol.* 27, 945–962.
- Hall, B.L., Thummel, C.S., 1998. The RXR homolog Ultraspiracle is an essential component of the *Drosophila* ecdysone receptor. *Development* 125, 4709–4717.
- Halling, B.P., Yuhas, D.A., Eldridge, R.R., Gilbery, S.N., Deutsch, V.A., *et al.*, 1999. Expression and purification of the hormone binding domain of the *Drosophila* ecdysone and ultraspiracle receptors. *Protein Expr. Purif.* 17, 373–386.
- Hannan, G.N., Hill, R.J., 1997. Cloning and characterization of LcEcR: a functional ecdysone receptor from the sheep blowfly *Lucilia cuprina*. *Insect Biochem. Mol. Biol.* 27, 479–488.
- Hannan, G.N., Hill, R.J., 2001. Lcusp, an ultraspiracle gene from the sheep blowfly, *Lucilia cuprina*: cDNA cloning, developmental expression of RNA and confirmation of function. *Insect Biochem. Mol. Biol.* 31, 771–781.
- Harmon, M.A., Boehm, M.F., Heyman, R.A., Mangelsdorf, D.J., 1995. Activation of mammalian retinoid X receptors by the insect growth regulator methoprene. *Proc. Natl Acad. Sci. USA* 92, 6157–6160.
- Hayward, D.C., Bastiani, M.J., Truman, J.W., Riddiford, L.M., Ball, E.E., 1999. The sequence of *Locusta* RXR, homologous to *Drosophila* Ultraspiracle, and its evolutionary implications. *Devel. Genes Evol.* 209, 564–571.
- Hayward, D.C., Dhadialla, T.S., Zhou, S., Kuiper, M.J., Ball, E.E., *et al.*, 2003. Ligand specificity and developmental expression of RXR and ecdysone receptor in the migratory locust. *J. Insect Physiol.* 49, 1135–1144.
- Henrich, V.C., Brown, N.E., 1995. Insect nuclear receptors; a developmental and comparative perspective. *Insect Biochem. Mol. Biol.* 25, 881–897.
- Henrich, V.C., Burns, E., Yelverton, D.P., Christensen, E., Weinberger, C., 2003. Juvenile hormone potentiates ecdysone receptor-dependent transcription in a mammalian cell culture system. *Insect Biochem. Mol. Biol.* 33, 1239–1247.
- Henrich, V.C., Rybczynski, R., Gilbert, L.I., 1999. Peptide hormones, steroid hormones, and puffs: mechanisms and models in insect development. In: Litwack, G. (Ed.), *Vitamins and Hormones*, Vol. 55. Academic Press, San Diego, pp. 73–125.
- Henrich, V.C., Sliter, T.J., Lubahn, D.B., MacIntyre, A., Gilbert, L.I., 1990. A steroid-thyroid hormone receptor superfamily member in *Drosophila melanogaster* that shares extensive sequence similarity with a mammalian homologue. *Nucleic Acids Res.* 18, 4143–4148.
- Henrich, V.C., Szekely, A.A., Kim, S.J., Brown, N., Antoniewski, C., *et al.*, 1994. Expression and function of the ultraspiracle (*usp*) gene locus during development in *Drosophila melanogaster*. *Devel. Biol.* 165, 38–52.
- Henrich, V.C., Vogtli, M., Grebe, M., Przibilla, S., Spindler-Barth, M., *et al.*, 2000. Developmental effects of chimeric ultraspiracle gene derived from *Drosophila* and *Chironomus*. *Genesis* 28, 125–133.
- Hill, R.J., Segraves, W.A., Choi, D., Underwood, P.A., Macavoy, E., 1993. The reaction with polytene chromosomes of antibodies raised against *Drosophila* E75A protein. *Insect Biochem. Mol. Biol.* 23, 99–104.
- Hirai, M., Shinoda, T., Kamimura, M., Tomita, S., Shiotsuki, T., 2002. *Bombyx mori* orphan receptor,

- BmHR78: cDNA cloning, testis abundant expression, and putative dimerization partner for *Bombyx* ultraspiracle. *Mol. Cell. Endocrinol.* 189, 201–211.
- Hiruma, K., Carter, M.S., Riddiford, L.M., 1995. Characterization of the dopa decarboxylase gene of *Manduca sexta* and its suppression by 20-hydroxyecdysone. *Devel. Biol.* 169, 195–209.
- Hiruma, K., Shinoda, T., Malone, F., Riddiford, L.M., 1999. Juvenile hormone modulates 20-hydroxyecdysone-inducible ecdysone receptor and *ultraspiracle* gene expression in the tobacco hornworm, *Manduca sexta*. *Devel. Genes Evol.* 209, 18–30.
- Hock, T., Cottrill, T., Keegan, J., Garza, D., 2000. The E23 early gene of *Drosophila* encodes an ecdysone-inducible ATP-binding cassette transporter capable of repressing ecdysone-mediated gene activation. *Proc. Natl Acad. Sci. USA* 15, 9519–9524.
- Hollenberg, S.M., Weinberger, C., Ong, E.S., Cerelli, G., Oro, A., et al., 1985. Domain structure of human glucocorticoid receptor and its relationship to the v-erb-A oncogene product. *Nature* 318, 635–641.
- Hoppe, U.C., Marban, E., Johns, D.C., 2000. Adenovirus-mediated inducible gene expression *in vivo* by a hybrid ecdysone receptor. *Mol. Therap.* 1, 159–164.
- Horner, M.A., Chen, T., Thummel, C.S., 1995. Ecdysteroid regulation and DNA binding properties of *Drosophila* nuclear hormone receptor superfamily members. *Devel. Biol.* 168, 490–502.
- Hu, W., Cook, B.J., Ampasala, D.R., Zheng, S., Caputo, G., et al., 2004. Morphological and molecular effects of 20-hydroxyecdysone and its agonist tebufenozide on CF-203, a midgut derived cell line from the spruce budworm, *Choristoneura fumiferana*. *Arch. Insect Biochem. Physiol.* 55, 68–78.
- Hu, X., Cherbas, L., Cherbas, P., 2003. Transcription activation by the ecdysone receptor (EcR/Usp): Identification of activation functions. *Mol. Endocrinol.* 17, 716–731.
- Huet, F., Ruiz, C., Richards, G., 1993. Puffs and PCR: the *in vivo* dynamics of early gene expression during ecdysone responses in *Drosophila*. *Development* 118, 613–627.
- Hurban, P., Thummel, C.S., 1993. Isolation and characterization of fifteen ecdysone-inducible *Drosophila* genes reveal unexpected complexities in ecdysone regulation. *Mol. Cell. Biol.* 13, 7101–7111.
- Imhof, M.O., Rusconi, S., Lezzi, M., 1993. Cloning of a *Chironomus tentans* cDNA encoding a protein (cEcRH) homologous to the *Drosophila melanogaster* ecdysteroid receptor (dEcR). *Insect Biochem. Mol. Biol.* 23, 115–124.
- Jayachandran, G., Fallon, A.M., 2000. Evidence for expression of EcR and Usp components of the 20-hydroxyecdysone receptor by a mosquito cell line. *Arch. Insect Biochem. Physiol.* 43, 87–96.
- Jayachandran, G., Fallon, A.M., 2001. Antisense expression of the 20-hydroxyecdysone receptor (EcR) in transfected mosquito cells uncovers a new EcR isoform that varies at the C-terminal end. *In Vitro Cell Devel. Biol. Anim.* 37, 522–529.
- Jindra, M., Sehnal, F., Riddiford, L.M., 1994a. Isolation, characterization, and developmental expression of the ecdysteroid-induced E75 gene of the wax moth *Galleria mellonella*. *Eur. J. Biochem.* 221, 665–675.
- Jindra, M., Sehnal, F., Riddiford, L.M., 1994b. Isolation and developmental expression of the ecdysteroid-induced GHR3 gene of the wax moth *Galleria mellonella*. *Insect Biochem. Mol. Biol.* 24, 763–773.
- Jindra, M., Sehnal, F., Riddiford, L.M., 1995. Ecdysteroid-induced expression of the GmE75 and GHR3 orphan receptor genes in *Galleria mellonella* (Lepidoptera: Pyralidae) larvae and cultured silk glands. *Eur. J. Entomol.* 92, 235–236.
- Jindra, M., Huang, J.-Y., Malone, F., Asahina, M., Riddiford, L.M., 1997. Identification and developmental profiles of two ultraspiracle isoforms in the epidermis and wings of *Manduca sexta*. *Insect Mol. Biol.* 6, 41–53.
- Jindra, M., Malone, F., Hiruma, K., Riddiford, L.M., 1996. Developmental profiles and ecdysteroid regulation of the mRNAs for two ecdysone receptor isoforms in the epidermis and wings of the tobacco hornworm, *Manduca sexta*. *Devel. Biol.* 180, 258–272.
- Jones, G., Sharp, P.A., 1997. Ultraspiracle: An invertebrate nuclear receptor for juvenile hormones. *Proc. Natl Acad. Sci. USA* 94, 13499–13503.
- Jones, G., Wozniak, M., Chu, Y., Dhar, S., Jones, D., 2001. Juvenile hormone III-dependent conformational changes of the nuclear receptor ultraspiracle. *Insect Biochem. Mol. Biol.* 32, 33–49.
- Kamimura, M., Tomita, S., Kiuchi, M., Fujiwara, H., 1997. Tissue-specific and stage-specific expression of two silkworm ecdysone receptor isoforms – ecdysteroid-dependent transcription in cultured anterior silk glands. *Eur. J. Biochem.* 248, 786–793.
- Kapitskaya, M.Z., Li, C., Miura, K., Segraves, W., Raikhel, A.S., 2000. Expression of the early-late gene encoding the nuclear receptor HR3 suggests its involvement in regulating the vitellogenic response to ecdysone in the adult mosquito. *Mol. Cell. Endocrinol.* 160, 25–37.
- Kapitskaya, M., Wang, S., Cress, D.E., Dhadialla, T.S., Raikhel, A.S., 1996. The mosquito ultraspiracle homologue, a partner of ecdysteroid receptor heterodimer: cloning and characterization of isoforms expressed during vitellogenesis. *Mol. Cell Endocrinol.* 12, 119–132.
- Karim, F.D., Thummel, C.S., 1991. Ecdysone coordinates the timing and amounts of E74B and E74A transcription in *Drosophila*. *Genes Devel.* 5, 1067–1079.
- Kethidi, D.R., Perera, S.C., Zheng, S.C., Feng, Q.L., Krell, P.J., et al., 2004. Identification and characterization of a JH response region in the juvenile hormone esterase gene from the spruce budworm, *Choristoneura fumiferana*. *J. Biol. Chem.* 279, 19634–19642.
- Kiss, I., Beaton, A.H., Tardiff, J., Fristrom, D., Fristrom, J.W., 1988. Interactions and developmental effects of mutations in the Broad-Complex of *Drosophila melanogaster*. *Genetics* 118, 247–259.

- Kitareewan, S., Burka, L.T., Tomer, K.B., Parker, C.E., Deterding, L.J., *et al.*, 1996. Phytol metabolites are circulating dietary factors that activate the nuclear receptor RXR. *Mol. Biol. Cell* 7, 1153–1166.
- Koch, P.B., Merk, R., Reinhardt, R., Weber, P., 2003. Localization of ecdysone receptor protein during colour pattern formation in wings of the butterfly *Precis coenia* (Lepidoptera: Nymphalidae) and co-expression with Distal-less protein. *Devel. Genes Evol.* 212, 571–584.
- Koelle, M.R., Segraves, W.A., Hogness, D.S., 1992. DHR3: A *Drosophila* steroid receptor homolog. *Proc. Natl Acad. Sci. USA* 89, 6167–6171.
- Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P., *et al.*, 1991. The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* 4, 59–77.
- Koo, J.C., Asurmendi, S., Bick, J., Woodford-Thomas, T., Beachy, R.N., 2004. Ecdysone agonist-inducible expression of a coat protein gene from tobacco mosaic virus confers viral resistance in transgenic *Arapidopsis*. *Plant J.* 37, 439–448.
- Kostrouchova, M., Karause, M., Kostrouch, Z., Rall, J.E., 1998. CHR3: a *Caenorhabditis elegans* orphan nuclear hormone receptor required for proper epidermal development and molting. *Development* 125, 1617–1626.
- Kothopalli, R., Palli, S.R., Ladd, T.R., Sohi, S.S., Cress, D., *et al.*, 1995. Cloning and developmental expression of the ecdysone receptor gene from the spruce budworm, *Choristoneura fumiferana*. *Devel. Genet.* 17, 319–330.
- Kozlova, T., Pokholkova, G.V., Tzertzinis, G., Sutherland, J.D., *et al.*, 1998. *Drosophila* hormone receptor 38 functions in metamorphosis: a role in adult cuticle formation. *Genetics* 149, 1465–1475.
- Kozlova, T., Thummel, C.S., 2002. Spatial patterns of ecdysteroid receptor activation during the onset of *Drosophila* metamorphosis. *Development* 129, 1739–1750.
- Kozlova, T., Thummel, C.S., 2003a. Essential roles for ecdysone signaling during *Drosophila* mid-embryonic development. *Science* 301, 1911–1914.
- Kozlova, T., Thummel, C.S., 2003b. Methods to characterize *Drosophila* nuclear receptor activation and function *in vivo*. *Methods Enzymol.* 364, 475–490.
- Kumar, M.B., Fujimoto, T., Potter, D.W., Deng, Q., Palli, S.R., 2002. A single point mutation in ecdysone receptor leads to increased specificity: implications for gene switch applications. *Proc. Natl Acad. Sci. USA* 99, 14710–14715.
- Lafont, R., Connat, J-L., 1989. Pathways of ecdysone metabolism. In: Koolman, J. (Ed.), *Ecdysone*. Thieme, Stuttgart, pp. 167–173.
- Lam, G., Hall, B.L., Bender, M., Thummel, C.S., 1999. DHR3 is required for the prepupal–pupal transition and differentiation of adult structures during *Drosophila* metamorphosis. *Devel. Biol.* 212, 204–216.
- Lan, Q., Hiruma, K., Hu, X., Jindra, M., Riddiford, L.M., 1999. Activation of a delayed early gene encoding MHR3 by the ecdysone receptor heterodimer EcR-B1-Usp-1 but not by EcR-B1-Usp-2. *Mol. Cell. Biol.* 19, 4897–4906.
- Lan, Q., Wu, Z-N., Riddiford, L.M., 1997. Regulation of the ecdysone receptor, Usp, E75, and MHR3 genes by 20-hydroxyecdysone in the GV1 cell line of the tobacco hornworm, *Manduca sexta*. *Insect Mol. Biol.* 6, 3–10.
- Lavorgna, G., Ueda, H., Clos, J., Wu, C., 1991. FTZ-F1, a steroid hormone receptor-like protein implicated in the activation of fushi tarazu. *Science* 252, 848–851.
- Lee, C.Y., Simon, C.R., Woodard, C.T., Baehrecke, E.H., 2002. Genetic mechanism for the stage and tissue-specific regulation of steroid triggered programmed cell death in *Drosophila*. *Devel. Biol.* 252, 138–148.
- Lee, T., Marticke, S., Sung, C., Robinow, S., Luo, L., 2000. Cell-autonomous requirement of the Usp/EcR-B ecdysone receptor for mushroom body neuronal remodeling in *Drosophila*. *Neuron* 28, 807–818.
- Lehman, M., 1995. *Drosophila* Sgs genes: stage and tissue specificity of hormone responsiveness. *BioEssays* 18, 47–54.
- Lehmann, M., Korge, G., 1995. Ecdysone regulation of the *Drosophila* Sgs-4 gene is mediated by the synergistic action of ecdysone receptor and SEBP 3. *EMBO J.* 14, 716–726.
- Lezzi, M., Bergman, T., Henrich, V.C., Vogtli, M., Fromel, C., *et al.*, 2002. Ligand-induced heterodimerization between the ligand binding domains of the *Drosophila* ecdysteroid receptor and ultraspiracle. *Eur. J. Biochem.* 269, 3237–3245.
- Li, F-Q., Ueda, H., Hirose, S., 1994. Mediators of activation of fushi tarazu gene transcription by BmFTZ-F1. *Mol. Cell. Biol.* 14, 3013–3021.
- Li, T-R., Bender, M., 2000. A conditional rescue system reveals essential functions for the ecdysone receptor (EcR) gene during molting and metamorphosis in *Drosophila*. *Development* 127, 2897–2905.
- Liang, C., Spitzer, J.D., Smith, H.S., Gerbi, S.A., 1993. Replication initiates at a confined region during DNA amplification in *Sciara* DNA puff II/9A. *Genes Devel.* 7, 1072–1084.
- Luo, Y., Amin, J., Voellmy, R., 1991. Ecdysterone receptor is a sequence-specific transcription factor involved in the developmental regulation of heat shock genes. *Mol. Cell. Biol.* 11, 3660–3675.
- Mangelsdorf, D.J., Ong, E.S., Dyck, J.A., Evans, R.M., 1990. Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature* 345, 224–229.
- Martinez, A., Scanlon, D., Gross, B., Perera, S.C., Palli, S.R., *et al.*, 1999a. Transcriptional activation of the cloned *Heliothis virescens* (Lepidoptera) ecdysone receptor (HvEcR) by muristerone A. *Insect Biochem. Mol. Biol.* 29, 915–930.
- Martinez, A., Sparks, C., Drayton, P., Thompson, J., Greenland, A., *et al.*, 1999b. Creation of ecdysone receptor chimeras in plants for controlled regulation of gene expression. *Mol. Gen. Genet.* 261, 546–552.
- Maschat, F., Dubertret, M.L., Lepesant, J.A., 1991. Transformation mapping of the regulatory elements of the

- ecdysone-inducible P1 gene of *Drosophila melanogaster*. *Mol. Cell. Biol.* 11, 2913–2917.
- Matusuoka, T., Fujiwara, H., 2000. Expression of ecdysteroid-regulated genes is reduced specifically in the wing discs of the wing-deficient mutant (fl) of *Bombyx mori*. *Devel. Genes Evol.* 210, 120–128.
- Mikitani, K., 1996. A new nonsteroidal chemical class of ligand for the ecdysteroid receptor 3, 5-di-*tert*-butyl-4-hydroxy-*N*-isobutyl-benzamid shows apparent insect molting hormone activities at molecular and cellular levels. *Biochem. Biophys. Res. Comm.* 227, 427–432.
- Minakuchi, C., Nakagawa, Y., Kiuchi, M., Tomita, S., Kamimura, M., 2002. Molecular cloning, expression analysis and functional confirmation of two ecdysone receptor isoforms from the rice stem borer, *Chilo suppressalis*. *Insect Biochem. Mol. Biol.* 33, 561–562.
- Miura, K., Zhu, J., Dittmer, N.T., Chen, L., Raikhel, A.S., 2002. A COUP-TF/Svp homolog is highly expressed during vitellogenesis in the mosquito *Aedes aegypti*. *J. Mol. Endocrinol.* 29, 223–238.
- Mlodzik, M., Hiromi, Y., Weber, U., Goodman, C.S., Rubin, G.M., 1990. The *Drosophila* seven-up gene, a member of the steroid receptor gene superfamily, controls photoreceptor cell fates. *Cell* 60, 211–224.
- Mouillet, J.F., Bousquet, F., Sedano, N., Alabouvette, J., Nicolai, M., *et al.*, 1999. Cloning and characterization of new orphan nuclear receptors and their developmental profiles during *Tenebrio* metamorphosis. *Eur. J. Biochem.* 265, 972–981.
- Mouillet, J.F., Delbecque, J.P., Quenedey, B., Delachambre, J., 1997. Cloning of two putative ecdysteroid receptor isoforms from *Tenebrio molitor* and their developmental expression in the epidermis during metamorphosis. *Eur. J. Biochem.* 248, 856–863.
- Mouillet, J.F., Henrich, V.C., Lezzi, M., Vogtli, M., 2001. Differential control of gene activity by isoforms A, B1, and B2 of the *Drosophila* ecdysone receptor. *Eur. J. Biochem.* 268, 1811–1819.
- Mugat, B., Brodu, V., Kejzloarova-Lepesant, J., Antoniewski, C., Bayer, C.A., *et al.*, 2000. Dynamic expression of broad-complex isoforms mediates temporal control of an ecdysteroid target gene at the onset of *Drosophila* metamorphosis. *Devel. Biol.* 227, 104–117.
- Murata, T., Kageyama, Y., Hirome, S., Ueda, H., 1996. Regulation of the EDG84A gene by FTZ-F1 during metamorphosis in *Drosophila melanogaster*. *Mol. Cell. Biol.* 16, 6509–6515.
- Myohara, M., Okada, M., 1987. Induction of ecdysone-stimulated chromosomal puffs in permeabilized *Drosophila* salivary glands: A new method for assaying the gene-regulating activity of cytoplasm. *Devel. Biol.* 122, 396–406.
- Nakagawa, Y., Minakuchi, C., Takahashi, K., Ueno, T., 2002. Inhibition of [³H] ponasterone A binding by ecdysone agonists in the intact Kc cell line. *Insect Biochem. Mol. Biol.* 32, 175–180.
- Nauber, U., Pankratz, M.J., Kienlin, A., Seifert, E., Klemm, U., *et al.*, 1988. Abdominal segmentation of the *Drosophila* embryo requires a hormone receptor-like protein encoded by the gap gene knirps. *Nature* 336, 489–492.
- Neal, S.J., Gibson, M.L., Westwood, J.T., 2003. Construction of a cDNA-based microarray for *Drosophila melanogaster*: a comparison of gene transcription profiles from SL2 and Kc167 cells. *Genome* 46, 879–892.
- Niedziela-Majka, A., Kochman, M., Ozyhar, A., 2000. Polarity of the ecdysone receptor complex interaction with the palindromic response element from the hsp27 gene promoter. *Eur. J. Biochem.* 267, 507–519.
- Niedziela-Majka, A., Rymarczyk, G., Kochman, M., Ozyhar, A., 1998. Pure, bacterially expressed DNA-binding domains of the functional ecdysteroid receptor capable of interacting synergistically with the hsp27 20-hydroxyecdysone response element. *Protein Exp. Purif.* 14, 208–220.
- No, D., Yao, T-P., Evans, R.M., 1996. Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proc. Natl Acad. Sci. USA* 93, 3346–3351.
- Oberdorster, E., Clay, M.A., Cottam, D.M., Wilmot, F.A., McLachlan, J.A., *et al.*, 2001. Common phytochemicals are ecdysteroid agonists and antagonists: a possible evolutionary link between vertebrate and invertebrate steroid hormones. *J. Steroid Biochem. Mol. Biol.* 77, 229–238.
- Oberdorster, E., Cottam, D.M., Wilmot, F.A., Milner, M.J., McLachlan, J.A., 1999. Interaction of PAHs and PCBs with ecdysone-dependent gene expression and cell proliferation. *Toxicol. Appl. Pharmacol.* 160, 101–108.
- O'Connor, J.D., 1985. Ecdysteroid action at the molecular level. In: Kerkur, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry, and Pharmacology*, Vol. 8, Endocrinology II. Pergamon, Oxford, pp. 85–98.
- Oro, A.E., McKeown, M., Evans, R.M., 1990. Relationship between the product of the *Drosophila ultraspiracle* locus and the vertebrate retinoid X receptor. *Nature* 347, 298–301.
- Oro, A.E., McKeown, M., Evans, R.M., 1992. The *Drosophila* retinoid X receptor homolog ultraspiracle functions in both female reproduction and eye morphogenesis. *Development* 115, 449–462.
- Oro, A.E., Ong, E.S., Margolis, J.S., Posakony, J.W., McKeown, M., *et al.*, 1988. The *Drosophila* gene knirps-related is a member of the steroid-receptor gene superfamily. *Nature* 336, 493–496.
- Ozyhar, A., Gries, M., Kiltz, H-H., Pongs, O., 1992. Magnetic DNA affinity purification of ecdysteroid receptor. *J. Steroid Biochem. Mol. Biol.* 43, 629–634.
- Ozyhar, A., Pongs, O., 1993. Mutational analysis of the interaction between ecdysteroid receptor and its response element. *J. Steroid Biochem. Mol. Biol.* 46, 135–145.
- Padidam, M., Gore, M., Lu, D.L., Smirnova, O., 2003. Chemical inducible, ecdysone receptor based gene expression system for plants. *Transgenic Res.* 12, 101–109.

- Palli, S.R., Hiruma, K., Riddiford, L.M., 1992. An ecdysteroid-inducible *Manduca* gene similar to the *Drosophila* DHR3 gene, a member of the steroid hormone receptor superfamily. *Devel. Biol.* 150, 305–318.
- Palli, S.R., Kaptiskaya, M.Z., Kumar, M.B., Cress, D.E., 2003. Improved ecdysone receptor based inducible gene regulation system. *Eur. J. Biochem.* 270, 1308–1315.
- Palli, S.R., Ladd, T.R., Retnakaran, A., 1997. Cloning and characterization of a new isoform of *Choristoneura* hormone receptor 3 from the spruce budworm. *Arch. Insect Biochem. Physiol.* 35, 33–44.
- Palli, S.R., Sohi, S.S., Cook, B.J., Lambert, D., Ladd, T.R., et al., 1995. Analysis of ecdysteroid action in *Malacosoma disstria* cells: cloning selected regions of E75 and MHR3-like genes. *Insect Biochem. Mol. Biol.* 25, 697–707.
- Palmer, M.J., Warren, J.T., Jin, X., Guo, X., Gilbert, L.I., 2002. Developmental profiles of ecdysteroids, ecdysteroid receptor mRNAs and DNA-binding properties of ecdysteroid receptors in the Ixodid tick *Amblyomma americanum* (L.). *Insect Biochem. Mol. Biol.* 32, 465–476.
- Perera, S.C., Ladd, T.R., Dhadialla, T.S., Krell, P.J., Sohi, S.S., et al., 1999a. Studies on two ecdysone receptor isoforms of the spruce budworm, *Choristoneura fumiferana*. *Mol. Cell. Endocrinol.* 152, 73–84.
- Perera, S.C., Palli, S.R., Ladd, T.R., Krell, P.J., Retnakaran, A., 1998. The ultraspiracle gene of the spruce budworm, *Choristoneura fumiferana*: cloning of cDNA and developmental expression of mRNA. *Devel. Genet.* 22, 169–179.
- Perera, S.C., Sundaram, M., Krell, P.J., Retnakaran, A., Dhadialla, T.S., et al., 1999b. An analysis of ecdysone receptor domains required for heterodimerization with Ultraspiracle. *Arch. Insect Biochem. Physiol.* 41, 61–70.
- Perlmann, T., Umesono, K., Rangarajan, P.N., Froman, B.M., Evans, R.M., 1996. Two distinct dimerization interfaces differentially modulate target gene specificity of nuclear hormone receptors. *Mol. Endocrinol.* 10, 958–966.
- Perrimon, N., Engstrom, L., Mahowald, A.P., 1985. Developmental genetics of the 2C-D region of the *Drosophila* X chromosome. *Genetics* 126, 4581–4589.
- Przibilla, S., Hitchcock, W.W., Szecsi, M., Grebe, M., Beatty, J., et al., 2004. Functional studies on the ligand-binding domain of Ultraspiracle from *Drosophila melanogaster*. *Biol. Chem.* 385, 21–30.
- Rauch, P., Grebe, M., Elke, C., Spindler, K-D., Spindler-Barth, M., 1998. Ecdysteroid receptor and ultraspiracle from *Chironomus tentans* (Insecta) are phosphoproteins and are regulated differently by molting hormone. *Insect Biochem. Mol. Biol.* 28, 265–275.
- Rebers, J., 1999. Overlapping antiparallel transcripts induced by ecdysone in a *Drosophila* cell line. *Insect Biochem. Mol. Biol.* 29, 293–302.
- Ren, B., Robert, F., Wyrick, J.J., Aparicio, O., Jennings, E.G., et al., 2000. Genome-wide location and function of DNA binding proteins. *Science* 290, 2306–2309.
- Retnakaran, A., Gelbic, L., Sundaram, M., Tomkins, W., Ladd, T., et al., 2001. Mode of action of the ecdysone agonist tebufenozide (RH-5992), and an exclusion mechanism to explain resistance to it. *Pest. Manag. Sci.* 57, 951–957.
- Restifo, L.L., Wilson, T.G., 1998. A juvenile hormone agonist reveals distinct developmental pathways mediated by ecdysone-inducible Broad Complex transcription factors. *Devel. Genet.* 22, 141–159.
- Richards, G.P., 1976. The control of prepupal puffing patterns *in vitro*; implications for prepupal ecdysone titres in *Drosophila melanogaster*. *Devel. Biol.* 48, 91–95.
- Richards, G., 1982. Sequential gene activation by ecdysteroids in polytene chromosomes of *Drosophila melanogaster* VII. Tissue specific puffing. *Wilhelm Roux's Arch.* 191, 103–111.
- Riddiford, L., 1985. Hormone action at the cellular level. In: Kerkur, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry, and Pharmacology*, Vol. 8, Endocrinology II. Pergamon, Oxford, pp. 37–84.
- Riddiford, L.M., Cherbas, P., Truman, J.W., 2000. Ecdysone receptors and their biological actions. *Vitam. Horm.* 60, 1–73.
- Riddiford, L.M., Hiruma, K., Zhou, X., Nelson, C.A., 2003. Insights into the molecular basis of the hormonal control of molting and metamorphosis from *Manduca sexta* and *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* 33, 1327–1338.
- Riddihough, G., Pelham, H.R.B., 1987. An ecdysone response element in the *Drosophila* hsp27 promoter. *EMBO J.* 6, 3729–3734.
- Robinow, S., Talbot, W.S., Hogness, D.S., Truman, J.W., 1993. Programmed cell death in the *Drosophila* CNS is ecdysone-regulated and coupled with a specific ecdysone receptor isoform. *Development* 119, 1251–1259.
- Rymarczyk, G., Grad, I., Rusek, A., Oswiecimska-Rusin, K., Niedziela-Majli, A., et al., 2003. Purification of *Drosophila melanogaster* ultraspiracle protein and analysis of its a/B region-dependent dimerization *in vitro*. *Biol. Chem.* 384, 59–69.
- Saez, E., Nelson, M.C., Eshelman, B., Banayo Koder, E.A., Cho, G.J., et al., 2000. Identification of ligands and coligands for the ecdysone-regulated gene switch. *Proc. Natl Acad. Sci. USA* 97, 14512–14517.
- Saleh, D.S., Zhang, J., Wyatt, G.R., Walker, V.K., 1998. Cloning and characterization of an ecdysone receptor cDNA from *Locusta migratoria*. *Mol. Cell. Endocrinol.* 143, 91–99.
- Sasorith, S., Billas, I., Iwema, T., Moras, D., Wurtz, J-M., 2002. Structure-based analysis of the ultraspiracle protein and docking studies of putative ligands. *J. Insect Sci.* 2, 25–36.

- Savakis, C., Demetri, G., Cherbas, P., 1980. Ecdysteroid-inducible polypeptides in a *Drosophila* cell line. *Cell* 22, 665–674.
- Schmidt, A., Endo, N., Rutledge, S.J., Vogel, R., Shinar, D., et al., 1992. Identification of a new member of the steroid hormone receptor superfamily that is activated by a peroxisome proliferator and fatty acids. *Mol. Endocrinol.* 6, 1634–1641.
- Schubiger, M., Tomita, S., Sung, C., Robinow, S., Truman, J.W., 2003. Isoform specific control of gene activity *in vivo* by the *Drosophila* ecdysone receptor. *Mech. Devel.* 120, 909–918.
- Schubiger, M., Truman, J.W., 2000. The RXR ortholog Usp suppresses early metamorphic processes in *Drosophila* in the absence of ecdysteroids. *Development* 127, 1151–1159.
- Schubiger, M., Wade, A.A., Carney, G.E., Truman, J.W., Bender, M., 1998. *Drosophila* EcR-B ecdysone receptor isoforms are required for larval molting and for neuron remodeling during metamorphosis. *Development* 125, 2053–2062.
- Schulz, R.A., Cherbas, L., Cherbas, P., 1986. Alternative splicing generates two distinct Eip28/29 gene transcripts in *Drosophila* Kc cells. *Proc. Natl Acad. Sci. USA* 83, 9428–9432.
- Sedkov, Y., Cho, E., Petruk, S., Cherbas, L., Smith, S.T., et al., 2003. Methylation at lysine 4 of histone H3 in ecdysone-dependent development of *Drosophila*. *Nature* 426, 78–83.
- Segal, D., Cherbas, L., Cherbas, P., 1996. Genetic transformation of *Drosophila* cells in culture by P-element mediated transposition. *Somat. Cell Mol. Genet.* 22, 159–165.
- Segraves, W.A., Hogness, D.S., 1990. The E75 ecdysone-inducible gene responsible for the 75B early puff in *Drosophila* encodes two new members of the steroid receptor superfamily. *Genes Devel.* 4, 204–219.
- Segraves, W.A., Woldin, C., 1993. The E75 gene of *Manduca sexta* and comparison with its *Drosophila* homolog. *Insect Biochem. Mol. Biol.* 23, 91–97.
- Sempere, L.F., Sokol, N.S., Dubrovsky, E.B., Berger, E.M., Ambros, V., 2003. Temporal regulation of microRNA expression in *Drosophila melanogaster* mediated by hormonal signals and broad-Complex gene activity. *Devel. Biol.* 259, 9–18.
- Shea, C., Hough, D., Xiao, J., Tzertzinis, G., Maina, C.V., 2004. An rxr/usp homolog from the parasitic nematode, *Dirofilaria immitis*. *Gene* 324, 171–182.
- Shea, M.J., King, D.L., Conboy, M.J., Mariani, B.D., Kafatos, F.C., 1990. Proteins that bind to *Drosophila* chorion cis-regulatory elements: A new C2H2 zinc finger protein and a C2C2 steroid receptor-like component. *Genes Devel.* 4, 1128–1140.
- Shemshidini, L., Wilson, T.G., 1990. Resistance to juvenile hormone and an insect growth regulator in *Drosophila* is associated with an altered cytosolic juvenile hormone-binding protein. *Proc. Natl Acad. Sci. USA* 87, 2072–2086.
- Simon, A.F., Shih, C., Mack, A., Benzer, S., 2003. Steroid control of longevity in *Drosophila melanogaster*. *Science* 299, 1407–1410.
- Sobrier, M.L., Chapel, S., Couderc, J.L., Micard, D., Lecher, P., et al., 1989. 20-OH-ecdysone regulates 60C beta tubulin gene expression in Kc cells and during *Drosophila* development. *Exp. Cell Res.* 184, 241–249.
- Somme-Martin, G., Colardeau, J., Beydon, P., Blais, C., Lepesant, J.A., et al., 1990. P1 gene expression in *Drosophila* larval fat body: induction by various ecdysteroids. *Arch. Insect Biochem. Physiol.* 15, 43–56.
- Somme-Martin, G., Colardeau, J., Lafont, R., 1988. Conversion of ecdysone and 20-hydroxyecdysone into 3-dehydroecdysteroids is a major pathway in third instar *Drosophila melanogaster* larvae. *Insect Biochem.* 395, 398–402.
- Song, Q., Alnemri, E.S., Litwack, G., Gilbert, L.I., 1997. An immunophilin is a component of the insect ecdysone receptor (EcR) complex. *Insect Biochem. Mol. Biol.* 27, 973–982.
- Song, Q., Gilbert, L.I., 1998. Alterations in ultraspiracle (Usp) content and phosphorylation state accompany feedback regulation of ecdysone synthesis in the insect prothoracic gland. *Insect Biochem. Mol. Biol.* 28, 849–860.
- Song, Q., Sun, X., Jin, X.Y., 2003. 20E regulated Usp expression and phosphorylation in *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* 1211–1218.
- Spindler-Barth, M., Spindler, K-D., 1998. Ecdysteroid resistant subclones of the epithelial cell line from *Chironomus tentans* (Insecta, Diptera). I. Selection and characterization of resistant clones. *In Vitro Cell Devel. Biol. Anim.* 36, 116–122.
- Stallcup, M.R., 2001. Role of protein methylation in chromatin remodeling and transcriptional regulation. *Oncogene* 20, 3014–3020.
- Stathopoulos, A., Levine, M., 2002. Whole-genome expression profiles identify gene batteries in *Drosophila*. *Devel. Cell* 3, 464–465.
- Staudinger, J., Goodwin, B., Jones, S.A., Hawkins-Brown, D., MacKenzie, K.I., et al., 2001. The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc. Natl Acad. Sci. USA* 98, 3369–3374.
- Stocker, A.J., Pavan, C., 1977. Developmental puffing patterns in salivary gland chromosomes of *Rhynchosciara hollanderi*. *Chromosoma* 62, 17–47.
- Stone, B.L., Thummel, C.S., 1993. The *Drosophila* 78C early late puff contains E78, an ecdysone-inducible gene that encodes a novel member of the nuclear hormone receptor superfamily. *Cell* 75, 307–320.
- Strangmann-Diekmann, M., Klone, A., Ozyhar, A., Kreklau, F., Kiltz, H-H., et al., 1990. Affinity labeling of a partially purified ecdysteroid receptor with a bromoacetylated 20-OH ecdysone derivative. *Eur. J. Biochem.* 189, 137–145.
- Sung, C., Robinow, S., 2000. Characterization of the regulatory elements controlling neuronal expression of the

- A-isoform of the ecdysone receptor gene of *Drosophila melanogaster*. *Mech. Devel.* 91, 237–248.
- Suhr, S.T., Gil, E.B., Senut, M.-C., Gage, F.H., 1998. High level transactivation by a modified *Bombyx* ecdysone receptor in mammalian cells without exogenous retinoid X receptor. *Proc. Natl Acad. Sci. USA* 95, 7999–8004.
- Sullivan, A.A., Thummel, C.S., 2003. Temporal profiles of nuclear receptor gene expression reveal coordinate transcriptional responses during *Drosophila* development. *Mol. Endocrinol.* 17, 2125–2137.
- Sutherland, J.D., Kozlova, T., Tzertzinis, G., Kafatos, F.C., 1995. *Drosophila* hormone receptor 38: a second partner for *Drosophila* Usp suggests an unexpected role for nuclear receptors of the nerve growth factor-induced protein B type. *Proc. Natl Acad. Sci. USA* 92, 7966–7970.
- Swevers, L., Cherbas, L., Cherbas, P., Iatrou, K., 1996. *Bombyx* EcR (BmEcR) and *Bombyx* Usp (BmCF1) combine to form a functional ecdysone receptor. *Insect Biochem. Mol. Biol.* 26, 217–221.
- Swevers, L., Drevet, J.R., Lunke, M.D., Iatrou, K., 1995. The silkworm homolog of the *Drosophila* ecdysone receptor (B1 isoform): cloning and analysis of expression during follicular cell differentiation. *Insect Biochem. Mol. Biol.* 25, 857–866.
- Swevers, L., Iatrou, K., 2003. The ecdysone regulatory cascade and ovarian development in lepidopteran insects: insights from the silkworm paradigm. *Insect Biochem. Mol. Biol.* 33, 1285–1297.
- Talbot, W.S., Swyryd, E.A., Hogness, D.S., 1993. *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. *Cell* 73, 1323–1337.
- Thomas, H.E., Stuttenberg, H.G., Stewart, A.F., 1993. Heterodimerization of the *Drosophila* ecdysone receptor with retinoid X receptors and ultraspiracle. *Nature* 362, 471–475.
- Thummel, C.S., 2002. Ecdysone-regulated puff genes 2000. *Insect Biochem. Mol. Biol.* 32, 113–120.
- Toya, T., Fukasawa, H., Masui, A., Endo, Y., 2002. Potent and selective partial ecdysone agonist activity of chromafenozide in Sf9 cells. *Biochem. Biophys. Res. Commun.* 292, 1087–1091.
- Tran, H.T., Askari, H.B., Shaaban, S., Price, L., Palli, S.R., et al., 2001b. Reconstruction of ligand-dependent transactivation of *Choristoneura fumiferana* ecdysone receptor in yeast. *Mol. Endocrinol.* 15, 1140–1153.
- Tran, H.T., Shaaban, S., Askari, H.B., Walfish, P.G., Raikhel, A.S., et al., 2001a. Requirement of co-factors for the ligand-mediated activity of the insect ecdysteroid receptor in yeast. *J. Mol. Endocrinol.* 27, 191–209.
- Trisyono, A., Goodman, C.L., Grasela, J.J., McIntosh, A.H., Chippendale, G.M., 2000. Establishment and characterization of an *Ostrinia nubilalis* cell line and its response to ecdysone agonists. *In Vitro Cell. Devel. Biol.* 36, 300–404.
- Truman, J.T., Talbot, W.S., Fahrbach, S.E., Hogness, D.S., 1994. Ecdysone receptor expression in the CNS correlates with stage-specific responses to ecdysteroids during *Drosophila* and *Manduca* development. *Development* 120, 219–234.
- Tsai, C.C., Kao, H.Y., Yao, T.P., McKeown, M., Evans, R.M., 1999. SMRTER, a *Drosophila* nuclear receptor coregulator, reveals that EcR mediated repression is critical for development. *Mol. Cell.* 4, 175–186.
- Tzertzinis, G., Malecki, A., Kafatos, F.C., 1994. BmCF1, a *Bombyx mori* RXR-type receptor related to the *Drosophila* ultraspiracle. *J. Mol. Biol.* 238, 479–486.
- Uhlirova, M., Foy, B.D., Beaty, B.J., Olson, K.E., Riddiford, L.M., et al., 2003. Use of Sindbis virus-mediated RNA interference to demonstrate a conserved role of Broad-Complex in insect metamorphosis. *Proc. Natl Acad. Sci. USA* 23, 15607–15612.
- Unger, E., Cigan, A.M., Trimmell, M., Xu, R.J., Kendall, T., et al., 2002. A chimeric ecdysone receptor facilitates methoxfenozide-dependent restoration of male fertility in ms45 maize. *Transgenic Res.* 11, 455–465.
- Verras, M., Gourzi, P., Zacharapoulou, A., Mintzas, A.C., 2002. Developmental profiles and ecdysone regulation of the mRNAs for two ecdysone receptor isoforms in the Mediterranean fruit fly *Ceratitis capitata*. *Insect Mol. Biol.* 11, 553–565.
- Vogtli, M., Elke, C., Imhof, M.O., Lezzi, M., 1998. High level transactivation by the ecdysone receptor complex at the core recognition motif. *Nucleic Acids Res.* 10, 2407–2414.
- Vogtli, M., Imhof, M.O., Brown, N.E., Rauch, P., Spindler-Barth, M., et al., 1999. Functional characterization of two Ultraspiracle forms (CtUsp-1 and CtUsp-2) from *Chironomus tentans*. *Insect Biochem. Mol. Biol.* 29, 931–942.
- vonKalm, L., Crossgrove, K., Von Seggern, D., Guild, G.M., Beckendorf, S.K., 1994. The Broad-Complex directly controls a tissue-specific response to the steroid hormone ecdysone at the onset of *Drosophila* metamorphosis. *EMBO J.* 13, 3505–3516.
- Walker, V.K., Ashburner, M., 1981. The control of ecdysterone-regulated puffs in *Drosophila* salivary glands. *Cell* 26, 269–277.
- Wang, S-F., Ayer, S., Segreaves, W.A., Williams, D.R., Raikhel, A.S., 2000. Molecular determinants of differential ligand sensitivities of insect ecdysteroid receptors. *Mol. Cell. Biol.* 20, 3870–3879.
- Wang, S.F., Li, C., Sun, G., Zhu, J., Raikhel, A.S., 2002. Differential expression and regulation by 20-hydroxyecdysone of mosquito ecdysteroid receptor isoforms A and B. *Mol. Cell. Endocrinol.* 196, 29–42.
- Wang, S-F., Miura, K., Miksicek, R.J., Segreaves, W.A., Raikhel, A.S., 1998. DNA-binding and transactivation characteristics of the mosquito ecdysone receptor-Ultraspiracle complex. *J. Biol. Chem.* 273, 27531–27540.
- Weller, J., Sun, G.C., Zhou, B., Lan, Q., Hiruma, K., et al., 2001. Isolation and developmental expression of two nuclear receptors, MHR4 and β FTZ-F1, in the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol.* 22, 827–837.
- Wegmann, I.S., Quack, S., Spindler, K-D., Dorsch-Hasler, K., Vogtli, M., et al., 1995. Immunological studies on

- the developmental and chromosomal distribution of ecdysteroid receptor proteins in *Chironomus tentans*. *Arch. Insect Biochem. Physiol.* 30, 95–114.
- White, K.P., Hurban, P., Watanabe, T., Hogness, D.S., 1997. Coordination of *Drosophila* metamorphosis by two ecdysone-induced nuclear receptors. *Science* 276, 114–117.
- White, K.P., Rifkin, S.A., Hurban, P., Hogness, D.S., 1999. Microarray analysis of *Drosophila* development during metamorphosis. *Science* 286, 2179–2184.
- Willy, P.J., Umesono, K., Ong, E.S., Evans, R.M., Heyman, R.A., et al., 1995. LXR, a nuclear receptor that defines a distinct retinoid response pathway. *Genes Devel.* 9, 1033–1045.
- Wing, K.D., 1988. RH5849, a nonsteroidal ecdysone agonist: effects on a *Drosophila* cell line. *Science* 241, 467–469.
- Woodard, C.T., Baehrecke, E.H., Thummel, C.S., 1994. A molecular mechanism for the stage specificity of the *Drosophila* prepupal genetic response to ecdysone. *Cell* 18, 607–615.
- Wurtz, J.-M., Guilot, B., Fagart, J., Moras, D., Tietjen, K., et al., 2000. A new model for 20-hydroxyecdysone and dibenzoylhydrazine binding: a homology modeling and docking approach. *Protein Sci.* 9, 1073–1084.
- Yao, T., Forman, B.M., Jiang, Z., Cherbas, L., Chen, J.D., et al., 1992. *Drosophila* ultraspiracle modulates ecdysone receptor function via heterodimer formation. *Cell* 71, 63–72.
- Yao, T.P., Forman, B.M., Jiang, Z., Cherbas, L., Chen, J.D., et al., 1993. Functional ecdysone receptor is the product of EcR and ultraspiracle genes. *Nature* 366, 476–479.
- Yund, M.A., Osterbur, D.L., 1985. Ecdysteroid receptors and binding proteins. In: Kerkur, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry, and Pharmacology*, Vol. 7, Endocrinology I. Pergamon, Oxford, pp. 473–490.
- Zelhof, A.C., Ghbeish, N., Tsai, C., Evans, R.M., McKeown, M., 1997. A role for Ultraspiracle, the *Drosophila* RXR, in morphogenetic furrow movement and photoreceptor cluster formation. *Development* 124, 2499–2505.
- Zelhof, A.C., Yao, T.P., Chen, J.D., Evans, R.M., McKeown, M., 1995a. Seven-up inhibits ultraspiracle-based signaling pathways *in vitro* and *in vivo*. *Mol. Cell. Biol.* 15, 6736–6745.
- Zelhof, A.C., Yao, T., Evans, R.M., McKeown, M., 1995b. Identification and characterization of a *Drosophila* nuclear receptor with the ability to inhibit the ecdysone response. *Proc. Natl Acad. Sci. USA* 92, 10477–10481.
- Zheng, X., Wang, J., Haerry, T.E., Wu, A.Y-H., Martin, J., et al., 2003. TGF- β signaling activates steroid hormone receptor expression during neuronal remodeling in the *Drosophila* brain. *Cell* 112, 303–315.
- Zhou, Z., Riddiford, L., 2002. Broad specifies pupal development and mediates the ‘status quo’ action of juvenile hormone on the pupal-adult transformation in *Drosophila* and *Manduca*. *Development* 129, 2259–2269.
- Zhu, J., Miura, K., Chen, L., Raikhel, A.S., 2000. AHR38, a homolog of NGF1-B, inhibits formation of the functional ecdysteroid receptor in the mosquito *Aedes aegypti*. *EMBO J.* 19, 253–262.
- Zhu, J., Miura, K., Chen, L., Raikhel, A.S., 2003a. Cyclicity of mosquito vitellogenic ecdysteroid-mediated signaling is modulated by alternative dimerization of the RXR homologue, Ultraspiracle. *Proc. Natl Acad. Sci. USA* 100, 544–549.
- Zhu, J., Chen, L., Raikhel, A.S., 2003b. Posttranscriptional control of the competence factor β FTZ-F1 by juvenile hormone in the mosquito *Aedes aegypti*. *Proc. Natl Acad. Sci. USA* 100, 13338–13343.
- Zinke, I., Schutz, C.S., Katzenberger, J.D., Bauer, M., Pankratz, M.J., 2002. Nutrient control of gene expression in *Drosophila*: microarray analysis of starvation and sugar-dependent response. *EMBO J.* 21, 6162–6173.

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8 The Juvenile Hormones

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8.1. Introduction

The juvenile hormones (JHs) represent a family of acyclic sesquiterpenoids that are, with a single known exception, unique to the class Insecta. They are the principal products of the corpora allata (CA), retrocerebral paired or unpaired endocrine glands of ectodermal origin (Wigglesworth, 1970). One or more JHs have been identified in approximately 100 insect species spanning at least 10 insect orders, from the most ancestral to the most highly derived. From an evolutionary standpoint, the JHs may have originally been involved in orchestration of reproductive processes such as control of gonadal development and vitellogenin synthesis. The role of JH in the more highly derived insect orders has expanded considerably to include regulation of metamorphosis, caste determination, behavior, diapause, and various polyphenisms (Nijhout, 1994). This chapter will present the current status of our knowledge about the JHs, their evolution, roles in embryological and larval development, biosynthesis, transport, catabolism, and molecular mode of action. The role of JH in reproduction is covered elsewhere.

8.2. Chemistry of the Juvenile Hormones

8.2.1. Discovery of the Major Juvenile Hormone Homologs

Elucidation of the chemical structures of the JHs began with Röller and colleagues, who identified the principal JH in lipid extracts of *Hyalophora cecropia* (Röller *et al.*, 1967). This first JH, methyl (2*E*,6*E* 10-*cis*)-10,11-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate, was termed JHI, and since that time, eight molecules with similar aliphatic sesquiterpene structures have been identified in insects (Figure 1). The structure was confirmed as the 2*E*,6*E*, 10 *cis* isomer (Dahm *et al.*, 1968), while the absolute configuration of JHI at its chiral centers (C10 and C11) was determined to be 10*R*,11*S* (Faulkner and Petersen, 1971; Nakanishi *et al.*, 1971; Meyer *et al.*, 1971).

Meyer *et al.* (1968) subsequently identified a minor component in the *H. cecropia* extracts that differed from JHI by a methyl, instead of an ethyl

group at C7 (Figure 1). Termed JHII, methyl (2*E*, 6*E* 10-*cis*)-10,11-epoxy-3,7,11-trimethyl-2,6-tridecadienoate, like JHI, displays an *E,E* configuration at C2, C3 and C6, C7. The absolute configuration at the C10, C11 positions for the naturally occurring JHII has not been rigorously determined (Baker, 1990); however, in binding studies, (10*R*,11*S*)-JHII bound with nearly the same affinity to the *Manduca sexta* hemolymph juvenile hormone binding protein as (10*R*,11*S*)-JHI (Park *et al.*, 1993). Thus, it is likely that the naturally occurring enantiomers of JHI and II display the same 10*R*,11*S* configuration.

The third JH homolog, JHIII, methyl 10,11 epoxyfarnesoate, was identified from medium in which CA of the tobacco hornworm, *M. sexta*, had been maintained (Judy *et al.*, 1973) (Figure 1). JHIII differs from the higher homologs in that the three branches of the carbon skeleton at C3, C7, and C11 are methyl groups; however, it displays the same 2*E*,6*E* geometry. The hormone has only one chiral carbon, C10, which, in the naturally occurring hormone, displays the 10*R* configuration. JHIII is the only, or predominant, JH in all insects except Lepidoptera (Schooley *et al.*, 1984). Interestingly, JHIII had been chemically synthesized a decade earlier, before it was recognized as a naturally occurring hormone (Bowers *et al.*, 1965). A fourth and fifth JH (Figure 1), the trihomosesquiterpenoids JH0 and its isomer, 4-methyl JHI (iso-JH0), were identified in *M. sexta* eggs (Bergot *et al.*, 1981a), but nothing is currently known of their functions.

More recently, JHIII with a second epoxide substitution at C6,C7 was isolated and identified from *in vitro* cultures of larval ring glands of *Drosophila melanogaster* (Richard *et al.*, 1989) (Figure 1). This homolog, termed JHIII bisepoxy (JHB₃), is active in a *D. melanogaster* bioassay and has been found in other higher Diptera (Borovsky *et al.*, 1994a; Moshitzky and Applebaum, 1995; Yin *et al.*, 1995).

8.2.2. Hydroxylated Juvenile Hormones

Hydroxylation reactions are frequently associated with the inactivation of bioreactive molecules but in certain cases, hydroxylation can actually increase the biological potency of a compound. This important biochemical reaction appears to be involved in

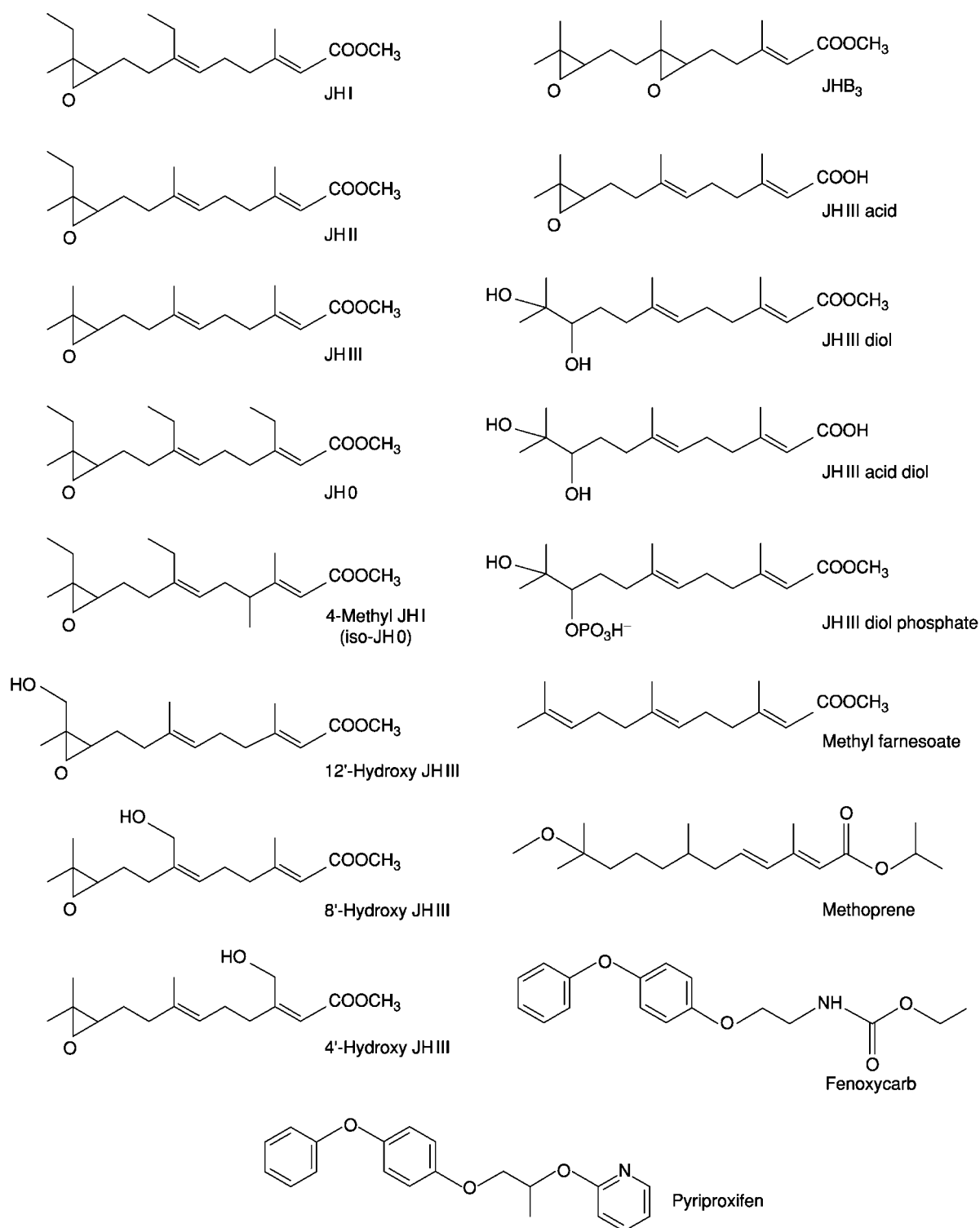


Figure 1 Chemical structures of the naturally occurring juvenile hormone (JH) homologs, hydroxylated JHs, JH metabolites, and commonly used JH analogs.

the production of a new group of JH homologs, the hydroxylated JHs (HJHs). These HJHs, 4-OH, 8-OH, and 12-OH JH III, are synthesized and released by the CA of the African locust, *Locusta migratoria* (Darrouzet *et al.*, 1997, 1998; Mauchamp *et al.*,

1999) (Figure 1). The biological activity of synthetic 12-OH JH III was bioassayed utilizing *Tenebrio molitor* and was found to be 100-fold more active than JH III (Darrouzet *et al.*, 1997). This result may reflect the cross-species nature of the bioassay,

since the assay using *L. migratoria* is not sufficiently sensitive (see Section 8.5.1).

While the biosynthetic pathway for the synthesis of the HJHs has not been determined, two pathways have been suggested: (1) hydroxylation of the JH III structure itself or (2) incorporation of hydroxylated precursors into the JH backbone via the isoprenoid biosynthetic pathway (Darrouzet *et al.*, 1998). A cytochrome P450, *CYP4C7*, identified in the cockroach *Diploptera punctata*, can catalyze the formation of 12-OH JH III and (10E)-12-OH farnesol, indicating that hydroxylation of JH III appears to be a primary reaction in the CA (Sutherland *et al.*, 1998). However, there is a sharp increase in the expression of this enzyme when JH is absent or when there is an intrinsic repression of JH synthesis, suggesting that hydroxylation of JH is a mechanism for inactivating the hormones. The pathways of synthesis and the physiological roles of the HJHs remain a fertile area for investigation.

8.3. Other Naturally Occurring Juvenile Hormones

Davey (2000a), in a thought-provoking essay, posed an intriguing question: "How many JHs are there?" Using a chemical definition, i.e., that JH is a farnesoid molecule secreted by the CA, one can count but a handful. However, if a JH is defined by its biological activity, the JHs could include HJHs, catabolic products, and nontraditional molecules, increasing the number significantly and creating the difficult problem of sorting out which molecules are actually JHs.

8.3.1. Metabolites of Juvenile Hormones as Hormones?

An open mind is needed when considering whether metabolites of JH could serve as substrate for conversion to the biologically active hormone or perhaps could act as hormones themselves. A group of molecules that has long been overlooked and frequently relegated to a mere biochemical curiosity are the JH conjugates (Roe and Venkatesh, 1990). Polar metabolites of JHI that are cleaved by glucosidases and sulfatases were identified in flies nearly three decades ago (Yu and Terriere, 1978), and more recent studies hint at the existence of very polar metabolites of JH. A major metabolite resulting from the injection or application of JH II into wandering larvae and prepupae of the cabbage looper, *Trichoplusia ni*, was found to be an unidentified, water-soluble polar product of the hormone (Kallapur *et al.*, 1996). Identification by radio

high-performance liquid chromatography (HPLC), as well as mass spectrometry, has confirmed that the molecule is a novel polar metabolite and that it is not a JH diol phosphate (M. Roe, personal communication). *In vitro* studies on the biosynthetic products of the CA from *M. sexta* and two other lepidopteran species indicate that the glands are synthesizing JH conjugates not recognized by antibodies highly specific for the JHs and their acids; nevertheless, when these compounds are hydrolyzed by esterases, JH III acid is formed (Granger *et al.*, 1995a). This product also appears to exist in the hemolymph, and the results of this study suggest the moiety is either a glucuronide of JH III or an acylglycerol with JH III as the side chain(s).

Schooley's group identified other polar metabolites in the hemolymph of *M. sexta* as phosphate conjugates of JHI and JH III diol and found that JHI diol phosphate (Figure 1) is the principal end product of JHI metabolism in *M. sexta* (Halarnkar and Schooley, 1990; Halarnkar *et al.*, 1993). More recently, Maxwell *et al.* (2002a, 2002b) identified the enzyme responsible for catalyzing the conversion of the JH diol to the JH diol phosphate, JH diol kinase (JHDK), and proposed that cellular JH epoxide hydrolase (JHEH) and JHDK are primarily responsible for the irreversible inactivation of JH early in the last stadium of *M. sexta*, when the JH titer rapidly decreases (Baker *et al.*, 1987) (see Section 8.9).

At the present time, there appear to be only two definitive pathways for the catabolism of JH: (1) hydrolysis of the methyl ester of JH by hemolymph esterases, yielding JH acid, and (2) hydration of the epoxide by JHEH, yielding JH diol (Roe and Venkatesh, 1990) (Figures 1 and 2). Can any of these catabolites act as a JH? There is certainly evidence that JH acid can be converted to JH and that it may also act as a hormone. It has been known for a number of years that JH acids are secreted by the CA of *M. sexta* beginning early in the last larval stadium (Janzen *et al.*, 1991) and that they are apparently the sole product of the CA by the wandering stage in this stadium (Sparagana *et al.*, 1984; Janzen *et al.*, 1991) (Figure 3). JH acid is also detected in the hemolymph of *M. sexta* (Baker *et al.*, 1987) and of the silkworm *Bombyx mori*, where, at certain critical stages, the titer of JH acid surpasses that of JH (Niimi and Sakurai, 1997). JH acid is found as a product of the CA in other lepidopterans including the adult male loyeyi leafworm, *Mythimna loyeyi* (Ho *et al.*, 1995a, 1995b), the adult male black cutworm, *Agrotis ipsilon* (Duportets *et al.*, 1998), and the larval tomato moth, *Lacanobia oleracea* (Audsley *et al.*, 2000).

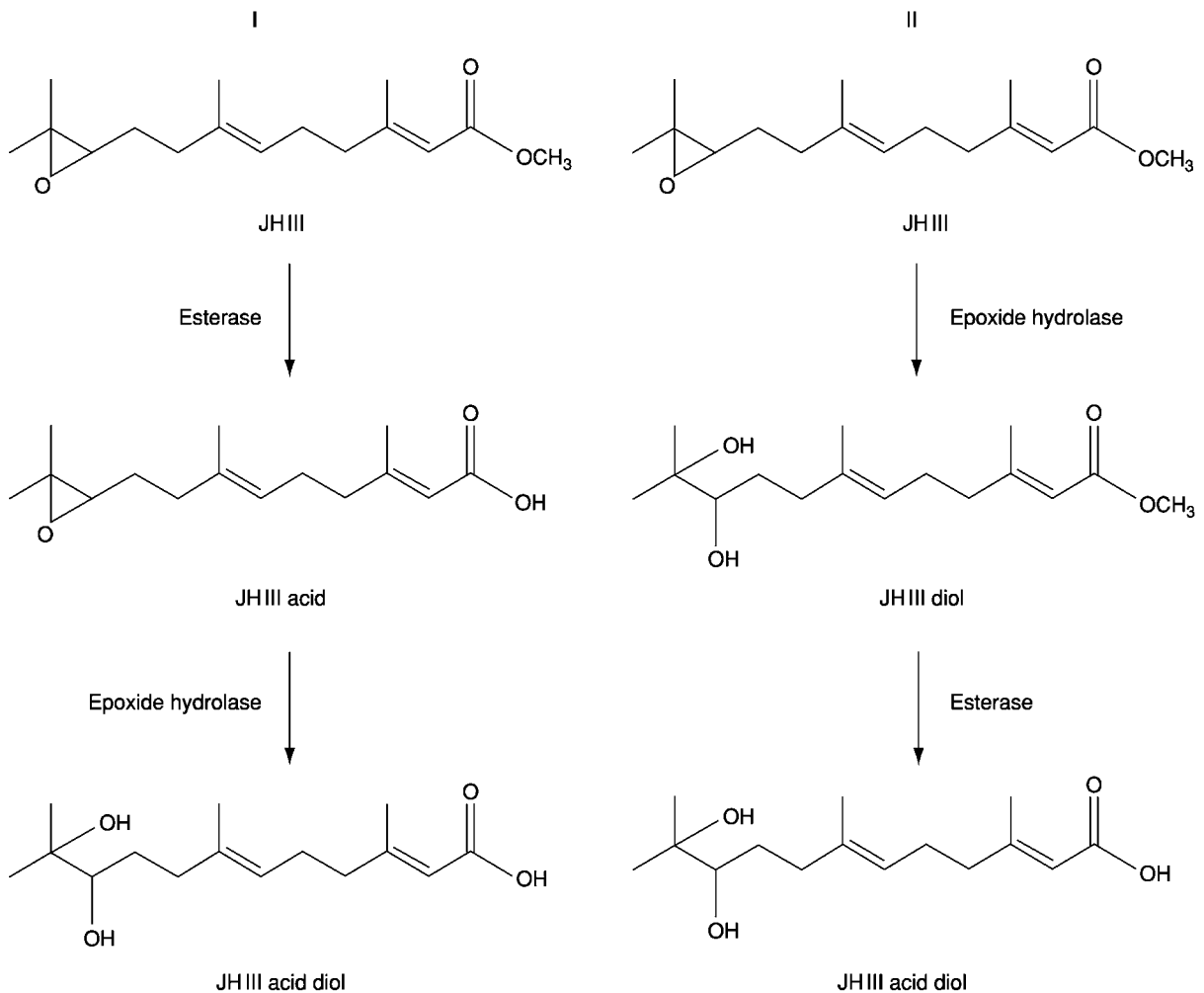


Figure 2 Primary routes of JH metabolism. Route I represents hydrolysis of the methyl ester via an esterase followed by hydrolysis of the epoxide ring via an epoxide hydrolase. Route II represents hydrolysis of the epoxide ring via an epoxide hydrolase followed by hydrolysis of the methyl ester via an esterase. The end product in both routes is JH acid diol.

Studies by Bhaskaran and his colleagues suggest that JH acid may be a hormone in its own right. The ability of *M. sexta* fat body cells to respond to the JH analog methoprene (Figure 1) with enhanced production of yolk protein and its mRNA is acquired after ecdysteroid-initiated commitment to pupation, but it also requires prior exposure to JH II acid or methoprene acid (Ismail *et al.*, 1998). In subsequent work, Ismail *et al.* (2000) demonstrated that for two metamorphic events, the production of pupal proteins by Verson's gland, and the loss of the ability of crochets epidermis to produce larval crochets, exposure to JH acid or methoprene acid plus a low dose of RH5992 (an ecdysteroid analog) is required. Use of RH5992 or methoprene acid alone does not induce these changes. Gilbert *et al.* (2000) recently outlined unpublished experiments by Bhaskaran and colleagues to determine whether JH acids played a role in the acquisition

of metamorphic competence in abdominal rings of first stadium *D. melanogaster*. Preliminary results indicate that JH acid plus ecdysteroid induce in first instar *D. melanogaster* both Broad, a transcription factor that appears in response to ecdysteroids early in the last larval stadium (see Section 8.12.3), and the adult promoter of alcohol dehydrogenase. However, attempts to repeat this work have been inconclusive, and the results of the previous works have been questioned on technical grounds.

8.3.2. Other Juvenile Hormones

It is a curious fact that the JH of *Rhodnius prolixus*, the hemipteran used by Wigglesworth in his pioneering experiments on the nature of the hormones controlling growth and reproduction (Wigglesworth, 1936), does not correspond to any other known JH (Wyatt and Davey, 1996). Early

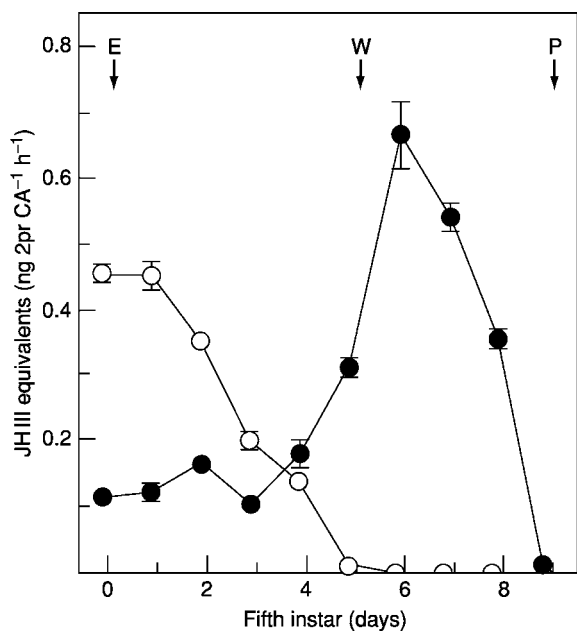


Figure 3 Biosynthesis of JH and JH acid by *Manduca sexta* corpora allata (CA) *in vitro*. Biosynthesis of JH I (open circles) and JH I acid (closed circles) by CA *in vitro* from fifth instars as measured by radioimmunoassay after separation of JH and JH acid by phase partition. Synthesis is expressed as ng JH I radioimmunoassay equivalents. E, the time of larval ecdysis; W, the onset of wandering behavior; P, the time of pupal ecdysis. Each datum point is the mean of 9–12 incubations (\pm SEM). (Reproduced from Janzen, W.P., Menold, M., Granger, N.A., 1991. Effects of endogenous esterases and an allatostatin on the products of *Manduca sexta* corpora allata *in vitro*. *Physiol. Entomol.* 16, 283–293.)

attempts to identify the hemipteran JHs did not meet with success (Baker *et al.*, 1988), but a more recent report suggests that JH I is the JH of Hemiptera (Numata *et al.*, 1992). Unfortunately, this latter identification seems doubtful in light of the results of Kotaki (1993, 1996). These studies revealed that while the product of the CA of the stink bug, *Plautia stali*, has a sesquiterpenoid skeleton similar to JH III, the addition of the JH III precursors farnesoic acid or farnesol to incubations of CA stimulates the production of neither JH III nor JHB₃, but one that differs chromatographically from any known JH (Kotaki, 1997).

Among the possible groups of known, but nontraditional JHs, are the biosynthetic precursors of JH (Figures 4 and 5). Methyl farnesoate (MF) (Figure 1) may be the crustacean form of the insect JH (see Section 8.6), but it is also present during embryogenesis in the cockroach *Nauphoeta cinerea* (Bürgin and Lanzrein, 1988) (see Section 8.10.2) and is synthesized by the embryonic CA of *D. punctata* (Cusson *et al.*, 1991a) and the adult CA of the dipteran *Phormia regina* (Yin *et al.*, 1995). In *P.*

regina, MF alone has limited JH-like biological activity, but appears to work best in a coordinated role together with JH III and JHB₃.

Farnesoic acid (FA) (Figure 4), another JH precursor, has also been identified as a product of the *D. punctata* nymphal CA; moreover, it is proposed to have a hormonal or prohormonal function during the latter half of the fourth stadium, when release of JH III ceases. Alternatively, it may be secreted as a by-product of O-methyl transferase activity in the terminal steps of JH III biosynthesis (Yagi *et al.*, 1991). Cusson *et al.* (1991a) speculate that continued synthesis of FA would allow for the rapid resumption of JH III synthesis when it is needed.

Plants possess compounds that exhibit JH-like activity in insect bioassays, and these molecules are considered to play a defensive role. For the most part, these “phytojuvenoids” are structurally distinct from the insect JHs and, with the exception of farnesol, which occurs widely in flowering plants, do not resemble the JH homologs (Bergamasco and Horn, 1983). Curiously, (10R)-JH III and MF have been conclusively identified in the sedges *Cyperus iria* and *C. aromaticus* (Toong *et al.*, 1988). Recently, it has been possible to produce JH III and its precursors and to define its biosynthetic pathway in cell suspension cultures of *C. iria* (Bede *et al.*, 1999, 2001). Given the large amount of JH III present in the plants, one might suspect that it acts as a naturally occurring insect growth regulator to deter feeding. However, it is possible that the plant uses the hormone as a plant growth inhibitor to retard the growth of other nearby species.

There exist a few compounds with no structural similarity to the JHs that display hormonal activity. The ecdysteroids top this short list. Ovarian ecdysteroids have long been known to have JH functions in adult females, stimulating the synthesis of vitellogenin in some species, and terminating previtellogenic reproductive diapause and promoting uptake of yolk proteins by oocytes in others, all roles traditionally associated with JH (Hagedorn, 1983; Girardie and Girardie, 1996; Richard *et al.*, 1998). There may also exist peptides that mimic the action of JH. In *R. prolixus*, JH stimulates protein synthesis in the male accessory gland *in vitro*, a function also supported by an uncharacterized neuropeptide from the brain (Barker and Davey, 1983; Gold and Davey, 1989). In addition, there are reports of peptides stimulating the synthesis of vitellogenin in *L. migratoria*, a role usually attributed to JH (Girardie and Girardie, 1996; Girardie *et al.*, 1998). Finally, there is the proposal of Davey (2000b) that thyroid hormones may function in

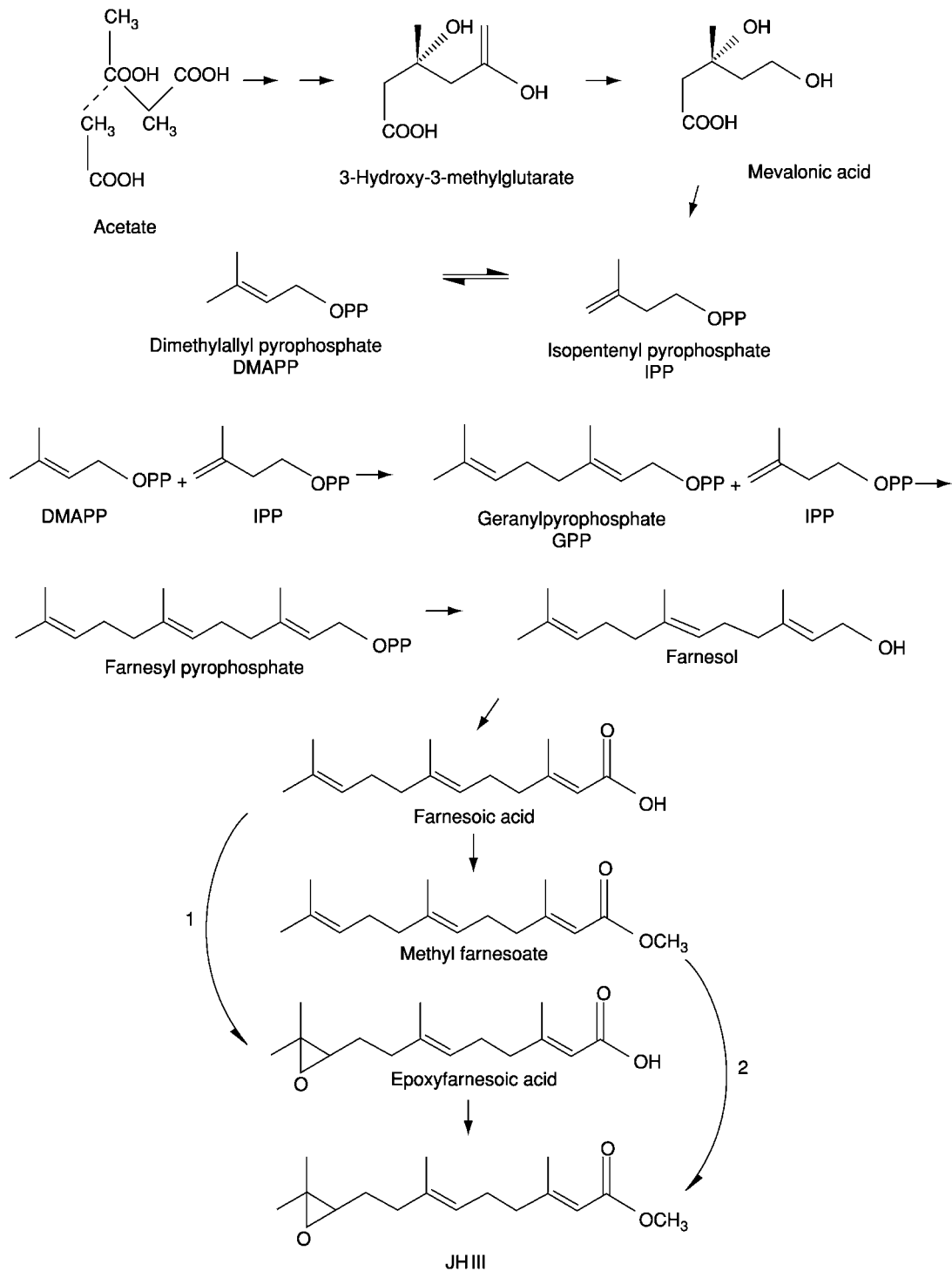


Figure 4 Biosynthetic pathway for JH III. In Lepidoptera, the last two steps in the biosynthetic pathway convert farnesoic acid to JH acid, which is then methylated to form JH III (1). In Orthoptera and Dictyoptera, the last two steps in the biosynthetic pathway convert farnesoic acid to methyl farnesoate, which is then epoxidized to form JH III (2).

insects. Phenoxy-phenyl compounds such as the JH analog fenoxycarb (Figure 1), thyroxine (T4) and triiodothyronine (T3) mimic the effects of JH III in reducing the volume of follicle cells in *L. migratoria* (Davey and Gordon, 1996; Kim *et al.*, 1999). In

many species, follicle cells respond to JH by altering their cytoskeletal structure, resulting in the appearance of lateral spaces between the cells (patency) via which vitellogenin gains access to the oocyte surface (Davey *et al.*, 1993). T3 binds to the same receptor

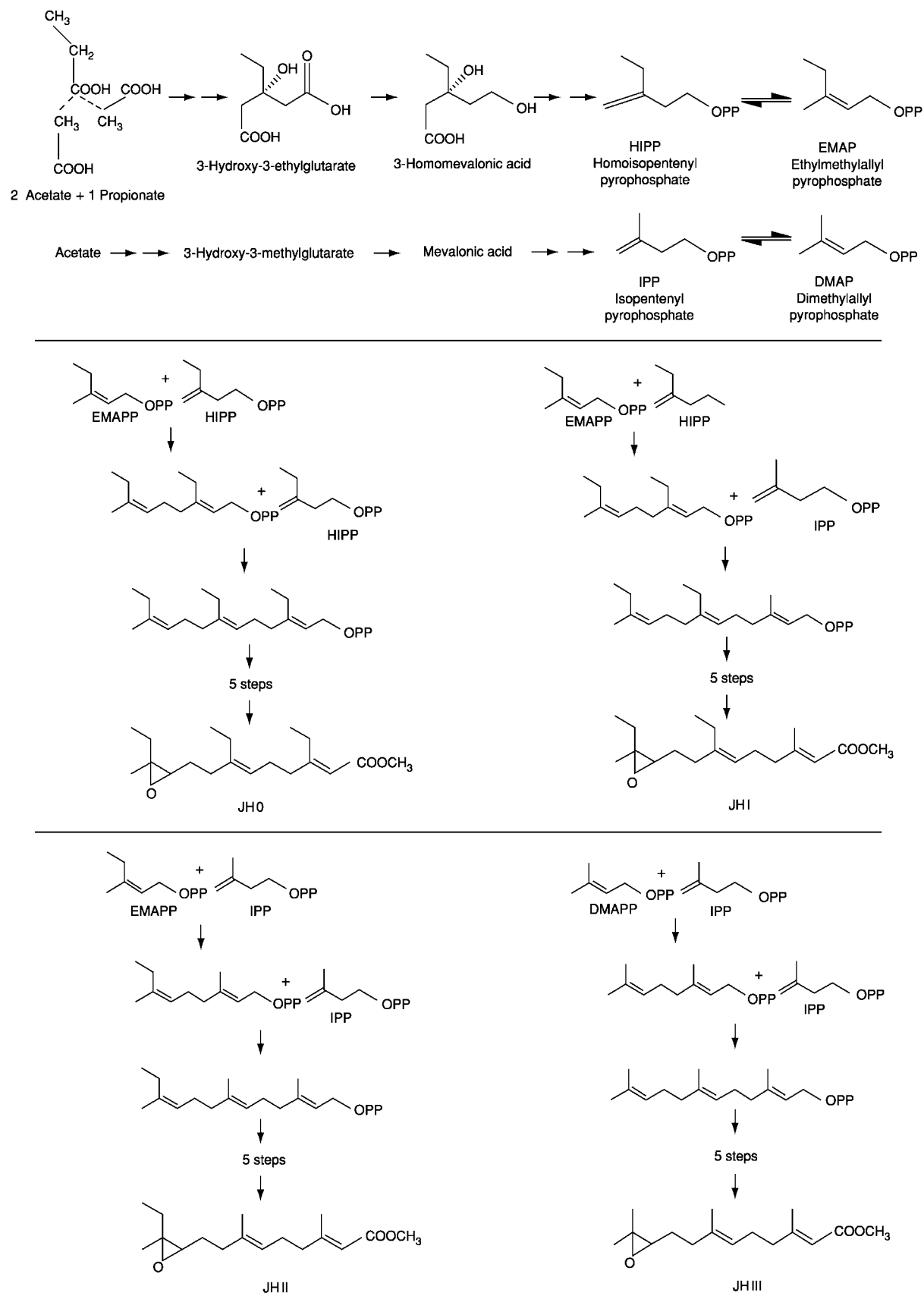


Figure 5 Biosynthetic pathways for JH 0, I, II, and III.

on locust follicle cells as JHIII (Davey and Gordon, 1996; Kim *et al.*, 1999), and T3 immunoreactivity can be found in locust hemolymph (Davey, 2000a, 2000b). Locust food (wheat shoots and bran) was found to contain both T3 and T4, thus suggesting that insect diets could serve as supplementary sources of compounds which act as JH (Davey, 2000b). The fact that T3 apparently binds to a receptor that also binds JH has interesting implications in the search for potential JH receptor(s) (see Section 8.12).

The increased sensitivity afforded by modern analytical tools should make easier the difficult task of deciphering the biological roles of these new JH-like molecules. Are they biologically relevant or are they the result of promiscuous biochemical reactions that yield end products with no real biological activity? It remains to be determined whether the results of studies using incubations *in vitro* or homogenates of CA to generate new JH-like molecules under experimental conditions bear any resemblance to the situation *in vivo*. There is good evidence from vertebrate endocrinology to believe that it is a real problem. A very early study on corticosteroid biosynthesis in the adrenal gland (Heftmann and Mosettig, 1960) lists more than 40 corticosteroids present in the human adrenal glands, even with the limited technology of that time. Of those, only a handful have ever been detected in the blood. A similar situation has been found with the insect ecdysteroids (Rees, 1985).

8.4. Biosynthetic Pathways for the Juvenile Hormones

The biosynthetic pathway for the JHs, now known for many years, has been described in exquisite detail by Schooley and Baker (1985). Briefly, biosynthesis of the most ubiquitous JH, JHIII, proceeds by the normal terpenoid pathway: the formation of five-carbon (5C) isoprenoid units from acetate via mevalonic acid, with the sequential head to tail condensation of three 5C units to form farnesyl pyrophosphate (Figure 4). Farnesyl pyrophosphate then undergoes esteratic cleavage to farnesol, which is then oxidized to FA. The last two steps in the biosynthetic pathway diverge depending upon the insect order. In Lepidoptera, a C10,C11 epoxidase converts the FA to the epoxy acid (JH acid), which is then methylated by an *O*-methyl transferase to form the methyl ester. In Orthoptera and Dictyoptera, the converse appears to be the case: epoxidation follows methylation (Schooley and Baker, 1985; Figure 4).

Synthesis of the higher homologs, JH0, I, and II, appears to be a unique biochemical feature limited to certain insect species (Schooley *et al.*, 1984). These insects can synthesize homoisoprenoid (6C) units from acetate and propionate via 3'-homomevalonate, and from a mixture of these isoprenoid (5C) and homoisoprenoid (6C) units form the respective homologs (Figure 5). Thus, three homoisoprenoid units (ethylmethylallyl pyrophosphates) form the skeleton of JH0, two homoisoprenoid units and one isoprenoid unit (dimethylallyl pyrophosphate) form JHI, and one homoisoprenoid unit and two isoprenoid units form JHII.

With regard to the pathway for JHB₃, Moshitzky and Applebaum (1995) have presented evidence that synthesis in *D. melanogaster* proceeds from FA, which is epoxidized twice, at C6,C7 and C10, C11 to form 6,7;10,11-bisepoxyfarnesoic acid (FABE). FABE is then methylated to yield JHB₃ (Figure 6). It should be noted that JHIII is not epoxidized to JHB₃.

8.4.1. Specificity of the Biosynthetic Pathways

The coexistence of multiple JH homologs in a single species at the same developmental stage, together with the discovery of new forms of JH in insects previously thought to have only one, presents an enigma not only as to their functions, but also as to the substrate specificity of the pathways by which they are synthesized and the regulatory mechanisms that effect those steps. Schooley and Baker (1985) concluded that biogenesis of the JH skeleton occurs via the condensation of isoprenoid and homoisoprenoid units and that overwhelming evidence suggests low substrate specificity at most steps in the biosynthetic pathway, even in enzyme preparations from CA, or incubations of CA, from insects known to produce JHIII only. This is particularly evident in the two terminal steps, where lack of substrate specificity has been demonstrated in a number of species. For example, the CA of the locust *Schistocerca gregaria*, which normally synthesize only JHIII *in vitro*, will produce JHI as their sole product if incubated with dihomofarnesoic acid (Schooley *et al.*, 1978a). Schooley and Baker (1985) concluded that the proportions of the precursors (propionate, mevalonate, and homomevalonate) added to incubation medium in which CA are maintained dictate the proportions of the JH homologs produced.

The precursor propionate is essential for the formation of the ethyl side chain of JH0, I and II (Figure 5), and within the CA, propionate is derived principally from the branched-chain amino acids

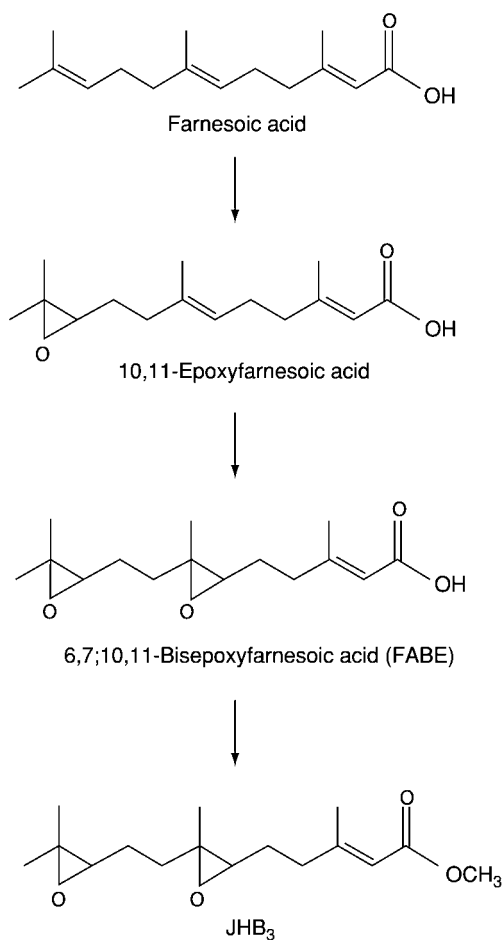


Figure 6 Biosynthetic pathway for JHB₃.

isoleucine and valine (Brindle *et al.*, 1988). The CA of *D. punctata* produce only trace amounts of JH II *in vitro* when propionate is the sole carbon donor, while continuing to produce copious amounts of JH III, the sole naturally occurring JH in this species (Feyereisen and Farnsworth, 1988). The CA of such insects are unable to convert branched-chain amino acids into propionate due to the lack of an active branched-chain amino acid transaminase (Brindle *et al.*, 1987, 1992). Cusson *et al.* (1996) tested the hypothesis that shifts in the proportions of the different JHs and JH acids released by lepidopteran CA are associated with changes in the activity of the transaminase. They found that the age-related increase in the biosynthesis of JH I acid by adult male CA of the moth *Pseudaletia unipuncta* was linked to an age-related increase in transaminase activity in CA homogenates. This increase was not observed with female CA, which synthesized more JH II than JH I during this same time period. The authors propose the existence of a factor regulating propionate production in lepidopteran CA. This factor is not

the *M. sexta* allatostatin (see Section 8.7.2) that inhibits JH biosynthesis by female *P. unipuncta* CA, since this peptide has no effect on the rate of isoleucine metabolism by either male or female glands.

Thus, as predicted, the availability of propionate within the CA and the activity of the transaminase has a significant impact on the proportions of JH I and II and their acids, but has no effect on the production of JH III, which is synthesized from the nonbranched isoprenoid units (Figure 5). The specificity and activity of other enzymes may therefore be involved in creating the blend of JH homologs and their acids synthesized by lepidopteran CA. As noted by Schooley *et al.* (1976) and Cusson *et al.* (1996), these enzymes may be the isopentenyl diphosphate isomerase and the farnesyl diphosphate synthase that catalyze the condensation of the isoprenoid and homoisoprenoid units to form the backbone of the JH homologs. Due to their low abundance, the substrate specificity of these enzymes has not been studied in enzyme preparations from CA homogenates. The cloning and sequencing of farnesyl diphosphate synthase in *A. ipsilon* (GenBank Accession no. AJ009962) and *B. mori* (GenBank Accession no. AB072589) have been reported (Castillo-Gracia and Couillaud, 1999; Kikuchi *et al.*, 2001), and recently Sen and Sperry (2002) have partially purified native farnesyl diphosphate synthase from whole-body preparations of *M. sexta*. Thus, substrate specificity for this enzyme may be known in the near future.

Sen and her colleagues have also examined other enzymes in the JH biosynthetic pathway, including the specificity of prenyltransferase activity of the larval CA of *M. sexta* (Sen and Ewing, 1997). This enzyme catalyzes the head-to-tail condensation of dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) units to form the skeleton of JH III, and of homoisopentenyl phosphate and ethylmethylallyl diphosphate (EMAPP) to form the skeletons of JH 0, I, II and 4-methyl JH I (Figures 5 and 6). Using homodimethylallyl diphosphate (HDMAPP) and the natural and unnatural homologs of geranyl diphosphate (GPP) (formed from the first coupling of one DMAPP and one IPP in the synthesis of JH III) (Figure 5), they found that this enzyme has a high tolerance for homologous substrates. EMAPP was a better substrate than DMAPP, while the unnatural geranyl homolog was a poorer substrate for the prenyltransferase than the natural homolog, suggesting that this enzyme contributes to the specificity of the JH skeleton.

Sen and Garvin (1995) examined the specificity of the farnesol dehydrogenase, an enzyme that

catalyzes the oxidation of farnesol to farnesal. Their results suggest that this enzyme is a unique dehydrogenase with high specificity for alcohols with Δ -2,3 unsaturation and alkyl chain hydrophobicity corresponding to at least three isoprenoid units, and hence is a selective enzyme for JH biosynthesis. In a subsequent study, Sperry and Sen (2001) concluded that, in fact, the oxidation of farnesol to farnesal did not involve a unique dehydrogenase, but rather a specific oxygen-dependent enzyme, possibly a flavin- or nonheme iron-dependent oxidase. Further studies on these enzymes will identify targets for compounds with anti-JH activity, as well as foster a better understanding of the key enzymes that are targets of allatotropins and allatostatins (see Section 8.7).

8.4.2. Possible Feedback Loops in the Biosynthetic Pathways

The formation of farnesyl pyrophosphate is an intermediate step in the synthesis of JH from mevalonate. A mammalian “orphan” nuclear receptor activated by farnesyl pyrophosphate metabolites has been identified (Forman *et al.*, 1995) and termed farnesoid X receptor (FXR) (Weinberger, 1996). FXR is activated by farnesol, farnesal, FA, and methyl farnesoate (Figure 4), and is expressed in isoprenoidogenic tissues. It has been suggested that these farnesyl pyrophosphate metabolites (farnesoids) may be signals for transcriptional feedback control of cholesterol biosynthesis, since cholesterol is synthesized from isoprenoids (Weinberger, 1996). As noted by Gilbert *et al.* (2000), Weinberger and his colleagues used a transactivation assay in Chinese hamster ovary cells by transfecting with plasmid DNAs expressing FXR, RXR (retinoid X receptor) and a farnesol responsive chloramphenicol acetyltransferase (CAT) reporter to demonstrate that JH, methoprene, and farnesol can all affect mammalian FXR. Farnesoids are JH biosynthetic agonists; they stimulate the formation of JH when added to incubations of CA and are derived from farnesyl pyrophosphate by a metabolic pathway that may serve to excrete surplus isoprenoid precursors (Schooley and Baker, 1985). Since insects cannot synthesize cholesterol *de novo* and require a dietary source of sterols (Rees, 1985), FXR may be involved in the feedback control of JH biosynthesis via these farnesoids. This possibility certainly deserves further study and opens the door to an exciting field of investigation of the role of orphan nuclear receptors in the regulation of JH biosynthesis (Chawla *et al.*, 2001) (see Section 8.7.3)

8.4.3. What Constitutes a Juvenile Hormone Titer?

As discussed by Darrouzet *et al.* (1998), the identification of new JHs, whether metabolites, conjugates, precursors, or hydroxylated forms, illustrates the variability of compounds released as end products from the CA; these end products are what Darrouzet *et al.* (1998) term “bouquets” of compounds. The “bouquets” are different in different orders, and undoubtedly vary between species within orders, and even within species by sex and stage in the life cycle (Couillaud, 1995). This is certainly true for *M. sexta*, where JHI, II, and III and their acids have been measured in larval hemolymph (Baker *et al.*, 1987), and for *P. regina*, in which the CA secrete JHIII, JHB₃, and MF (Yin *et al.*, 1995). Thus, it may be naive to think of the circulating titer of a single JH as responsible for the production of a JH effect. Rather, it may be a particular ratio or balance of homologs that is important for biological activity.

Sites outside the CA may also contribute to the circulating titers of the different hormones by metabolism, catabolism, hydroxylation, or conjugation. The JHI originally isolated and identified from the abdomens of male *H. cecropia* pupae (Röller *et al.*, 1967) was, in fact, synthesized by male accessory glands from JH acid secreted by the CA (Dahm *et al.*, 1976; Peter *et al.*, 1981). The male accessory glands in the mosquito *Aedes aegypti* synthesizes JHI, JHIII, JHB₃, and MF from acetate (Borovsky *et al.*, 1994a), and the mosquito ovary synthesizes JHIII from FA (Borovsky *et al.*, 1994b). Non-JHs such as the thyroid hormones, which can be ingested by the insect, may function as a JH in some JH-linked events. Are we safe in assuming that an event is truly JH independent if it occurs in an insect from which the CA have been removed (Davey, 2000a)? Is the JH titer in the hemolymph truly reflective of what elicits a JH-dependent event?

Thus, in considering JH titers and any temporally linked JH-dependent event, the event must be viewed from the perspective of a coordinated interplay between products of the CA, the products of other tissues in the body, the catabolism of these products (see Section 8.9), and the receptors for these products on the target tissues (see Section 8.12.1). The list of products possibly involved in this interplay is long and getting longer.

8.5. Measurement of the Juvenile Hormones

The lipophilic nature of JH, its occurrence in exceptionally low concentrations in biological samples

(parts per billion or lower), its lability, and its tendencies to bind nonspecifically to substrates, contribute to making this one of the most difficult of all hormones to measure accurately. There are three methods employed to quantitatively assess JH titers: bioassays, physicochemical assays, and radioimmunoassays. Relative JH titers can be determined using the radiochemical assay.

8.5.1. Bioassays

The first techniques used to measure JH titers were an assortment of bioassays that quantified a JH effect on a morphological or physiological process (Sláma *et al.*, 1974). Initially, these bioassays were used to assess the activity of transplanted CA, but are now more commonly used to quantify JH indirectly by comparing the biological response of an unknown preparation to a known amount of JH. These types of assays have been well described and critiqued in previous reviews (Feyereisen, 1985; Baker, 1990). Their use is in decline for a number of reasons: (1) they are cumbersome; (2) they are time-consuming, requiring maintenance of a test insect colony; (3) they are subjective in their scoring; (4) they lack specificity, because they measure only total JH; and (5) they show differential sensitivity to the various homologs. Nevertheless, several assays including the *Galleria* wax test (Gilbert and Schneiderman, 1961; deWilde *et al.*, 1968) and the *Manduca black* larval assay (Fain and Riddiford, 1975) are still used, since there are certain instances where bioassays play an important role. These include testing biological extracts for JH activity, especially when the active ingredient is thought to have a non-JH-like structure, or monitoring biological activity of a JH during its purification process.

8.5.2. Physicochemical Assays

With growing sophistication in the technology for studying organic compounds, a number of physicochemical methods have been developed for measuring JH in biological samples. Unfortunately, none of these methods is rapid, simple, or inexpensive. Moreover, the literature surrounding the physicochemical detection methods is awash in methods that can be bewildering for a biologist. The current physicochemical methods reflect several decades of experimentation that emphasized specificity and ultrasensitivity at the expense of simplicity. There are normally two phases to the physicochemical assay; the sample extraction/clean-up phase, and the quantification phase. Because JH represents only a minute fraction of the extractable lipids,

especially in whole-body extracts, protocols generally require some type of clean-up procedure(s) such as column chromatography, thin layer chromatography, separatory cartridge purification, HPLC, and/or gas chromatography (GC). Preparation of a biological sample for JH analysis has been discussed in several excellent reviews that cover extraction methods, solvents, and partition techniques (Baker, 1990; Halarnkar and Schooley, 1990). Further cautions with regard to degradation, volatility, and nonspecific adsorption have also been extensively outlined (Granger and Goodman, 1983, 1988; Goodman *et al.*, 1995).

Once the clean-up procedure is completed, JH can be quantified by a number of different procedures that employ a gas chromatograph interfaced with an electron-capture detection unit or mass spectrometer (Baker, 1990). In the early analyses of the hormones, GC separation of underivatized JH was coupled with electron impact (EI) or chemical ionization (CI) detection systems to monitor levels of the hormone. While these methods required no derivatization, they often resulted in complex spectra, poor recoveries, and an accumulation of breakdown products (Baker, 1990). Moreover, JH is not particularly stable under most GC conditions, due to instability of the epoxide ring. The use of CI, with its softer ionization process and its improved sensitivity and specificity, yields fewer fragment ions than the harsher EI ionization mode. Nevertheless, problems remain with the misidentification of the JH homologs (Baker, 1990).

To enhance sensitivity and specificity, methods were developed that generated JH derivatives with unique chemical tags. The first to be developed were the organohalide derivatives that are detected by a GC interfaced with an electron capture detection (ECD) system. While this method greatly increased sensitivity of the assay, it had serious drawbacks in uniformity of derivatization and in identification of the homologs. To overcome these difficulties, Rembold *et al.* (1980) and Bergot *et al.* (1981b) developed GC-mass spectrometry (MS) methods which, while still requiring derivatization of the hormones, were far more efficient and sufficiently selective to allow each JH homolog to be identified using selected ion monitoring. GC-MS-EI and GC-MS-CI analyses are now performed with slight variations on these procedures (Neese *et al.*, 2000; Smith *et al.*, 2000; Cole *et al.*, 2002).

Teal *et al.* (2000) have recently developed a method for quantifying JH from biological samples that allows for direct analysis of hormone without derivatization. These investigators use GC interfaced with ion-trap MS and CI. This method

requires no clean-up procedure or sample derivatization and is highly sensitive, and thus may be the assay of choice for future studies. While this procedure has been used to titer JH in hemolymph (Burns *et al.*, 2002) and culture medium (Bede *et al.*, 2001), in which lipid content is relatively low, it will be interesting to see if the assay can be successfully used with whole-body extracts.

The advantage of the physicochemical assays lies in their sensitivity and their unequivocal identification of the different JHs and metabolites. Their drawback lies in accessibility to the prohibitively costly equipment and the relatively low throughput.

8.5.3. Radioimmunoassays

Radioimmunoassays (RIAs) are a rapid, sensitive, and inexpensive alternative to measuring JH, both in biological samples and in medium from incubations of CA. The JH RIA is a competitive protein binding assay in which JH from a biological sample competes with a fixed amount of radiolabeled JH for a limited number of binding sites on JH-directed antibodies. The radiolabeled JH bound to the antibody in the presence of the unknown is compared to a standard curve derived with known amounts of radioinert JH.

Since JH itself is not antigenic, antibodies against JH are produced by chemically linking JH to a protein such as human serum albumin or thyroglobulin. The hormone can be conjugated to the protein via the C1 (ester) terminus of JH or through C10, C11 (see Granger and Goodman, 1983, 1988; Baker, 1990; Goodman, 1990, for reviews of the technique). The resulting antiserum is carefully screened for specificity, since it may recognize a particular JH homolog exclusively (Granger *et al.*, 1979, 1982; Goodman, 1990), two or more homologs to differing extents (Granger and Goodman, unpublished data), or all homologs equivalently (Goodman *et al.*, 1993, 1995).

Like the physicochemical methods, JH from whole-body or hemolymph extracts must be partially purified prior to conducting the RIA. The partial purification process is essential for a successful assay since a number of lipids can nonspecifically interfere with the assay. Inattention to this process invariably results in excessively large and inaccurate JH titers due to micelle formation of nonspecific lipids that either trap JH or inactivate the JH-directed antibodies. Misidentification of the JH homologs and overestimation of titers can also occur when chromatographic systems contaminated with high levels of JH standards are also used for the purification of biological samples (Baker *et al.*,

1984). It is for these reasons that RIA technology has rightly been criticized (Feyereisen, 1985; Tobe and Stay, 1985). A number of excellent clean-up procedures have been suggested (Strambi, 1981; Strambi *et al.*, 1981; Goodman *et al.*, 1995; Niimi and Sakurai, 1997; Noriega *et al.*, 2001). It is important to note that despite differences in extraction methods and JH-directed antibodies, excellent agreement between assays can be achieved (Goodman *et al.*, 1993).

JH RIAs have been used most effectively to measure CA activity *in vitro*, since the products are secreted into defined incubation medium that lacks the massive amount of lipid found in whole-body or hemolymph extracts. RIAs have been used extensively in studies on CA activity and in control of JH biosynthesis in the tobacco hornworm (Bollenbacher *et al.*, 1987; Granger and Janzen, 1987; Janzen *et al.*, 1991; Granger *et al.*, 1994), locusts (Couillaud *et al.*, 1984; Baehr *et al.*, 1986), honeybees (Huang and Robinson, 1995), and bumblebees (Cnaani *et al.*, 2000). Furthermore, certain extant RIAs recognize JH and JH acid equivalently (Janzen *et al.*, 1991), which can be a benefit in light of the fact that the CA of a number of species secrete JH acid at certain times in their life cycle (Sparagana *et al.*, 1984; Janzen *et al.*, 1991; Ho *et al.*, 1995a, 1995b; Niimi and Sakurai, 1997; Duportets *et al.*, 1998; Audsley *et al.*, 2000).

One of the major problems that has plagued research on the chemistry and biology of JH is the lack of commercially available, enantiomerically pure JH; nowhere is this more evident than in RIA technology. Depending upon the antigen used for immunization and immunocompetence of the rabbit, antibodies can be highly specific and capable of recognizing a single enantiomer of each homolog. Thus, using racemic JH as a standard may pose difficulties in establishing an accurate titer. A very complex binding reaction is generated when the radiotracer is racemic, but JH from the biological sample is enantiomerically pure; the analysis becomes even more complex when the radioinert standards are racemic. This problem has been eliminated by the use of a chiral HPLC matrix that can resolve the JH enantiomers (Cusson *et al.*, 1997). Moreover, the use of the appropriate enantiomers leads to increased sensitivity of the assay. Based on the ED₅₀ values (effective dose required to inhibit binding of 50% of the radiolabeled tracer), the naturally occurring enantiomers of JHI, JHII, and JHIII in this study were between 30 and 87 times more immunoreactive than the unnatural isomers. In summary, the JH RIA is a relatively rapid, inexpensive, and accurate method for titrating JH. If

measurement of more than one homolog is required, then confirmation by physicochemical methods is recommended.

8.5.4. Radiochemical Assays

The radiochemical assay (RCA), originally developed by Pratt and Tobe (Pratt and Tobe, 1974; Tobe and Pratt, 1974), has been employed for many years to measure JH biosynthesis *in vitro* by the CA of a wide variety of insects (Feyereisen and Tobe, 1981; Tobe and Feyereisen, 1983; Tobe and Stay, 1985; Yagi and Tobe, 2001). The RCA measures the rate of incorporation of the methyl group from either [¹⁴C]methyl methionine or [³H]methyl methionine into JH in isolated CA. The availability of assay components, the relative simplicity of the protocol, its sensitivity (0.1 pmol), and the short incubation time (1–3 h), have made this assay popular among insect endocrinologists.

There is a general assumption that isolated glands lacking neural connections function in the same fashion as glands *in vivo*. This assumption has been challenged by Horseman *et al.* (1994), who demonstrated that nerve transection in adult *L. migratoria* led to a rapid, 16-fold increase in JH production. A similar result has also been found in isolated CA of *A. aegypti* (Noriega, personal communication). Thus, the RCA may, at certain times, seriously overestimate the expected *in vivo* production of JH (Pratt *et al.*, 1990). Another potential problem is the use of [³H]methyl methionine as the methyl donor (Yagi and Tobe, 2001). The lack of definitive data from the manufacturers on the purity and specific activity of this compound can make determination of the biosynthesized JH difficult. Yagi and Tobe (2001) recommend either comparing incorporation of the [³H]methyl group with the [¹⁴C]methyl group in a dual-label experiment or switching altogether to [¹⁴C]methyl methionine. It appears that isotopic discrimination by the O-methyl transferase leads to preferential incorporation of the [¹⁴C]-labeled methyl group vs. the [³H]-labeled methyl group, but only when concentrations of [³H]methyl methionine above the normal range for the RCA are used.

The measurements of JH biosynthesis using the RCA can also be affected by the incubation medium, which in most instances is TC199. TC199 has a high Na⁺:K⁺ ratio (17.8) and thus is a suitable medium for the CA from insects where the hemolymph ratio of Na⁺:K⁺ is similar, for example, *Leptinotarsa decemlineata* or *T. molitor* (Granger *et al.*, 1986). This medium has been used extensively in the RCA, irrespective of insect

species. For insects such as *M. sexta*, where the hemolymph Na⁺:K⁺ ratio is low (0.1) (Nowock and Gilbert, 1976), TC199 has been found to depress JH synthesis in comparison to Grace's medium, in which the Na⁺:K⁺ ratio is 0.33 (Granger *et al.*, 1986; Watson *et al.*, 1986). More recently, L-15B, a medium widely used in the culture of arthropod tissues and found to be isoosmotic with cockroach hemolymph, was demonstrated to be superior to TC199 for both long- and short-term cultures of cockroach CA (Holbrook *et al.*, 1997).

8.6. Evolution of the Juvenile Hormones

8.6.1. Juvenile Hormone Homolog Distribution among the Insect Orders

One or more of the JH homologs have been identified in nearly all species of insects in which they have been sought (Sehnal, 1984) and have even been found in Collembola, an order once thought basal to insects but now considered to represent a more ancestral group, distinct from insects (Nardi *et al.*, 2003). A JH effect on ecomorphosis of a collembolan species, *Hypogastrura tulbergi*, has been reported (Lauga-Reyrel, 1985), and on this basis, it seems likely that JH or a JH-like molecule functioned in the ancestors of modern insects at least 400 million years ago. Figure 7 illustrates the evolutionary time line in which the various orders of insects were thought to have appeared, the orders in which JHs or JH-like molecules have been identified, and the identity of these compounds.

8.6.2. Evolutionary Connections among the Arthropods

Arthropods, which appeared more than 500 million years ago during the Cambrian period, include four extant classes: Hexapoda, Chelicerata, Myriapoda, and Crustacea. Of these, the Hexapoda and Crustacea appear to be the most closely related, based on molecular data, and are now considered together as Pancrustacea (Boore *et al.*, 1995; Friedrich and Tautz, 1995; Tobe and Bendena, 1999). Figure 8 illustrates a time line of the appearance of the extant classes of Arthropoda and those classes in which JH effects have been demonstrated.

Outside the class Insecta, JH-like molecules have been identified in Crustacea, and functions for these molecules have been proposed. Both FA and MF, the immediate precursors of JH III (Figure 4), are major biosynthetic products of the mandibular organs of many crustaceans. The similarities in function and embryological origin of the mandibular organs and

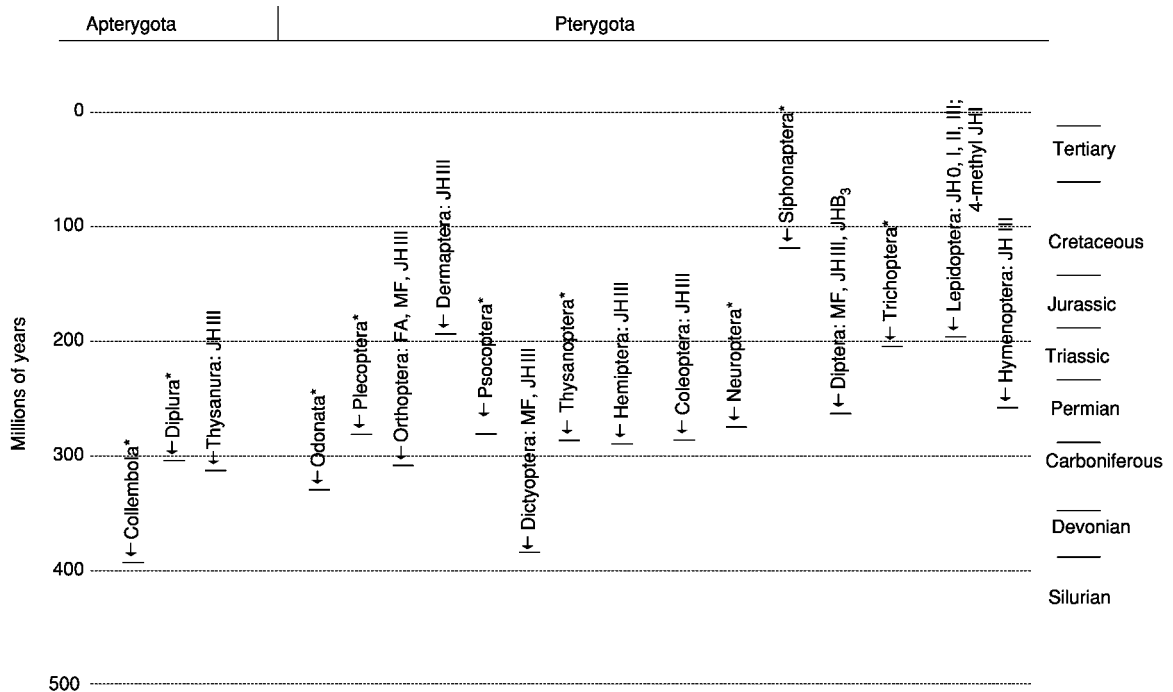


Figure 7 Evolutionary timeline for the appearance of the insect orders based on the earliest documented fossil record (Heming, 2003). Orders in which JH effects have been demonstrated are noted with an asterisk (*). Orders in which JH or JH-like molecules have been conclusively identified are noted by the homolog.

CA, and the fact that the biosynthetic pathway for MF and FA are the same in both crustaceans and insects (see Section 8.4.1), suggests that these products may be the JHs of Crustacea. FA can act as a JH in crustacean reproduction (Laufer *et al.*, 1993), and MF has a juvenilizing role in the prawn (Abdu *et al.*, 1998) and the barnacle (Smith *et al.*, 2000) during metamorphosis from the larval to the juvenile form. MF also delays metamorphosis in larvae of the lobster, *Homerus americanus* (Borst *et al.*, 1987). Nevertheless, definitive physiological and developmental roles for these two JH precursors remain vague (Homola and Chang, 1997), and further work is needed to determine their exact functions.

Although it is not clear whether FA or MF are prohormones, hormones, or simply side products of JH biosynthesis in insects, the esterification of JH acids in the accessory glands of male *H. cecropia* (Shirk *et al.*, 1983) and the imaginal discs of *M. sexta* (Sparagana *et al.*, 1985) certainly suggests that FA can be converted to MF, and MF esterified to JH III, in tissues outside the CA. Research is needed on the existence, location, and activity of the enzymes O-methyl transferase and MF epoxidase to elucidate whether FA and MF are prohormones or hormones in their own right.

8.6.3. Evolutionary Inferences

FA is released from the nymphal CA of the cockroach, *D. punctata*, while homo/dihomo MF and FA appear to be products of the CA of adult female and male *P. unipuncta* (Cusson *et al.*, 1991a). It has recently been shown that in addition to JH III, the embryonic CA of *D. punctata* produce MF, with a shift to predominantly JH production as development proceeds (Stay *et al.*, 2002) (see Section 8.10.2). This evidence has led Stay *et al.* (2002) to postulate that the production of MF by the early embryonic CA is an ancient trait in Arthropoda, and that the original metamorphic and reproductive hormone in this more basal order was MF rather than JH. In light of the proposal that the ancestral role of JH was involved in embryogenesis (Truman and Riddiford, 1999), MF may have fulfilled this role in the early evolution of the Arthropoda, with a shift to JH III production as metamorphosis in the Insecta evolved.

Sehnal *et al.* (1996) have suggested that the post-embryonic development in the ancestors of ametabolous insects occurred in the absence of JH and that the original function of JH in postembryonic life was the regulation of reproduction. As the occurrence of metamorphic molts evolved in ancient

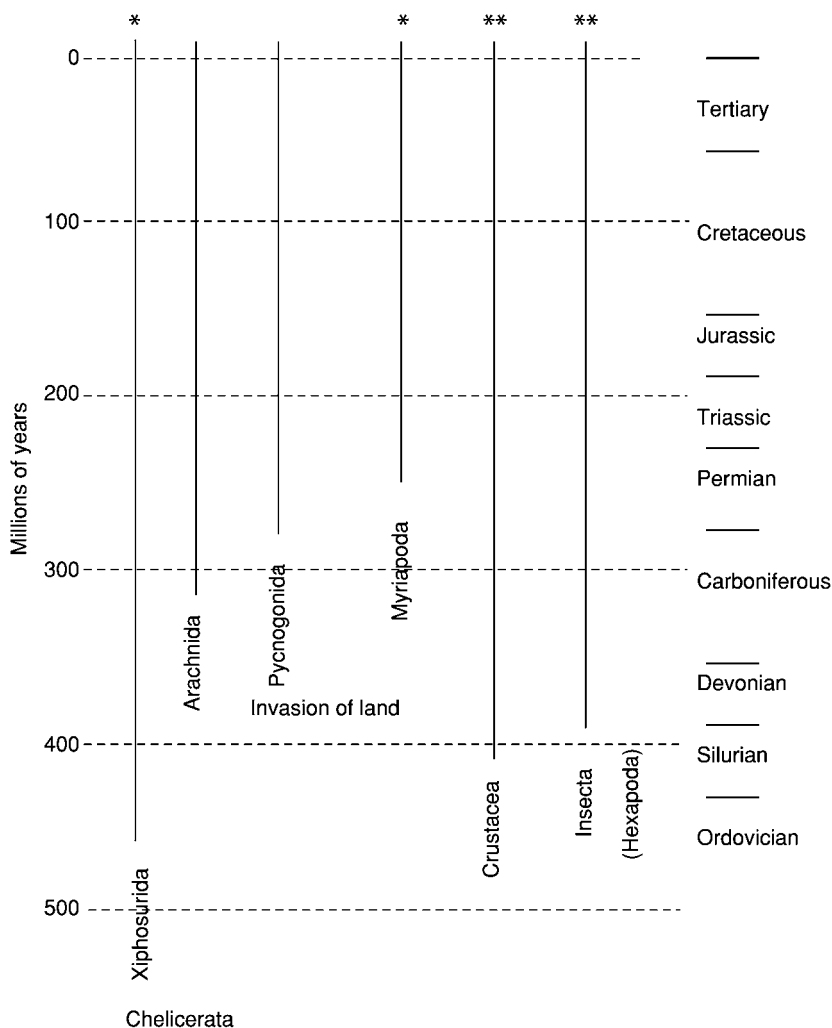


Figure 8 Evolutionary timeline of the appearance of the extant classes of Arthropoda. Classes in which JH effects have been demonstrated are noted with an asterisk (*). Classes in which JH or JH-like molecules have been identified are noted with a double asterisk (**).

arthropods, it is possible that a hormone (JH) that functioned in the embryo and the mature adult, but played no role in postembryological development, was coopted to regulate development before and after these metamorphic molts (Tobe and Bendena, 1999). In some insect species, JH appears to regulate the types of responses that would facilitate evolution of a larval stage, such as premature differentiation of the embryo and alterations in morphology (Truman and Riddiford, 1999). As outlined below (see Section 8.10.7), Truman and Riddiford (1999, 2002) have proposed that an advancement in the time of appearance of JH during embryogenesis may have been key to the evolutionary transformation of a pronymphal stage to the larval stage of holometabolous insects.

The production of more complex forms of JH may have occurred with the evolution of holometabolous

development. At the present time, JHIII is the only known form of JH in most insect species, particularly the Hemimetabola. With the exception of the HJHs (see Section 8.2.2), the more complex forms of JH, JH0, iso-JH0, JHI, methyl-JHI, JHIII, and JHB₃, appear to occur only in two highly derived orders of holometabolous insect, the Lepidoptera and the Diptera. Furthermore, only Diptera appear to have evolved the ability to produce JHB₃. The production of the higher homologs and their multiple occurrence in some species appears to be related to the evolutionary development of intricate physiological events and their regulation. The exact physiological or developmental role of the different JHs or JH-like molecules in each class and order needs to be determined to further elucidate the role of JH in arthropod and insect evolution and by extension, the evolution of these molecules themselves.

8.7. Regulation of Juvenile Hormone Biosynthesis

A great deal of progress has been made since 1985 in understanding the regulation of JH biosynthesis by neurohormones, neuromodulators, and neurotransmitters (Feyereisen, 1985). At that time it was generally believed, based on a considerable body of *in vivo* studies, that physiological factors, mainly from the central nervous system, either stimulated (allatotropins) or inhibited (allatostatins) JH synthesis by the CA. Their chemical nature was unknown, but their existence was accepted, based on distinct and predictable changes in the hemolymph JH titer that related directly to physiological events, such as ecdysis of the larva and reproduction in the adult. By 1985, methods for maintenance of the CA *in vitro* had been established for a number of species, and two approaches to the measurement of JH biosynthesized *in vitro* had been developed: the radiochemical assay (Pratt and Tobe, 1974) (see Section 8.5.4) and the radioimmunoassay (Baehr *et al.*, 1976, 1979; Strambi, 1981) (see Section 8.5.3). Using these methods, the JH homologs synthesized by the CA *in vitro* have been found to be the same as those in hemolymph, although the homolog ratio may vary for CA that synthesize more than one JH. Moreover, developmental changes in JH biosynthetic activity by the CA *in vitro* parallel changes in the hemolymph JH titers (Feyereisen, 1985; Tobe and Stay, 1985). What factors elicit these changes in JH biosynthetic activity?

8.7.1. Neuropeptides

8.7.1.1. Allatotropins The first peptide to be isolated that affected JH synthesis *in vitro* was the allatotropin (AT) of *M. sexta* (Manse-AT) (Kataoka *et al.*, 1989). While this amidated tridecapeptide did not stimulate JH biosynthesis by larval or pupal CA *in vitro*, it did stimulate synthesis by adult CA at a concentration of 10^{-9} M. The gene for Manse-AT was isolated a decade later and was found to express three different mRNAs that differ from one another by alternative splicing (GenBank Accession no. U62100, U62101, U62102) (Taylor *et al.*, 1996). It has been suggested that the different mRNAs encode three distinct prohormones. Immunocytochemistry with polyclonal antibodies to Manse-AT and *in situ* hybridization with riboprobes for its mRNAs were used to show that Manse-AT exists in the central nervous system of *M. sexta* larvae. The former study found immunoreactivity in cerebral neurosecretory cells as well as axons in the corpora cardiaca (CC) and CA (Žitňan *et al.*, 1995), while the latter found only low levels in

nonneurosecretory cells of the larval brain and subesophageal ganglion (Bhatt and Horodyski, 1999). Although Manse-AT stimulates the adult CA in this species, its mRNAs were not detected in prepupal, pharate pupal, or adult brains (Bhatt and Horodyski, 1999).

Is Manse-AT a functional AT in other lepidopteran species? There is direct evidence from several different studies that this is indeed the case. Manse-AT may be the true allatotropin in *P. unipuncta*: it has been cloned in this species (GenBank Accession no. AF212926) and Manse-AT immunoreactivity has been found in the CA (Truesdell *et al.*, 2000). Manse-AT has also been cloned from the silkworm *B. mori* (Park *et al.*, 2002) and has been purified from methanolic brain extracts of the fall armyworm, *Spodoptera frugiperda*, in which it stimulates adult CA (Oeh *et al.*, 2000, 2001). A cDNA encoding 134 amino acids, including an allatotropic peptide, has recently been cloned in this species (GenBank Accession no. AJ488181, AJ508061) (Abdel-Latif *et al.*, 2003), and the mature peptide is identical to Manse-AT. The *Spodoptera* peptide precursor has 84%, 93%, and 83% amino acid sequence identity with those of *M. sexta*, *P. unipuncta*, and *B. mori*, respectively. Manse-AT also stimulates JH synthesis by both larval and adult CA of *L. oleracea* (Audsley *et al.*, 1999a, 1999b, 2000), and the adult CA of another moth, *Heliothis virescens* (Kataoka *et al.*, 1989; Teal, 2002).

Manse-AT may be a functional AT in other orders as well. Immunological analyses indicate it is present in the abdominal nervous system of the cockroach *Periplaneta americana* (Rudwall *et al.*, 2000) and in the brain of the fly *P. regina* (Tu *et al.*, 2001). Moreover, it stimulates JH synthesis by the larval CA of the honeybee, *Apis mellifera* (Rachinsky and Feldlaufer, 2000; Rachinsky *et al.*, 2000), and the adult female CA of *P. regina* (Tu *et al.*, 2002).

8.7.1.2. Other roles for Manse-AT Since Manse-AT does not affect larval *M. sexta* CA, could it have another function in the larva? Indeed, Manse-AT has been shown to be a multifunctional molecule in *M. sexta*, as well as in other species. It exerts an apparently species-specific inhibition of ion transport in the midgut of *M. sexta* fifth instars (Lee *et al.*, 1998) and accelerates the heart rate in pharate adults, but not larvae (Veenstra *et al.*, 1994). It stimulates foregut contractions in the moths *Helicoverpa armigera* and *L. oleracea* (Duve *et al.*, 1999, 2000); acts as a cardioaccelerator in *Leucophaea maderae*, *P. americana* (Rudwall *et al.*, 2000),

and *P. unipuncta* (Koladich *et al.*, 2002); and is involved in the photic entrainment of the circadian clock of *L. maderae* (Petri *et al.*, 2002). Thus, a phenomenon observed with a number of other insect neuropeptides is illustrated by Manse-AT: it has multiple functions and these functions are stage-, tissue-, and species-specific.

8.7.1.3. Molecular biology of the allatotropins Horodyski and his colleagues have undertaken a detailed study of the molecular biology of the ATs. They have found that while levels of Manse-AT mRNAs are low or nonexistent in the brain, the Manse-AT gene is expressed in a variety of cells in other regions of the central nervous system and frontal ganglion in larval, pupal, and adult stages of *M. sexta* and that the cellular expression pattern in the nerve cord, as indicated by levels of Manse-AT mRNA, changes during metamorphosis (Bhatt and Horodyski, 1999).

Manse-AT-like peptides can be derived from each of three distinct precursor proteins that are the predicted products of alternatively spliced mRNAs (Taylor *et al.*, 1996). The basis for differences in the spliced mRNAs is the inclusion of one or two exons at the same location within the open reading frame. These exons contain a sequence that codes for a peptide with limited structural identity to Manse-AT (Horodyski *et al.*, 2001; Lee and Horodyski, 2002). The three expressed peptides have overlapping but nonidentical activities in JH biosynthesis assays with adult CA *in vitro* and in the inhibition of active ion transport across larval posterior midgut epithelium *in vitro*. The presence of the mRNA isoforms resulting from the alternative splicing is regulated in a tissue- and developmentally specific manner, suggesting changing roles for the resulting peptides at different life stages (Lee *et al.*, 2002). Finally, the expression of one of these mRNA isoforms is increased in the ventral nerve cord of *M. sexta* last instars subjected to treatments that normally reduce feeding and increase levels of JH in the larva: starvation, parasitization, or ingestion of the ecdysteroid agonist RH5992 (K.Y. Lee and Horodyski, 2002). These discoveries are the first to identify the pathway by which these treatments first have their effect. Whether this effect involves downstream regulation of the endocrine system, midgut ion transport, or another target is yet unknown.

8.7.1.4. Other allatotropins Until recently, Manse-AT was the only AT identified. However, an allatotropic immunoreactive peptide has been isolated from the abdominal ganglia of the mosquito *A. aegypti* (Veenstra and Costes, 1999) and found to

have a unique sequence. It has now been shown that Aedae-AT has a stimulatory effect on adult female *A. aegypti* CA *in vitro*, with maximum stimulation at dosages of 10^{-8} – 10^{-9} M, suggesting that this moiety is the true AT in *A. aegypti* (Li *et al.*, 2003). Furthermore, while neither the JH precursor FA nor Aedae-AT stimulates JHIII synthesis by the CA of newly emerged females, when both are added to the incubation medium, high levels of JHIII production occur, indicating that Aedae-AT makes the CA competent to make JHIII from FA.

There are a number of studies that suggest the existence of at least seven other allatotropins (Stay, 2000; Li *et al.*, 2003). In the wax moth, *Galleria mellonella*, a protein fraction of larval brains was found to stimulate JHII synthesis by CA *in vitro*, and monoclonal antibodies raised to that fraction were used to identify a 20 kDa peptide by immunoblotting (Bogus and Scheller, 1996). Two pairs of immunoreactive neurosecretory cells in the *G. mellonella* brain and in the CC were identified with these monoclonal antibodies. One of the pairs of cerebral neurosecretory cells was immunoreactive with antibodies to Manse-AT as well, but because of the size of the putative Galme-AT, it is unlikely to be a homolog of Manse-AT. The investigators suggest that Manse-AT and Galme-AT have common epitopes due to splicing from the same preprohormone. Recent attempts to isolate the gene for this AT using an expression library and monoclonal antibodies have not been successful (Sehnal, personal communication).

A putative AT has been extracted from the subesophageal ganglion of the cricket *Gryllus bimaculatus*, and stimulates the adult CA of not only *Gryllus*, but also of another cricket, *Acheta domestica* (Lorenz and Hoffmann, 1995). Extracts of the brain–subesophageal ganglion–CC–CA from the true bug, *Pyrrhocoris apterus*, stimulate JH biosynthesis by the CA of adults raised under long-day photoperiodic conditions (Hodkova *et al.*, 1996), and methanolic extracts of brain or CC from adult vitellogenic females of the locust *L. migratoria* stimulate JHIII release from the CA of vitellogenic females 20–40-fold (Gadot *et al.*, 1987). These extracts also stimulate JH production by the CA of adult male *Locusta*, but not without some manipulation, since the CA must be chilled at 4 °C for 24 h to obtain the stimulatory response (Lehmberg *et al.*, 1992). Surgical manipulations and immunological studies suggest that the brain is the source of this factor (Couillaud *et al.*, 1984; Ulrich, 1985). Allatotropic activity was also extracted from the subesophageal ganglion and CC of adult male *M. loreyi*, and found to stimulate JHIII acid and iso-JHII

synthesis by male CA *in vitro* approximately two-fold (Kou and Chen, 2000).

8.7.1.5. Nonneural allatotropins The lack of effect of Manse-AT and other putative ATs on CA when one would assume an active stimulation of JH biosynthesis implies that there exist ATs of different structures than those isolated to date from neural tissue. Studies suggesting the existence of allatotropic molecules from nonneural sources represent the most intriguing line of research on the control of JH biosynthesis. There currently appear to be two sources for such factors: the ovary and the male accessory sex gland. Ovarian factors that effect JH production have been found in two species, the cockroach *D. punctata* and the cricket *G. bimaculatus*. In *D. punctata*, ovaries stimulate increased JH production in a stage-specific manner: ovaries just prior to ovulation are stimulatory while ovaries of pregnant females are not (Rankin and Stay, 1985). Stimulation is directly related to the size of the basal oocytes; ovaries with basal oocytes smaller than 0.8 mm or larger than 1.5 mm were not stimulatory. More recently, it has been demonstrated that *Diptera* ovaries, fat body, and muscle can all stimulate JH synthesis by CA *in vitro* (Unnithan *et al.*, 1998). Stimulation by the ovary is dose-dependent and sensitive, although the moiety responsible does not appear to be a peptide. In this species, however, the overall control of JH biosynthesis is very much dictated by allatostatins (see Section 8.7.2). In *G. bimaculatus* (Hoffmann *et al.*, 1996), an increase in JH biosynthesis follows the implantation of ovaries into adult males. Whether this effect is mediated by a true AT is not clear, since an extract of ovaries did not stimulate JH synthesis *in vitro*.

A 36 amino acid peptide of known sequence from the male accessory sex gland of *D. melanogaster* (Chen *et al.*, 1988; Schmidt *et al.*, 1993) stimulates JH synthesis when transferred to the female during mating and also increases JH synthesis *in vitro* by the CA of adult female *D. melanogaster* (Moshitzky *et al.*, 1996). The peptide has the same effect *in vitro* on CA from adult females of the moth *H. armigera* (Fan *et al.*, 1999). By contrast, fragments of the C-terminal and a truncated N-terminal peptide from this *Drosophila* sex peptide (SP) inhibit JH synthesis *in vitro* by CA from both species. Since two N-terminal peptides stimulate JH synthesis, similar to the full-length molecule, it appears that the first five N-terminal amino acid residues are essential for CA stimulation by SP in both species (Fan *et al.*, 2000). Moshitzky *et al.* (2003) recently reported that the *Drosophila* SP downregulates

JH III bisepoxide synthesis *in vitro* by CA of another dipteran, the medfly *Ceratitis capitata*.

8.7.1.6. Future directions of research on allatotropins As proposed by Gilbert *et al.* (2000), the future directions of research on ATs should concentrate in several areas: (1) the identification of ATs in other orders and the sequences of the Manse-AT-like molecules in those lepidopteran species where Manse-AT has an effect; (2) the exploration of other functions for identified ATs; (3) the elucidation of the interaction of ATs and allatostatins in the overall control of JH biosynthesis; and (4) studies of their receptors and ligand-receptor interactions leading to downstream events that modulate synthesis. To this list should be added research on nonneural ATs.

8.7.2. Allatostatins

Allatostatins (ASTs), compounds that inhibit JH biosynthesis by the CA, can be grouped into three families. More than 170 different peptides belong to the largest of the families, the FGLamide ASTs, or the AST-A group. This family is characterized by the presence of a highly conserved pentapeptide in the C-terminus, Y/F-X-F-G-L-amide but displays considerable variability in length and sequence in their N-termini (Tobe and Stay, 2004). These ASTs, first identified by Stay *et al.* (1991a) in *D. punctata*, have now been found in many different insect species (Gade *et al.*, 1997; Stay, 2000; Li and Noriega, personal communication). They have also been found in groups outside the insects, including Crustacea (Dircksen *et al.*, 1999; Skiebe, 1999; Duve *et al.*, 2002) and freshwater pulmonate snails (Rudolph and Stay, 1997). In *D. punctata* alone, there are 14 FGLamide ASTs, with one terminating in an isoleucine (Stay, 2000; Tobe and Stay, 2004). Although Lepidoptera have their own family of ASTs, an FGLamide AST was isolated and identified from *M. sexta* (Davis *et al.*, 1997). FGLamide immunoreactivity is found in the brain, abdominal ganglia, neurohemal organs, and thoracic motor neurons of *M. sexta* larvae, but the processes of immunoreactive neurons in the CA are sparse. The authors concluded that in this species, the FGLamides are probably neuromodulatory, myomodulatory, and myotrophic.

The second family of ASTs, or AST-B group, has been identified in crickets, locusts, and stick insects and contains a common amino acid sequence of W₂W₉amide, that is, tryptophan residues at the 2 and 9 positions of the N-terminus (Lorenz *et al.*, 1999, 2000; Tobe and Stay, 2004). Because these peptides in stick insects are variable in length, a

designation of W-X₆-Wamide has been assigned to this family (Tobe and Stay, 2004). At the present time there appear to be 17 of these peptides (Stay, personal communication): five in *G. bimaculatus*, six in *Carausius morosus*, with the remainder in orders as diverse as Lepidoptera (*M. sexta* and *B. mori*), Diptera (*D. melanogaster*), Orthoptera (*L. migratoria*), and Dictyoptera (*P. americana*) (Stay, 2000). A considerable number of these are not active in the species from which they were isolated, being identified solely on the basis of amino acid sequence.

The third family of ASTs, the AST-C group, has been identified in Lepidoptera and has been designated the PISCF family. The first to be discovered, an AST (Manse-AST) in *M. sexta*, was purified from pharate adult heads by Kramer *et al.* (1991) and shown to be a 15 residue, nonamidated peptide with a free C-terminus and with no homology to the cockroach ASTs. This neuropeptide is unique in another sense, in that it can completely inhibit JH production in *M. sexta*. Immunochemical and molecular techniques have shown that Manse-AST is present in other insects, including *P. unipuncta* (Jansons *et al.*, 1996), *L. oleracea* (Audsley *et al.*, 1998), *Spodoptera littoralis* (Audsley *et al.*, 1999a), and *D. melanogaster* (unpublished information cited in Stay, 2000). As with the other AST families, there is some question as to whether Manse-AST is the functional AST in all these species. Synthetic Manse-AST does not affect JH biosynthesis *in vitro* by *Drosophila* ring glands; in *P. unipuncta*, it does not inhibit larval CA or newly emerged adults, and inhibits adult female CA only 60% at a concentration of 10⁻⁶ M (Jansons *et al.*, 1996). Manse-AST inhibits JH synthesis *in vitro* by adult CA of *Heliothis (Helicoverpa) zea* (Kramer *et al.*, 1991) and *S. frugiperda* (Oeh *et al.*, 1999), but with the latter, only when the CA are first stimulated by Manse-AT. More recently, a synthetic Manse-AST was shown to inhibit JH II synthesis *in vitro* by CA from larval *L. oleracea* by approximately 70% (Audsley *et al.*, 2000). Manse-AST has also been demonstrated to modulate JH biosynthesis by the CA of insects of other orders, including *A. mellifera* (Rachinsky and Feldlaufer, 2000) and *P. regina* (Tu *et al.*, 2001). Thus, the PISCF ASTs do not appear to be limited to Lepidoptera, and may be the functional AST in other insect orders as well.

8.7.2.1. Effect of allatostatins on the juvenile hormone biosynthetic pathway Soon after the discovery of the ASTs, Pratt and his colleagues (Pratt *et al.*, 1989, 1991) showed that addition of exogenous farnesoate or mevalonate reverses the inhibition of

JH biosynthesis *in vitro* by AST, suggesting that the action of this neuropeptide occurs before the synthesis of the isoprenoid and homoisoprenoid units that condense to form the JH backbone (Granger, 2003) (see Section 8.4.1). Tobe and his colleagues (Wang *et al.*, 1994) provided evidence that *D. punctata* allatostatin 4 (Dippu-AST 4) inhibits the activity of O-methyl transferase, the enzyme catalyzing the penultimate step in JH III biosynthesis in this species (see Section 8.4.1), but only at the moderately high concentration of 10⁻⁸ M. The authors concluded that this effect is probably secondary since Dippu-AST 4 inhibits JH synthesis at a much lower concentration and because a variety of precursors such as FA and (*E,E*)-farnesol were found in this and other studies to reverse the inhibition by Dippu-AST 4. Wang *et al.* (1994) concluded that Dippu-AST 4 is acting primarily on the earlier, rather than later, stages of JH biosynthesis. More recent work has confirmed that the most probable targets for the ASTs are the first committed steps in the synthesis of JH III, that is, either the transfer of citrate to the cytoplasm across the mitochondrial membrane or the cleavage of citrate to yield cytoplasmic acetyl CoA, a building-block for the isoprenoid and homoisoprenoid units (Sutherland and Feyereisen, 1996).

8.7.2.2. Locations of allatostatins in the insect AST-positive cerebral neurosecretory cells have been shown for all three families of ASTs by immunocytochemistry and *in situ* hybridization. Immunocytochemistry has demonstrated that these cells in *M. sexta*, *D. punctata*, and *G. bimaculatus* are usually located in the lateral protocerebrum, project to the CA on the contralateral side, and arborize in the CC and the CA (see Stay, 2000 for a summary). In some cases, medial neurosecretory cells are immunoreactive, as are some cells in the tritocerebrum, and these cells, together with some of the lateral cells, have been observed to arborize within the brain and thus can be considered interneurons (Stay *et al.*, 1992; Žitňan *et al.*, 1995). With the use of immunocytochemistry, ASTs have been demonstrated in other regions of the central nervous system including the peripheral nerves, the gut, the ovary, and the oviducts (Stay, 2000; Witek and Hoffmann, 2001; Garside *et al.*, 2002) (see Section 8.7.2.4). *In situ* hybridization has been used less frequently to locate ASTs within the insect, usually in combination with immunocytochemistry – for example, the identification of ASTs in hemocytes of *D. punctata* (Skinner *et al.*, 1997). The wide distribution of the ASTs indicated by these results reinforces their pleiotropic roles.

8.7.2.3. Pleiotropic roles of allatostatins It is clear from the enormous bulk of work on the ASTs that, as with the ATs, their actions are not limited to the modulation of the production of JH and that they frequently do not have an allatostatic role in the insect from which they are isolated. The functions of ASTs have been loosely grouped into six categories: (1) inhibition of JH biosynthesis; (2) modulation of muscle contraction in the heart and gut; (3) modulation of neural activity in the central nervous system; (4) inhibition of vitellogenin synthesis/release; (5) inhibition of the production of ecdysteroids by prothoracic glands and ovaries *in vitro*; and (6) modulation of digestive enzyme activity (Bellés *et al.*, 1999; Stay, 2000; Gade, 2002; Tobe and Stay, 2004). The same AST can exert several different tissue-specific effects at various developmental stages in different insect species.

The ubiquitous nature of these peptides in insects and in other invertebrates, plus the conservation of core sequences within them, speaks to their ancient origin. It has been suggested that their function as regulators of JH biosynthesis evolved before the rise of the insects (Tobe and Stay, 2004). The great profusion of ASTs, even within a single species, suggests flexibility and overlap in their functions, although it is clear that in some instances, a single AST or structurally similar group can have a narrowly defined function and only minimal activity in other roles. It has been noted that in insect species more recently evolved than cockroaches, there are fewer ASTs (13–14 in cockroaches compared to 5–9 in Diptera and Lepidoptera), and a concurrent loss of their role as inhibitors of JH biosynthesis (Tobe and Stay, 2004). Since a single AST can have different effects at different times, it is not possible to conclude that the loss of AST function is the direct result of the loss of their numbers, and studies of the molecular evolution of the ASTs are needed to resolve this question (Donly *et al.*, 1993a; Ding *et al.*, 1995; Bellés *et al.*, 1999).

8.7.2.4. Other types of allatostatins A partially identified factor from the brains of late last instars of *M. sexta* has been found to inhibit JH production irreversibly in a combination *in vivo/in vitro* assay (Bhaskaran *et al.*, 1990; Unni *et al.*, 1993). CA exposed *in vitro* to the brain factor are then implanted into penultimate instars to assay their activity. Because this factor appears to be responsible for stable inhibition of JH biosynthesis until after metamorphosis, it was given the name allatinhibin to distinguish it from ASTs, whose effect is fast and reversible.

There are also nonneural factors of unknown structure that can inhibit JH synthesis by the CA. The ovary appears to be a prime source for these factors, which is not surprising, given the fact that JH regulates reproduction. Early work by Stay and Rankin (Stay *et al.*, 1980; Rankin and Stay, 1985) indicated that the ovary of *D. punctata* has a role in the decrease of JH synthesis at the end of vitellogenesis. Allatostatic peptides have been extracted from prechorionogenic ovaries of *G. bimaculatus* (Hoffmann *et al.*, 1996) and *L. migratoria* (Ferenz and Aden, 1993). The partially purified *Locusta* ovarian factor is small (1–1.3 kDa) and inhibits JH synthesis *in vitro* in a dose-dependent manner. The ovarian extract from *G. bimaculatus* also inhibits JH synthesis by CA *in vitro* in a dose-dependent manner, with the potency of inhibition dependent on the stage of the animal from which the CA are taken. Immunoassays using separate polyclonal antisera to *Gryllus* ASTs A1 (FGLamide) and B1 (W-X₆-Wamide) have recently been employed to demonstrate AST-A-like and -B-like immunoreactivity in ovary extracts and partially purified HPLC fractions (Witek and Hoffmann, 2001). The immunoreactive fractions inhibit JH biosynthesis *in vitro*. The AST- and AST-B-epitopes are immunolocalized to the cortical cytoplasm of oocytes in ovaries of both larval and adult crickets; no neural structures within the oocytes are stained. In *D. punctata*, where the ovary has a clear role in the decrease of JH biosynthesis at the end of vitellogenesis, the ovarian factor responsible could be either a neuropeptide sequestered by the ovary or an ovarian peptide with an AST epitope. With the use of a competitive reverse transcriptase polymerase chain reaction assay (RT-PCR) to quantify AST expression, it has been demonstrated that both the lateral and common oviducts and ovaries express message for ASTs and that there are changes in the pattern of expression during the reproductive cycle (Garside *et al.*, 2002). Unlike the situation in *G. bimaculatus*, however, there are no immunoreactive cell bodies in the oviducts or ovary, but there is immunoreactivity in the terminal abdominal ganglion and ventral nerve 7, branches of which innervate the oviducts. Thus, the transcripts quantified by RT-PCR may be generated in the axonal compartment of the allatostatin-containing nerves innervating these structures.

8.7.2.5. Molecular biology of the allatostatins The first AST gene to be cloned was that from *D. punctata*, which encoded for a 41.5 kDa precursor polypeptide (GenBank Accession no. U00444) (Donly *et al.*, 1993a, 1993b). This polypeptide

contains the 13-member family of AST peptides that exist in this species, with appropriate processing sites for endoproteolytic cleavage and amidation. The ASTs are clustered in the precursor, and separating the clusters are three acidic spacers. These spacers are believed to stabilize the precursor, but they have also been proposed to code for a related peptide (Donly *et al.*, 1993a; Ding *et al.*, 1995). Southern blot analysis revealed that there is a single copy of the AST gene per haploid genome. The prepro-ASTs for all cockroach species examined are similar in size, the organization of the ASTs within them (13 or 14) is conserved, and the separation of groups of peptides by acidic domains is maintained (Ding *et al.*, 1995; Bellés *et al.*, 1999). The different AST sequences in the gene of one species were derived from duplication events. Thus, the AST-A family in cockroaches is a good example of a family of peptides with similar sequences derived from a single precursor encoded by a single gene.

The *Drosophila* Genome Project database was screened for sequences corresponding to various insect ASTs. The resulting alignments enabled the cloning of the cDNA for a prepro-*Drosophila* AST containing four putative A-type ASTs (GenBank Accession no. AF263923) (Lenz *et al.*, 2000). A similar approach was taken to identify a prepro-B-type AST that contains two B-type ASTs (GenBank Accession no. AF312379) (Williamson *et al.*, 2001a, 2001c), and a prepro-C-type AST that contains a single C-type AST with one amino acid residue difference from the *M. sexta* C-type AST. The sequence of the C-type AST is identical to that of previously identified dipteran ASTs (GenBank Accession no. AF316433) (Williamson *et al.*, 2001b).

The mRNA for the *G. bimaculatus* AST encodes a hormone precursor that contains at least 14 putative hormones (GenBank Accession no. AJ302036) (Meyering-Vos *et al.*, 2001). Five of these were previously identified in this species, while the remaining ones are known from other species. The deduced prepro-AST sequence is similar to that in cockroaches, but shorter than that of locusts. Regions of the acidic spacers that separate clusters of hormones are conserved between cockroaches and crickets, providing additional evidence that the spacers have a real function.

Identification of the prepro-AST message has been made in two other species. In the mosquito *A. aegypti* the message is about 3×10^3 bases in length and encodes five ASTs (GenBank Accession no. U66841) (Veenstra *et al.*, 1997). The prepro-AST genes are expressed in both abdominal ganglia and midgut. Three cDNAs of 1506 bases encode

ASTs in the German cockroach, *Blattella germanica* (Yang *et al.*, 2000) and all have a nucleotide sequence with strong similarity (~80%) to that of other cockroach cDNAs for the prepro-AST.

8.7.2.6. Control of allatostatin titers The effect of ASTs on JH biosynthesis may occur in a paracrine fashion, i.e., local release within the CA from the axons of cerebral cells that produce these neuropeptides, or in a true endocrine fashion, via the hemolymph. By either means, receptors would mediate the effect of the neuropeptide (see Section 8.7.2.7) and it is ultimately the combination of titer and receptor that determines the effect of a neuropeptide. Recent work indicates that degradative mechanisms targeting ASTs can control the levels to which the CA are exposed. Initial work by Bendena *et al.* (1997) demonstrated that, like most neuropeptides, *Diptoptera* ASTs are susceptible to rapid degradation. One of the ASTs of *B. germanica* has a half-life of 3–6 min in the intact insect, thus explaining why high doses are required for biological effects *in vivo* (Peralta *et al.*, 2000). A study of the degradation of Manse-AST by enzymes in foregut extracts of *L. oleracea* revealed a half-life of 5 min for the AST. Its degradation resulted in two products, both of which are cleaved at the C-terminal side of arginine residues (Audsley *et al.*, 2002).

Dippu-AST 7 and Dippu-AST 9 are subject to two primary catabolic cleavage steps: cleavage by a putative endopeptidase, yielding a C-terminal hexapeptide, and subsequent cleavage of this product by an aminopeptidase to yield a C-terminal pentapeptide. Neither of these hemolymph enzymes inactivates the ASTs, since the C-terminal pentapeptide contains the minimal sequence necessary for the inhibition of JH synthesis (Garside *et al.*, 1997a). However, there are membrane-bound enzymes in the brain, gut, and CA that cleave ASTs at the C-terminus, inactivating them in a two-step process (Garside *et al.*, 1997b). Pseudopeptide mimetic analogs of Dippu-ASTs have been synthesized that are resistant to degradation by hemolymph and tissue-bound peptidases, and these can significantly inhibit JH biosynthesis by CA when injected into mated *Diptoptera* females, as measured by an *in vitro* assay using CA explanted from these females (Nachman *et al.*, 1999; Garside *et al.*, 2000). An outcome of this research is the creation of tools with which the actions of ASTs can be studied in detail, using degradation-resistant analogs.

8.7.2.7. Allatostatin receptors The type and number of receptors for ASTs in the CA control

the timing, duration, and potency these neuropeptides will have on JH biosynthesis. Work on AST receptors has also flourished, particularly with the discovery of such receptors in *D. melanogaster*, the model insect for genetic and molecular biological investigations. The first study to identify AST receptors was carried out by Cusson *et al.* (1991b), who used photoaffinity labeling to demonstrate two putative receptors in *Diploptera* CA. Subsequently, a single receptor for Dippu-AST 7 with a K_d of 7.2×10^{-10} M was found in the CA (Yu *et al.*, 1995), in addition to a single 37kDa receptor in adult female brains that recognized both Dippu-AST 5 and Dippu-AST 7. Since a single K_d was obtained with AST 5 (9×10^{-10} M), but two with AST 7 (1.5×10^{-9} and 3.8×10^{-9} M), it was suggested that there were two receptor sites for Dippu-AST 7 in the brain. A subsequent structure-activity study using synthetic analogs of Dippu-AST 2 revealed two receptor types in *Diploptera* CA (Pratt *et al.*, 1997). The investigators proposed that the C-terminal portion of ASTs contains both the “message” determining its full effect, as well as “address information” determining its binding affinity for the receptor, and that divergent evolution of receptor types occurred with the evolution of multiple ASTs from a common ancestor. More recently, a radioligand-binding assay was used to identify a single class of binding sites for Dippu-AST 7 in midgut membranes with a K_d of 2.1×10^{-10} M (Bowser and Tobe, 2000). In competitive binding assays, Dippu-AST 7 and AST 2 exhibit a higher affinity for the midgut receptor than AST 5, 9, 10, or 11, while the other ASTs did not compete. Clearly, cloning and sequencing of these receptors is needed to sort out the ubiquitous occurrence and varied functions of the multiple ASTs.

Two laboratories have cloned G-protein coupled receptors (see **Chapter 14**) from *D. melanogaster* that are structurally related to mammalian somatostatin/galanin/opioid receptors (GenBank Accession no. AF163775; Birgul *et al.*, 1999; GenBank Accession no. AF253526; Lenz *et al.*, 2000), but which bind a peptide related to the AST family and thus appear to be AST receptors. Lenz *et al.* (2000) termed their receptor DAR-1. Screening the *Drosophila* genome database, these investigators found a second G-protein coupled receptor, termed DAR-2, with a 47% amino acid residue similarity with DAR-1 (Lenz *et al.*, 2001). Expression studies of DAR-2 in Chinese hamster ovary cells indicated it is the cognate receptor for four *Drosophila* A-type ASTs that bind to the receptor differentially. The DAR-2 gene is expressed in embryos, larvae, pupae, and adults, and is mainly expressed in the

gut of third instars. Since the larval gut of *D. melanogaster* contains cells expressing the gene for the prepro-ASTs, the authors concluded that DAR-2 mediates AST-induced inhibition of gut motility. In a simultaneous study, both DAR-1 and DAR-2 were expressed in Chinese hamster ovary cells and tested for activation using synthetic *Drosophila* ASTs and selected *Diploptera* ASTs (Larsen *et al.*, 2001). Both types of ASTs activate DAR-1 and DAR-2, indicating ligand redundancy and cross-species activity.

An apparent A-type AST receptor has been cloned from the cockroach *P. americana*, with about 60% amino acid similarity in the transmembrane regions to the two identified *Drosophila* receptors. When functionally expressed in *Xenopus* oocytes, this receptor exhibits high affinity for cockroach ASTs (GenBank Accession no. AF336364) (Auerswald *et al.*, 2001). An A-type AST receptor has also been cloned from the silkworm *B. mori* (GenBank Accession no. AH011256) (Secher *et al.*, 2001). This receptor (BAR) shows 60% amino acid residue identity with DAR-1 and 48% with DAR-2. The genomic structure of BAR displays two introns that are coincident with, and have the same intron phasing as, two introns in DAR-1 and DAR-2, and the authors conclude that these three receptors are both structurally and genomically related. BAR mRNA is expressed mainly in the gut, and thus this receptor appears to be a gut peptide hormone receptor. Further studies should focus on identifying AST receptors in the CA to define the second messenger cascade that translates the AST signal in the gland, and to elucidate the evolutionary path by which this large class of peptides evolved to regulate JH biosynthesis.

8.7.2.8. Corpora allata sensitivity to allatostatins Humoral factors may play a role in regulating the sensitivity of the CA to ASTs. Unnithan and Feyereisen (1995) found that acquisition of sensitivity to AST by CA from adult mated *Diploptera* females occurs just before choriogenesis and is dependent on a humoral factor. The investigators could manipulate sensitivity by various experimental techniques, e.g., ovariectomy of vitellogenic females disrupts both JH biosynthesis and acquisition of sensitivity. This line of research deserves more investigation, since it could represent the upregulation of AST receptors by other circulating hormones.

8.7.3. Other Factors Regulating Juvenile Hormone Biosynthesis

8.7.3.1. Neurotransmitters It has long been thought that regulation of JH biosynthesis is exerted

via the axons of cerebral neurons that innervate the CA. Yet, it is clear from research on the ASTs that this control could occur via the hemolymph, with release from the CC, the neurohemal organ for cerebral neuropeptides, or other sites of AST-producing cells. Control of JH synthesis by neurotransmitters is also likely, since some of the nerve cells innervating the CA are ordinary neurons (Sedlak, 1985).

Octopamine, a primary insect neurotransmitter, was the first neurotransmitter to be identified in the CA of locusts and cockroaches (Evans, 1985). Studies of octopamine effects on JH biosynthesis *in vitro* have shown either stimulation or inhibition, depending on the species. Octopamine stimulates JH biosynthesis in adult *L. migratoria* (Lafon-Cazal and Baehr, 1988) and in larvae (Rachinsky, 1994) and adults (Kaatz *et al.*, 1994) of *A. mellifera*, but inhibits synthesis in adults of the cockroach *D. punctata* (Thompson *et al.*, 1990) and the cricket *G. bimaculatus* (Woodring and Hoffmann, 1994). In *D. punctata*, inhibition by octopamine is bimodal, with peaks occurring at 10^{-10} M and 10^{-4} M (Thompson *et al.*, 1990).

Recent work on adult females of *D. punctata* has provided the first indication that JH biosynthesis by the insect CA may be affected by the fast excitatory neurotransmitter L-glutamate, acting via ionotropic receptors to modulate the intracellular Ca^{2+} concentration (Pszczolkowski *et al.*, 1999) (see Section 8.7.4.3). Since high Mg^{2+} and kynurenate treatments inhibit the L-glutamate-induced stimulation of JH biosynthesis, it was proposed the NMDA (magnesium-sensitive) and non-NMDA (kynurenate-sensitive) receptors are present in the *Diploptera* CA and are involved in the regulation of JH synthesis. A subsequent study, monitoring rates of JH biosynthesis by *Diploptera* CA *in vitro* in response to L-glutamate agonists and antagonists, identified the receptors present as NMDA-, kainate-, and/or quisqualate-sensitive subtypes of ionotropic receptors. These receptors are coupled to calcium ion channels and are thus ionotropic channels rather than metabotropic channels, the latter being coupled to inositol (IP3)-stimulated Ca^{2+} release (Chiang *et al.*, 2002a).

The first suggestion that dopamine might be involved in the regulation of JH production was found in a study of dopamine in the brains of two cockroach species, where its levels fluctuated significantly in relation to events during the JH regulated ovarian cycle (Owen and Foster, 1988; Pastor *et al.*, 1991a). A similar situation has recently been described in the fire ant, *Solenopsis invicta* (Boulay *et al.*, 2001). Shortly after the study by

Pastor *et al.* (1991a), it was reported that dopamine affects JH synthesis *in vitro* by the CA of adult female *B. germanica*, and that its effect was either stimulatory or inhibitory, depending on the stage of the ovarian cycle (Pastor *et al.*, 1991b). A screen of neurotransmitters in the larval *Manduca* CA using electrochemical detection-HPLC revealed that dopamine was the only biogenic amine in the gland (Granger *et al.*, 1996). Immunocytochemical analysis further corroborated these studies and demonstrated that dopamine is present in both the brain and CA (Granger, unpublished data). Dopamine affects JH synthesis by larval *Manduca* CA *in vitro* differentially, stimulating both JH synthesis and cAMP production by CA in very early fifth stadium glands but inhibiting production of both JH and cAMP after day 2 (Granger *et al.*, 1996) (Figure 9).

Another line of evidence implicating dopamine in the regulation of larval development, possibly through an effect on the CA, derives from studies of the effects of parasitism of the armyworm *Pseudaletia separata* by the wasp *Cotesia kariyai*. Parasitism by this species elevates dopamine levels in the nerve cord and hemolymph, slows normal development, and delays pupation of the host (Noguchi *et al.*, 1995; Noguchi and Hayakawa, 1996). Dopamine levels are significantly elevated in the hemolymph of *M. sexta* larvae parasitized by the wasp *Cotesia congregata* (Hopkins *et al.*, 1998), although no developmental effects are related to the increase. Significantly higher levels of dopamine have also been found in the hemolymph and central nervous system of diapause-bound pupae of the armyworm *Mamestra brassicae* (Noguchi and Hayakawa, 1997). The relationship of elevated dopamine levels and delayed pupation is unclear, although persistent levels of JH are known to delay pupation in Lepidoptera. It is tempting to think that elevated levels of dopamine are stimulating CA activity. However, the converse could equally be true: persistent JH synthesis delaying pupation could elevate dopamine levels. A similar situation occurs in the honeybee: JH has been implicated in the control of honeybee division of labor, where elevated levels of octopamine and serotonin in the antennal lobes of adult workers are associated with foraging behavior (Schulz and Robinson, 2001). Treatment of honeybees with methoprene elevates octopamine levels, and these authors concluded that JH modulates octopamine levels in the honeybee brain, and that octopamine acts downstream of JH to influence behavior (Schulz *et al.*, 2002).

Neurotransmitter receptors exist in the CA, which is not surprising given that these glands

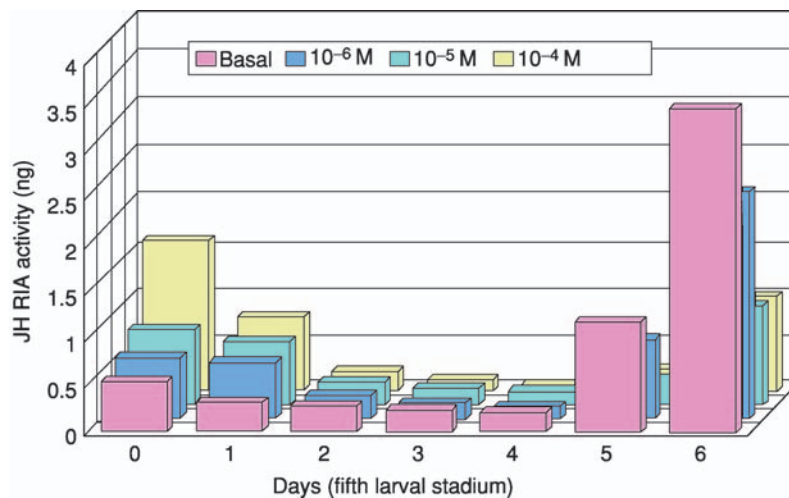


Figure 9 Effect of dopamine synthesis on JH biosynthesis *in vitro*. *Manduca sexta* CA from different days during the last larval stadium were incubated with increasing concentrations of dopamine. JH III/JH III acid synthesis was measured by radioimmunoassay (RIA) and expressed as ng of JH III radioimmunoassay equivalents. (Adapted from Granger, N.A., Sturgis, S.L., Ebersohl, R., Geng, C.X., Sparks, T.C., 1996. Dopaminergic control of corpora allata activity in the larval tobacco hornworm, *Manduca sexta*. *Arch. Insect Biochem. Physiol.* 32, 449–466.)

are extensively innervated by the axons of cerebral neurons and neurosecretory cells. The receptors for biogenic amines belong to a large superfamily of G-protein coupled receptors, and the nucleotide sequences of the octopamine, tyramine, dopamine D1 and serotonin receptors are known for *D. melanogaster*, *A. mellifera*, *H. virescens*, *B. mori*, and *L. migratoria* (von Nickisch-Roseneck *et al.*, 1996; Blenau and Baumann, 2001). Binding of dopamine to D1 receptors increases adenylyl cyclase activity, while binding to D2 receptors either has no effect or inhibits adenylyl cyclase (Gingrich and Caron, 1993; Vernier *et al.*, 1995; Huang *et al.*, 2001). D2-like receptors have not yet been cloned in any insect, although pharmacological studies suggest they exist (Blenau and Baumann, 2001), and receptors for biogenic amines in the CA have not been pharmacologically characterized except for the dopamine receptors in the CA of *M. sexta* last instars (Granger *et al.*, 2000). Because dopamine both stimulates and inhibits JH synthesis and adenylyl cyclase activity, depending on developmental stage, these glands undoubtedly possess both D1-like and D2-like receptors (Granger *et al.*, 1996). The CA D2-like receptor was found to have some pharmacological resemblance to vertebrate D2 receptors, but certain vertebrate D1 receptor agonists/antagonists were found to be equally effective as D2 receptor agonists/antagonists. By contrast, the CA D1-like receptor was found to be pharmacologically distinct from vertebrate D1 receptors (Granger *et al.*, 2000). This fits with the overall picture of dopamine receptors that have been cloned from insects—they display pharmacological properties that

set them apart from vertebrate receptors (Blenau and Baumann, 2001).

8.7.3.2. Interendocrine control by ecdysteroids

If, according to the axiom of Carroll Williams (1976), JH is the handmaiden of ecdysone, then one might ask: Do ecdysteroids regulate JH biosynthesis? The evidence that ecdysteroids may regulate CA activity comes primarily from Granger, Bollenbacher, and their colleagues, who determined that regulation is interendocrine and occurs via the brain. The increase in the ecdysteroid titer, specifically 20-hydroxyecdysone (20E), that elicits pupal commitment in the last stadium of *M. sexta* and other Lepidoptera, also stimulates the synthesis of JH acids by *Manduca* CA. This effect, exerted only when the CA are incubated as a complex with the brain–CC, occurs in response to physiological concentrations of 20E, and results in the postcommitment increase in the hemolymph titers of JH acids that have been shown to be necessary for development to the pupa (Whisenton *et al.*, 1985; Watson *et al.*, 1986; Granger *et al.*, 1987). A subsequent investigation of the kinetics of the 20E effect revealed that maximum stimulation was achieved in 1 h, supporting the idea of an indirect effect, probably via the brain, and suggesting the existence of a mediating factor (Whisenton *et al.*, 1987). Physiological concentrations of 20E were also shown to stimulate JH synthesis in *Manduca* fourth stadium CA, taken before the normal rise in CA activity at the end of the stadium. This suggests that interendocrine control of JH biosynthesis during larval molting is also exerted by 20E (Whisenton *et al.*, 1987), and that this effect

is specific for a biologically active ecdysteroid, since two biologically inactive ecdysteroids, 22-isoecdysone and 5- α -ecdysone, failed to stimulate the CA. The effective concentrations that stimulate the CA differ by about 200-fold, $\sim 1 \mu\text{g ml}^{-1}$ during larval development and $0.02 \mu\text{g ml}^{-1}$ during pupal commitment, in keeping with the hemolymph concentrations of 20E at those times (Bollenbacher *et al.*, 1981). Interestingly, both of these concentrations stimulate CA from last instars at the time of commitment, and there are no other maxima between these two concentrations (Figure 10) (Granger, unpublished data). Given the fact that Manse-AT does not affect *Manduca* larval CA, it would appear that 20E acts in lieu of an AT in larvae.

Incubation of brain-CC-CA complexes from pharate *Manduca* pupae with physiological concentrations of 20E resulted in a 40% decrease in the production of JH acids (Granger *et al.*, 1987). This decrease did not occur if the glands were incubated with, but separated from, the brain-CC complex. While the level of inhibition seen in these experiments is not sufficient to account for the inactivation of the CA by the end of the stadium, this mechanism could act in concert with Manse-AST, which does inhibit larval glands.

Granger *et al.* (1996) have demonstrated that the dopamine stimulation of JH synthesis by CA from day 0 last instars of *M. sexta* may be downregulated by 20E. Ecdysteroid receptors (see Chapter 7) have been detected in the nuclei of *Manduca* CA as early as day 3 of the last stadium (Bidmon *et al.*, 1992),

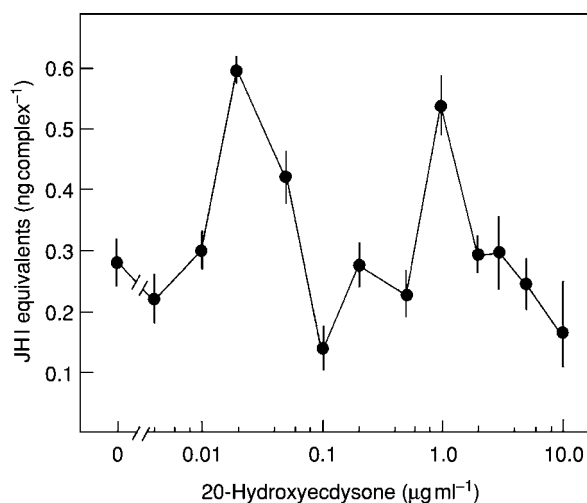


Figure 10 Effect of increasing concentrations of 20-hydroxyecdysone on JH acid biosynthesis *in vitro* by CA incubated as a brain-CC-CA complex, from gate II, day 3 fifth instars of *M. sexta*. Each datum point represents the mean (\pm) SEM, where $n = 5-6$ incubations. Results are expressed as ng JH I RIA equivalents synthesized per complex in a 6 h incubation. (From Granger, unpublished data.)

suggesting the possibility that the negative effect of ecdysteroids on JH biosynthesis involves nuclear receptors and transcriptional control. In a study of the ecdysteroid receptors in *Manduca* CA, it was found that both components of the heterodimeric receptor complex – ecdysteroid receptor (ECR) and Ultraspiracle protein (USP) – are expressed (Gilbert *et al.*, 2000; Granger and Rybczynski, unpublished data). The CA contain two or more USP isoforms that are probably the phosphorylation states of two primary translation products. Preliminary results show that the p49 USP isoform in the CA increases during the feeding period and after commitment to pupation in the last larval stadium (Figure 11). The larger USP isoforms show a maxima surrounding the time of the commitment peak in ecdysteroid titer and during the premolt ecdysteroid peak. This pattern directly contrasts with that of the ECRs which show few differences in relative abundance or isoform diversity in this stadium (Gilbert *et al.*, 2000). Culture *in vitro* of day 1 CA with ecdysteroids causes a decrease in the predominance of the larger USP isoforms and an increase in p49 (Figure 11). This response is also seen in prothoracic glands (Song and Gilbert, 1998). It may be that the changes in ecdysteroid titer are responsible for changes in USP isoform number and abundance during the last stadium and thus might modulate the number and/or kind of genes (for dopamine receptors, for example, which are affected by the ecdysteroid titer) activated by a given concentration of ecdysteroid receptor.

With regard to other possible molecular interactions of ecdysteroids and the JH biosynthetic pathway, the evidence is largely conjectural. As discussed above (see Section 8.4.2), a mammalian farnesoid receptor, FXR, is affected by JH, methoprene, and farnesol in a mammalian transactivation assay. Weinberger (1996) has demonstrated that bile acids, 3- α -hydroxysteroids, and oxysterols are endogenous FXR effectors. Is it possible that another class of steroids, the ecdysteroids, also affect FXR, and could this be a mechanism by which ecdysteroids affect JH biosynthesis?

8.7.3.3. Regulation of juvenile hormone biosynthesis by juvenile hormone There is a paucity of direct evidence that JH regulates its own synthesis. An early study by Granger *et al.* (1986) indicated that neither exogenous JH I nor JH III can inhibit JH I acid biosynthesis by CA from last instars of *M. sexta*. A later, more detailed study of JH feedback in this system confirmed this result (Granger, unpublished data). Richard and Gilbert (1991), using *Drosophila* ring glands, demonstrated that

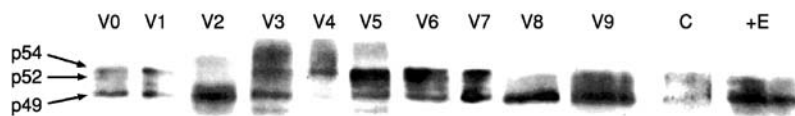


Figure 11 Ultraspiracle expression in the corpus allatum during the fifth larval stadium of *M. sexta*. The proportion of the p49 isoform increases as the stadium progresses, and this shift can be replicated *in vitro* by incubating CA with 20-hydroxyecdysone as described by Song and Gilbert (1998). CA were dissected on the indicated days and subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting with an anti-USP antibody (Song and Gilbert, 1998). V0–V9, days of the fifth larval stadium; C, control, V1 CA; E, V1 CA incubated with 10 μ M 20E for 18 h. Arrows indicate isoforms of USP. (Rybczynski, Moshitzky, and Granger, unpublished data.)

JHIII and JHB₃, but not MF, reversibly inhibited biosynthesis of JHIII, JHB₃, and MF. These results are in agreement with the generally accepted mechanism of negative feedback regulation of an endocrine gland by its product(s).

As discussed above (see Section 8.4.2), mammalian orphan nuclear receptors now appear to be involved in the coordination of the lipid-based metabolic signaling cascade (Chawla *et al.*, 2001). One of these nuclear receptors, RXR, is affected by farnesol and JH, and thus might be of significance in considering regulatory feedback loops of JH on JH biosynthesis. Molecular approaches have led to spectacular advances in our understanding of insect endocrinology, and the future holds great promise with respect to the elucidation of interendocrine control mechanisms.

8.7.4. Second Messengers

Receptors for neuropeptides and neurotransmitters are usually coupled to intracellular reaction cascades, regulating the level of second messengers that transduce these signals (see Chapter 6). The influence of a variety of second messenger systems on JH biosynthesis has been investigated both independently and as signal transducers, including cyclic AMP (cAMP), cyclic GMP (cGMP), phospholipid/protein kinase C, inositol phosphates, diacyl glycerol (DAG), and calcium (Ca²⁺).

8.7.4.1. Cyclic AMP The role of cAMP in the regulation of JH biosynthesis has been studied most extensively in the cockroach *D. punctata*. In an initial study, compounds that increased intracellular cAMP were found to inhibit JH synthesis *in vitro* (Meller *et al.*, 1985). These data suggest that the effect of the ASTs is mediated by cAMP. Of various compounds tested, 8-bromo-cAMP, which is resistant to hydrolysis by phosphodiesterases, and forskolin, a diterpene that activates adenylyl cyclases, were both potent inhibitors of JH synthesis *in vitro*. Furthermore, developmental changes in CA sensitivity to forskolin were found to parallel those to ASTs (Stay *et al.*, 1991a), and exposure of CA to crude brain extracts resulted in both an increase in cAMP

and a decrease in JH production (Aucoin *et al.*, 1987). However, a subsequent study by Tobe (1990) showed that cAMP levels were low in virgin female CA, whose JH biosynthesis is inhibited. More cGMP than cAMP was found in these CA, and cGMP was lowest when JH biosynthesis was at a maximum in mated females. Levels of cAMP and cGMP were then measured in the CA of both virgin and mated females exposed to synthetic Dippu-ASTs 1–4 (Cusson *et al.*, 1992). No significant changes in the levels of either cyclic nucleotide were found, strongly suggesting that neither of these cyclic nucleotides is involved in the signal transduction of the ASTs. Octopamine inhibition of JH biosynthesis in this species is bimodal (see Section 8.7.3.1), but only the inhibition in response to the higher concentration of octopamine (10⁻⁴ M) results in a rise in the levels of intracellular cAMP (Thompson *et al.*, 1990). Thus, the caveat expressed by Meller *et al.* (1985), that the effects of pharmacological agents might only mimic the cellular response to inhibitory factors, appears to hold in *D. punctata*, where only a moderately high dose of octopamine exerts an inhibitory effect via an octopamine-sensitive cAMP.

By contrast, cAMP has a stimulatory effect on larval CA activity in *M. sexta* and is involved in transduction of the dopamine signal. Granger *et al.* (1994) found that relatively inactive CA, such as those of day 4 of the last stadium or from the *black larval (bl)* mutant, were sensitive to a variety of compounds *in vitro* that elicited, or mimicked the effects of elevated intracellular cAMP levels. It was also discovered that the stage-specific effects of dopamine on JH/JH acid biosynthesis by *M. sexta* larval CA *in vitro* were mediated by a calcium/calmodulin sensitive adenylyl cyclase (Granger *et al.*, 1995b). Dopamine at concentrations of 10⁻⁶ and 10⁻⁷ M stimulated adenylyl cyclase activity in homogenates of glands taken from day 0 last instars, when this neurotransmitter stimulates JH biosynthesis, but inhibited enzyme activity in homogenates of day 6 glands, when dopamine inhibits CA activity (Granger *et al.*, 1995b, 1996). A similar approach demonstrated that octopamine had no effect on

enzyme activity in gland homogenates over a wide range of concentrations. It was subsequently found that effects of dopamine receptor agonists and antagonists on JH biosynthesis *in vitro* were mirrored by changes in adenyl cyclase activity (Granger *et al.*, 2000). Thus, in this species, cAMP appears to be important in neurotransmitter effects on the CA.

Transduction of a neurotransmitter signal appears to be mediated by cAMP in the CA of adult male *L. migratoria* as well (Lafon-Cazal and Baehr, 1988). In this species, stimulation of JH biosynthesis by dopamine correlates with an increase in adenyl cyclase activity. In honeybee larvae, both octopamine and serotonin elicit a dose-dependent increase in JH release from intraglandular stores of JH in the CA (Rachinsky, 1994). Incubation of these glands with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) and a threshold concentration of octopamine (10^{-8} M) potentiated the octopamine effect on JH release, indicating that the action of octopamine is mediated by cAMP.

8.7.4.2. Inositol phosphates and diacylglycerol

Other possible candidates for the transduction of neuropeptide signals in insects are IP₃ and diacylglycerol (DAG), which are products of phosphatidylinositol-4,5 bisphosphate hydrolysis. The binding of the neuropeptide to a cell membrane receptor, which then interacts with membrane-bound G proteins, initiates this cascade. Increased IP₃ then elicits the release of Ca²⁺ from intracellular storage sites, while DAG activates protein kinase C (PKC) (Berridge, 1993). The IP₃ second messenger pathway exists in insects (Berridge, 1993). Work by Feyereisen and Farnsworth (1987) demonstrated that phorbol esters, which activate PKC, are potent inhibitors of JH biosynthesis in *D. punctata*, suggesting this pathway as central to the transduction of the AST signal. In a subsequent study, Rachinsky *et al.* (1994) found that treatment of *Diploptera* CA *in vitro* with inhibitors of DAG kinase elevates the concentration of DAG, resulting in a significant, dose-dependent inhibition of JH biosynthesis. A further enhancement of this effect was obtained when the CA were exposed *in vitro* to both DAG kinase inhibitors and ASTs, suggesting that the DAG pathway is indeed involved in the cellular response to the AST signal. Treatment of the CA with thapsigargin, a drug that mobilizes calcium stores without generation of inositol phosphates, significantly stimulates JH biosynthesis by, and also reverses the effect of ASTs on, highly active CA from mated females. It does not affect inactive CA from virgin females. The authors concluded

that IP₃, the other product of phosphoinositide hydrolysis, could modulate JH biosynthesis at specific development time points and thus could be responsible for the decreasing sensitivity of the CA of mated females to ASTs (Stay *et al.*, 1991b). Furthermore, the stage-specific effects of IP₃ on intracellular free Ca²⁺ in turn modulate the antagonistic action of DAG (Rachinsky and Tobe, 1996).

In adult male and female *M. sexta*, Manse-AT induces the production of inositol phosphates, including IP₃, in CA *in vitro*, and two intracellular Ca²⁺ releasing agents were found to stimulate JH biosynthesis *in vitro*, similar to the effect of Manse-AT (Reagan *et al.*, 1992). On this basis, these investigators concluded that Manse-AT activates the IP₃ pathway in adult *Manduca* CA, increasing the intracellular concentration of free Ca²⁺ and thus stimulating JH biosynthesis. Staurosporine, a PKC inhibitor, had no effect on the inositol phosphates, but did stimulate the rate of JH biosynthesis *in vitro* and indicated a role for DAG as well. DAG involvement must be confirmed and the mechanism of staurosporine-induced stimulation must be established.

8.7.4.3. Calcium

Calcium is unique as a second messenger in the CA, in that a transmembrane channel is involved, which may open either in response to ligand-receptor binding or to a change in membrane potential. The resulting increase in cytosolic free Ca²⁺ then interacts with the same cascades of biochemical events as cyclic nucleotides and inositol phosphates. While JH biosynthesis by *Diploptera* CA *in vitro* can be stimulated by the release of intracellular Ca²⁺ stores (Rachinsky *et al.*, 1994; Rachinsky and Tobe, 1996), it appears that calcium stimulation of JH production occurs primarily by its influx from the extracellular environment. Calcium was first indicated as a player in the control of JH biosynthesis in *D. punctata* with the observation that elevated extracellular Ca²⁺ substantially reduces the effects of brain extract (ASTs) on JH biosynthesis (Aucoin *et al.*, 1987). It was then shown that JH synthesis is nearly totally inhibited in Ca²⁺-free medium but is stimulated in media with increasing concentrations of this ion (Kikukawa *et al.*, 1987). Studies by Thompson and Tobe (1986) indicated that Ca²⁺ acts as a second messenger to stimulate JH biosynthesis by entering CA cells through voltage-gated Ca²⁺ channels. Recent work by Chiang and colleagues demonstrated that another type of Ca²⁺-permeable membrane channel is involved in regulation of JH production by Ca²⁺; the receptor-operated, ionotropic L-glutamate receptor (Pszczolkowski *et al.*, 1999; Chiang *et al.*, 2002b).

Elevated Ca^{2+} concentrations in incubation medium stimulate the synthesis of JH *in vitro* by the ring glands of *D. melanogaster* (Richard *et al.*, 1990) and the larval blowfly *Lucilia cuprina* (East *et al.*, 1997), the larval CA of *M. sexta* (Allen *et al.*, 1992a), and adult male CA of the cricket *G. bimaculatus* (Klein *et al.*, 1993). By contrast, JH biosynthesis by the CA of *L. migratoria* appears to be relatively independent of changes in extracellular Ca^{2+} , although calcium ionophores can elicit and increase synthesis, depending on the physiological status of the adult source (gregarious but not solitary) (Dale and Tobe, 1988a).

With *M. sexta* CA, Ca^{2+} levels may vary with the stage of development. Larval CA were found to require extracellular $\text{Ca}^{2+} \geq 0.1 \text{ mM}$ for maximal JH biosynthesis *in vitro*, while JH acid synthesis by glands taken after pupal commitment continues in the absence of extracellular Ca^{2+} (Allen *et al.*, 1992a). Both calcium ionophores and caffeine, which initiate the release of Ca^{2+} from intracellular stores, stimulate JH acid synthesis by postcommitment CA, suggesting that intracellular Ca^{2+} may be the principal source of this ion after commitment. Calcium channel antagonists and calcium channel blockers decrease JH biosynthesis by both larval and postcommitment CA, indicating that Ca^{2+} channels exist in the CA cell membrane and that these channels may be both voltage-gated and receptor-operated.

Measurements of cytosolic free Ca^{2+} in isolated *Manduca* CA cells by use of the fluorescent Ca^{2+} indicator Fura-2 and digitized microscopy revealed that the highest levels of intracellular Ca^{2+} occur on days 0 and 6 of the last stadium, when *in vitro* synthesis of JH and JH acid is at the highest levels (Allen *et al.*, 1992a, 1992b) (Figure 12). Concentrations of free Ca^{2+} in *Manduca* hemolymph peak on days 0 and 7 of the last stadium (Allen *et al.*, 1992a); thus, there is an optimal concentration of Ca^{2+} in the hemolymph at the time when the CA require extracellular Ca^{2+} for maximal synthesis. This finding suggests that extracellular free Ca^{2+} could be responsible, at least in part, for the high activity of the gland at this time. Furthermore, free cytosolic Ca^{2+} levels decrease significantly by day 1, when the hemolymph titers in JH have dropped precipitously (Baker *et al.*, 1987). However, the concentrations of free Ca^{2+} in the hemolymph throughout the last stadium never drop below millimolar concentrations (Allen *et al.*, 1992a). Thus, it is clear that free Ca^{2+} is a significant player in the control of CA biosynthetic activity, but probably as a partner to another signal. It is interesting that steroid hormones are known to stimulate Ca^{2+} influx in certain

biological systems (Blackmore *et al.*, 1990) and that free Ca^{2+} is highest in the CA cells at times when the hemolymph ecdysteroid titer in *M. sexta* is high (Bollenbacher, 1988). Thus, another possible mechanism for interendocrine control of JH synthesis by ecdysteroids (see Section 8.7.3.2) could involve the calcium ion.

A similar approach to measuring free Ca^{2+} in isolated cells of *Diploptera* CA was used to study the effects of thapsigargin and Dippu-AST (Rachinsky and Tobe, 1996). It was found that thapsigargin elevated intracellular Ca^{2+} , confirming the stimulatory effect of thapsigargin on JH biosynthesis via elevated Ca^{2+} ; this effect could not be blocked by the later addition of Dippu-AST. The simultaneous addition of Dippu-AST with thapsigargin prevented the thapsigargin-induced increase in intracellular Ca^{2+} , although, as mentioned previously, the inhibition of JH biosynthesis by Dippu-AST is abolished by thapsigargin (Rachinsky *et al.*, 1994). These conflicting results are not yet resolved.

It has been known for many years that the calcium ion also can act indirectly as a second messenger, by binding to calmodulin (CaM) and eliciting a conformational modification of CaM (see Chapter 6). This enhances the affinity of the Ca^{2+} /CaM complex for target effectors, such as membrane-associated and cytosolic enzymes and transmembrane ion channels, including that for Ca^{2+} (Stoclet *et al.*, 1987). It is of interest to note that a Ca^{2+} /CaM-sensitive adenylyl cyclase has been identified in the CA of *Manduca* last instars (Granger *et al.*, 1995b).

8.7.4.4. Potassium Postassium also affects JH biosynthesis. Treatment of adult female *L. migratoria* CA *in vitro* with high levels of K^{+} stimulates JH biosynthesis, either by stimulating the release of ASTs from nerve endings within the CA or by a direct effect on the CA cells (Dale and Tobe, 1988a). A K^{+} -elicited inhibition of JH synthesis was also reported for *D. punctata* (Rankin *et al.*, 1986), but in both cases, the effect of high concentrations of K^{+} proved to be Ca^{2+} -dependent because it was abolished by Ca^{2+} channel blockers (Dale and Tobe, 1988b).

In summary, based on the evidence to date, it appears that the allatotrophic and allatostatic neurotransmitters, which are in evolutionary terms the more primitive intercellular signaling molecules, operate via cyclic nucleotide second messengers. The neuropeptidergic signals that evolved later are transduced by phosphoinositides and DAG. Calcium plays a central role in the function of both types

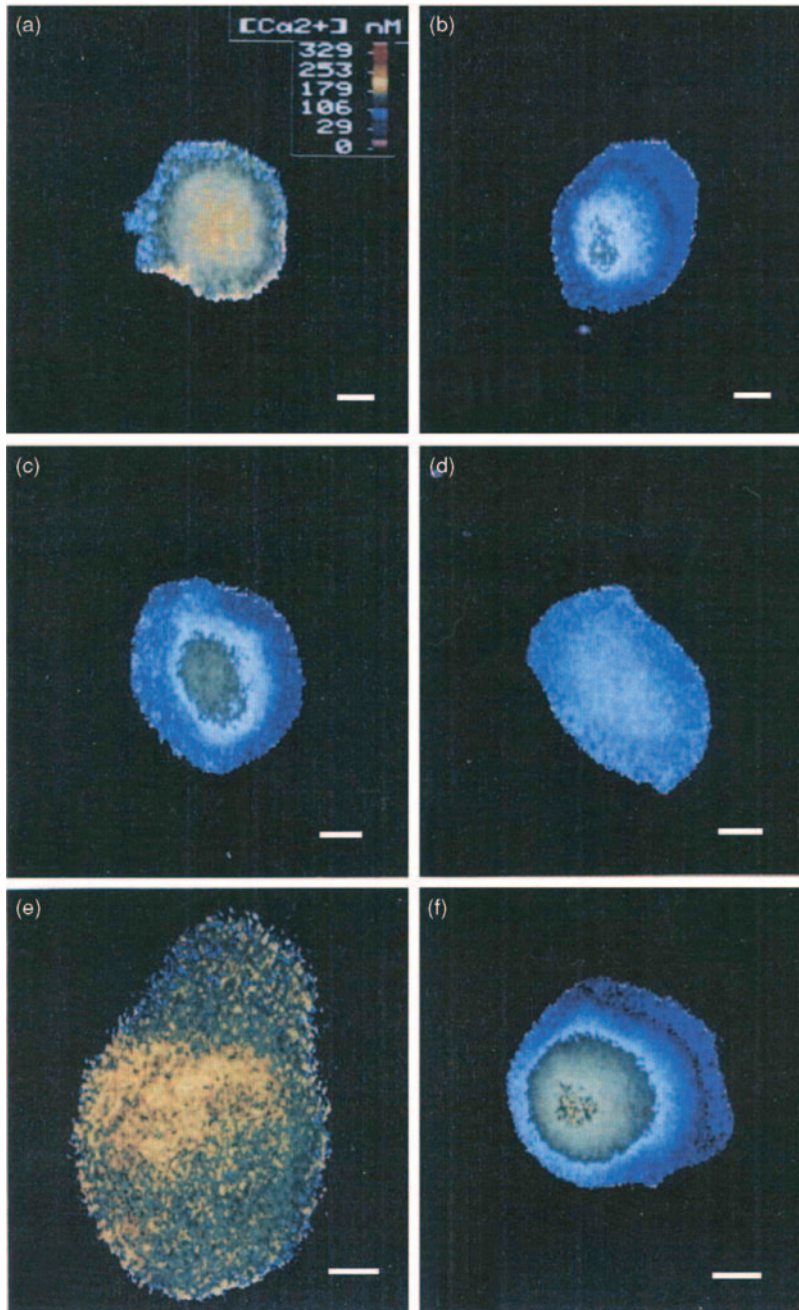


Figure 12 Measurements of cytosolic free Ca^{2+} in isolated *Manduca* CA cells. Pseudocolored ratio images of CA cells from fifth stadium larvae and early pupae. Cells loaded with Fura-2 and incubated in Grace's medium containing $0.1 \text{ mmol l}^{-1} \text{Ca}^{2+}$. (a) Day 0; (b) day 1; (c) day 2; (d) day 4; (e) day 6; (f) day 0 pupal period. The color spectrum in A corresponds to the concentration range (nmol l^{-1}) of Ca^{2+} . Scale bars represent $120 \mu\text{m}$. (Reproduced with permission from Allen, C.U., Janzen, W.P., Granger, N.A., **1992a**. Manipulation of intracellular calcium affects *in vitro* juvenile hormone synthesis by larval corpora allata of *Manduca sexta*. *Mol. Cell. Endocrinol.* 84, 227–241.)

of second messengers, and may itself contribute to the upregulation of gland activity at certain critical times when JH biosynthesis levels are high.

8.7.5. Putting It All Together

Given the long list of factors that can affect the biosynthetic activity of the CA, and perhaps also

its product(s) – something that has not yet been explored, attempts to create an overall mechanism of endocrine control is fraught with hazards. An excellent summary of the roles of calcium and phosphoinositides in second messenger signaling in the regulation of JH production was made by Rachinsky and Tobe (1996), using three different

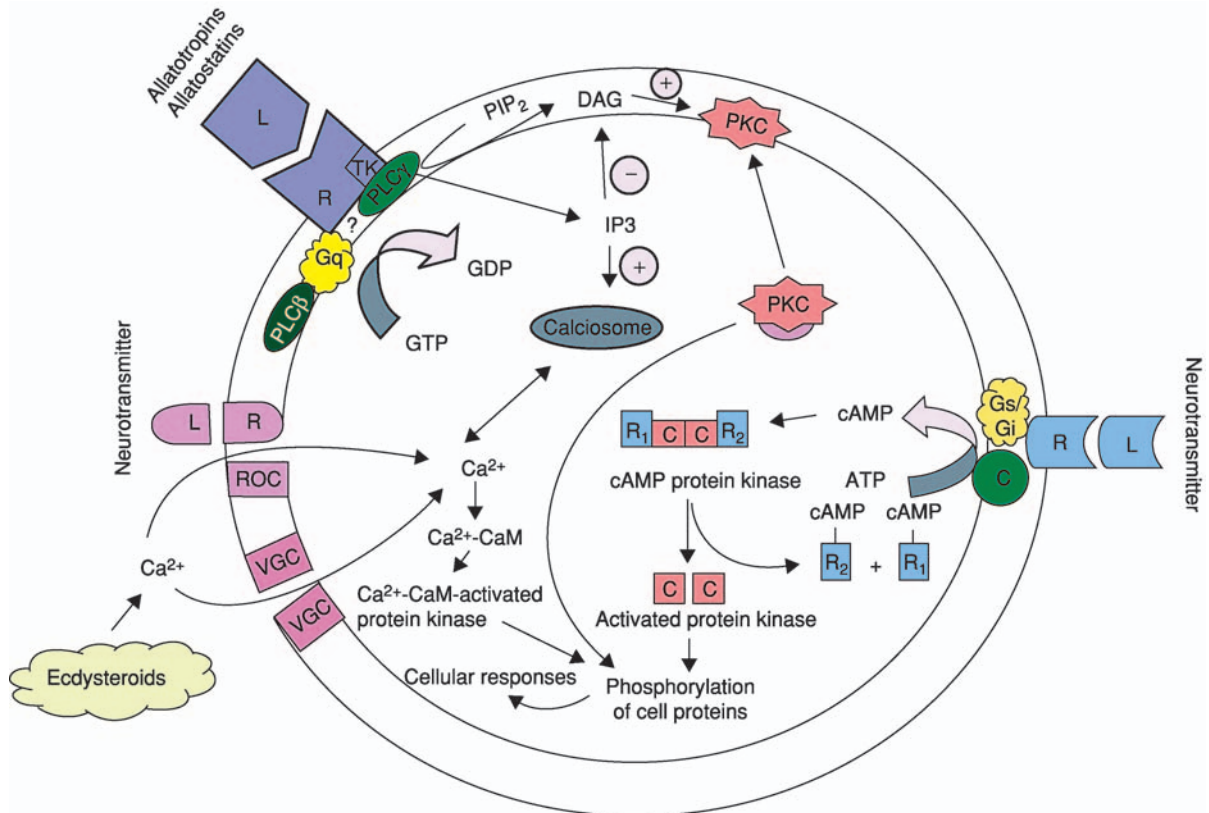


Figure 13 Hypothetical scheme for the regulation of JH biosynthesis by the CA. Factors thought to be involved include neurohormones, neurotransmitters, ecdysteroids, and second messengers. L, ligand; R, receptor; ROC, receptor-operated channel; VGC, voltage-gated channel; c, catalytic subunit; R_{1,2}, regulatory subunit; C, cyclase; G_s, stimulatory G protein; G_i, inhibitory G protein; G_q, G protein associated with phospholipase C; PKC, protein kinase C; DAG, diacylglycerol; IP₃, inositol triphosphate; PIP₂, phosphatidylinositol-4,5 bisphosphate; TK, tyrosine kinase; PLC, phospholipase C.

insects as model systems: *L. migratoria*, in which JH biosynthesis appears to be governed principally by allatotropic signals; *D. punctata*, where this role is filled by ASTs; and *M. sexta*, in which both types of signals occur, perhaps as the result of the complex nature of the products of the CA in this species and possibly coupled with its holometabolous development. They concluded that differences in signal transduction between species reflect, in part, differences in the signals themselves, and that these differences may be stage-specific.

With the complicated nature of this discussion, which excludes a consideration of signals other than neuropeptides, neurotransmitters, and selected hormones, we have chosen to create a scheme for a CA cell that is representative of all insects, responding to these various types of signals and containing all of the second messenger systems shown to exist thus far (Figure 13). Thus, this cell responds to ATs and ASTs, neurotransmitters, ecdysteroids, and Ca²⁺, operating via either voltage-gated or receptor-operated channels. It contains adenylyl cyclase, phosphoinositide/DAG, and Ca²⁺ second messenger

systems, involving PKC. The complexity of this scheme indicates how much work is yet to be done on the basic mechanisms regulating JH biosynthesis at the level of the gland.

8.8. Hemolymph Transport Proteins for the Juvenile Hormones

The physicochemical nature of JH presents the insect with a serious problem, that of dispersing a lipid hormone via an aqueous circulatory system. While the JH homologs are water-soluble at levels that far exceed the physiological titers normally encountered (Kramer *et al.*, 1976), their amphiphilic nature promotes surface binding (Law, 1980). When dispersed in an aqueous medium, JH displays a marked propensity for nonspecific binding to nearly any surface, making its distribution problematic. Early in the evolution of insects and crustaceans, hemolymph proteins arose that interacted with JH in a noncovalent fashion, yet allowed them to be dispersed in the aqueous environment of the hemolymph (Li and Borst, 1991; King *et al.*, 1995). These

transport proteins, with their hitch-hiking hormones buried in a hydrophobic pocket, provided early arthropods with a readily available source of hormone that could dissociate from the protein and interact with target tissues. These transport molecules have evolved into highly specific hormone carriers that have been extensively studied since their discovery in the 1970s (Trautmann, 1972; Whitmore and Gilbert, 1972).

The hemolymph of most insect species contains two classes of JH-binding macromolecules: (1) non-specific binding molecules characterized by a high equilibrium dissociation constant ($K_d = >10^{-6}$ M; low affinity), and (2) specific binding molecules exhibiting a low equilibrium dissociation constant ($K_d = <10^{-6}$ M; high affinity) (Goodman, 1990). JH binding proteins of both types have been reported in approximately 50 species (Goodman, 1983; Goodman *et al.*, 1991; Trowell, 1992). The association of JH with low affinity proteins may be biologically irrelevant, given the surface active nature of the hormone and the high probability of its metabolism. Examples of low affinity interactions between JH and proteins can be observed when photoaffinity-labeled JH probes are incubated *in vitro* with hemolymph proteins (Prestwich, 1987; Prestwich *et al.*, 1994). In contrast to the low affinity binders, the high affinity JH binding proteins have captured considerable experimental attention, and it is these hemolymph proteins that will be discussed.

8.8.1. High Affinity, High Molecular Weight Hemolymph Juvenile Hormone Binding Proteins

The circulatory system of a number of insect species contains high affinity, high molecular weight hormone binding proteins, here termed hemolymph juvenile hormone binding proteins (hJHBP) to distinguish them from intracellular hormone binding proteins. Their high molecular weight appellation stems from the fact that the native molecular weight of these binding proteins routinely exceeds 300 kDa. The high molecular weight hJHBPs can be divided into two subgroups, the lipophorins and the storage proteins or hexamerins. These proteins were originally considered low affinity binders but more recent evidence, using the appropriate homologs and enantiomers, indicates the affinity is much higher than first reported (Trowell, 1992; deKort and Granger, 1996).

8.8.1.1. Larval lipophorins as juvenile hormone transporters Lipophorins are multimeric hemolymph proteins that transport dietary lipids,

pheromones, and cuticular lipids to their sites of utilization (Soulages and Wells, 1994). These multifaceted transport proteins also transport JH in a number of insect species belonging to an evolutionarily diverse array of insect orders, including Dictyoptera, Isoptera, Coleoptera, Diptera, and Hymenoptera. Trowell (1992) examined this area extensively and we here focus briefly on lipophorin–JH transport proteins studies that have been reported since that review.

Much of the recent work on JH–lipophorin interaction has centered on the adult insects, and as noted by King and Tobe (1993), the information derived from the adult stage may not be applicable to the larval stage. Indeed, the situation in adults becomes more complicated as both lipophorins and vitellogenins interact with JH during the adult stage (Engelmann and Mala, 2000). Nevertheless, if one assumes that immunological identity between lipophorins of the nymphal and adult stages indicates these proteins have a similar if not identical structure (King and Tobe, 1993), then various characteristics of the larval JH binding lipophorin (JHBL) can be extrapolated from those of the adult. The JHBL from the adult female *D. punctata* is a multimer of 680 kDa, composed of subunits of apolipophorin I (230 kDa) and apolipophorin II (80 kDa) (King and Tobe, 1988). Covalent labeling with a JHIII photoaffinity probe indicates that the binding site resides on the larger subunit only. The protein appears to be specific for the naturally occurring enantiomer (10R)-JHIII and displays a dissociation constant of 3 nM (King and Tobe, 1988). These binding characteristics, high affinity, and selectivity towards the correct enantiomer are in keeping with the JHBLs from other species (Trowell, 1992; deKort and Granger, 1996). Since lipophorin levels in the hemolymph are typically high, ranging from 6 to 16 mg ml⁻¹ during the last nymphal stadium (10–40% of the total hemolymph protein), titers of the transporter are significant and yield a large excess of unoccupied binding sites (King and Tobe, 1993). These investigators also confirmed that JHBL offers protection from enzymatic degradation. As might be expected, titers of the JHBL follow total hemolymph protein levels, with a notable exception approximately one-third of the way through the stadium. At this time, levels of JHBL jump from 4 mg ml⁻¹ to 12 mg ml⁻¹ with no corresponding increase in total hemolymph protein. An even larger rise in JHBL is noted during a corresponding period in *L. maderae* (Engelmann and Mala, 2000). Unfortunately, the rapid increase in titer has yet to be linked to any biological event.

Trowell *et al.* (1994) characterized a JHBL in the hemolymph of larval *L. cuprina*. As is the case with other JHBLs, the blowfly protein is composed of two types of subunits, apolipohorin I (228 kDa) and apolipohorin II (70 kDa). Binding studies indicate that JH III is the preferred homolog followed by JH II > JH I > JH III acid > JH diol. Curiously, no displacement is observed with JHB₃ even though it is the predominant *in vitro* product of the blowfly CA (Trowell *et al.*, 1994). The dissociation constant for JH–JHBL is approximately 30 nM and, as with the other JHBLs studied to date, the fat body is the site of synthesis.

8.8.1.2. Larval hexamerins as juvenile hormone transporters A second type of high affinity, high molecular weight JH transport molecule has been discovered, having the characteristics of a hexamerin. The hexamerins, composed of six 70–80 kDa subunits, are widely distributed throughout the phylum Arthropoda and have been found in insects, crustaceans, and certain chelicerates (Burmester, 2002); however, they are not typically employed as hemolymph JH transporters. To date, only species in the order Orthoptera, including *L. migratoria* (Koopmanschap and deKort, 1988; Braun and Wyatt, 1996) and *Melanoplus sanguinipes* (Ismail and Gillott, 1995), are known to exploit hexamerins as JH transport proteins.

The most thoroughly studied of the JH-binding hexamerins (JHBHs) is that of *L. migratoria*. This protein, a 74.4 kDa protein, as deduced by cDNA analysis (Braun and Wyatt, 1996), contains 15% lipid (Koopmanschap and deKort, 1988). Binding analysis indicates the dissociation constant for (10R)-JH III–JHBH is 1–4 nM. According to Koopmanschap and deKort (1988), the hexamerin is present at relatively low concentrations that never exceed 2% of the total hemolymph protein, yet its hexameric structure allows a single native molecule to bind up to six molecules of hormone. As a result, this JHBH contains very large number of unoccupied hormone binding sites. The 4.3 kb hexamerin mRNA encodes a 668 amino acid protein and contains 2 kb of 3' untranslated region (GenBank Accession no. U74469) (Braun and Wyatt, 1996). Functional assays to locate the JH binding site suggest that it resides in the N-terminus, since a truncated JHBH lacking this region does not bind the hormone. A comparison of its amino acid sequence with other members of the hexamerin superfamily indicates that the JHBH from *L. migratoria* represents a new form that is most closely aligned with the hemocyanins (Braun and Wyatt, 1996).

8.8.2. High Affinity, Low Molecular Weight Hemolymph Juvenile Hormone Binding Proteins

While the high affinity, low molecular weight hJHBPs are limited to only Lepidoptera and Diptera (review: Trowell, 1992), they have, nevertheless, been characterized extensively because relatively large quantities of larval hemolymph can be easily obtained from just a few insects. The low molecular weight hJHBPs are usually monomeric and range in molecular weight from 25 to 35 kDa. Although low molecular weight hJHBPs have been identified in a number of lepidopterans, only those in *B. mori*, *G. mellonella*, and *M. sexta* have been extensively studied.

8.8.2.1. Chemical and physical characteristics The lepidopteran hJHBPs are monomers composed of approximately 220–240 amino acid residues. In species where the primary sequence of hJHBP has been deduced, four to six cysteine residues have been reported. *Manduca sexta* hJHBP, which has six cysteine residues (Park and Goodman, 1993) and *G. mellonella* hJHBP, which has four cysteine residues (Kolodziejczyk *et al.*, 2001), contain two disulfide bridges.

The results of CNBr cleavage, together with extrapolated data from the study of *G. mellonella* hJHBP, suggest that the disulfide bridges in *M. sexta* hJHBP are probably formed from the Cys9 to Cys16 and Cys151 to Cys195 residues. That the cysteine residues at position 9 and 16 are important to JH binding was demonstrated with the *H. virescens* hJHBP. This hJHBP displays a nearly identical alignment of cysteine residues with those in the *M. sexta* hJHBP (Wojtasek and Prestwich, 1995). Using the *H. virescens* hJHBP construct, Wojtasek and Prestwich (1995) generated mutant hJHBPs in which an alanine was substituted for a cysteine residue at each of the cysteine positions. They discovered that Cys9 and Cys16 are critical for JH binding but that cysteine residues at other sites are much less important. Park and Goodman (1993) demonstrated that of the two remaining cysteine residues not involved in disulfide bond formation, one is inaccessible to alkylating agents, implying an internal location within the protein. The other cysteine residue is easily modified, suggesting a location on the surface of the protein. The role of disulfide bridges in overall structure and hormone binding has yet to be determined.

Initial studies indicated that the *M. sexta* hJHBP is not glycosylated (Goodman *et al.*, 1978a); however, more recent evidence suggests hJHBPs from both

G. mellonella and *M. sexta* are indeed glycosylated. Using matrix assisted laser desorption ionization time of flight (MALDI-TOF) measurements to determine the molecular mass of the native protein and to calculate the deduced amino acid residue mass, it has been determined that carbohydrates account for approximately 10% of the molecular mass of the *M. sexta* hJHBP (Goodman, unpublished data). Computer analysis of potential glycosylation sites indicates only one potential motif, that surrounding the Asn66 residue of the mature, secreted protein. The carbohydrate moiety can be enzymatically cleaved from the hJHBP using *N*-glycosidase F; however, hJHBP is resistant to cleavage by other glycosidases such as neuraminidase and endoglycosidase H (Goodman, unpublished data). Computer analysis of potential glycosylation motifs in the hJHBP from *G. mellonella* indicate multiple sites for glycosylation, including an *O*-GalNAc site (Thr116), *N*-glycosylation sites (Asn3, Asn93) and *O*- β *N*-acetylglucosamine (*O*- β GLcNAc) sites (Ser12, Thr116, Thr186) (Duk *et al.*, 1996). Duk *et al.* (1996) confirmed this observation using lectin binding assays, gel staining, and mass spectrometry and suggested there are five major types of oligosaccharide chains, each containing two GlcNAc residues and two, three, or five mannose residues. The function of these carbohydrate moieties is unknown; however, glycosylation of hormone transport proteins in vertebrates is thought to limit proteolytic cleavage of the protein and thereby increase its half-life in the circulatory system (Westphal, 1986). While other potential posttranslational modifications can be predicted from computational programs, no experimental evidence for the modifications has been reported.

The lepidopteran hJHBPs interact with their ligands at concentrations in the low nanomolar range (Goodman, 1990; Trowell, 1992; deKort and Granger, 1996); however, the homologs are not all bound with the same affinity. It was originally thought that the least polar of the homologs was bound with the highest affinity (Goodman *et al.*, 1978b), following the polarity rule which states that within a series of small nonpolar homologous hormones, the least polar of the group will be bound with the highest affinity (Westphal, 1986). Park *et al.* (1993), in an extensive study of *M. sexta* hJHBP binding kinetics, demonstrated that the polarity of the JH homologs was not as important to the binding equilibrium as first proposed. This study revealed that the dissociation constants for the binding protein hormone complex are approximately the same for JHI or II, but as previously observed, the interaction with JHIII is considerably weaker.

Although the polarity rule may not apply to the equilibrium constants, it is quite clear that the more polar the homolog, the shorter the half-life of the hormone-protein complex (Park *et al.*, 1993). Thus, JHI, the least polar of the homologs tested, has the longest half-life (29 s), while JHIII, the most polar homolog, has the shortest (13 s). These half-times of dissociation are consistent with vertebrate hormone transport proteins (Mendel, 1989).

The hJHBP of *M. sexta* preferentially binds the naturally occurring enantiomers of (10*R*)-JHIII (Schooley *et al.*, 1978b), (10*R*,11*S*)-JHI, and (10*R*,11*S*)-JHII (Park *et al.*, 1993). This enantiomeric specificity appears universal among the high affinity JH binding proteins, whether they are lipophorins or hexamerins (Trowell, 1992; deKort and Granger, 1996), indicating the binding site for the hormone is reasonably selective.

In addition to binding studies using the JH homologs and their respective enantiomers, studies have been conducted using geometrical isomers of the hormones, hormone metabolites, and biologically active analogs (Goodman *et al.*, 1976; Peterson *et al.*, 1977, 1982). The results indicate that highly active JH analogs and JH metabolites do not interact with hJHBP. Armed with this information, several investigators have speculated on the nature of the ligand binding domain and its constituent residues. Goodman *et al.* (1978b) suggested the binding site may be a hydrophobic cleft on the surface of the protein. The primary interactions between the hormone and the binding site would occur along the alkyl side chains of the hormone and the methyl ester moiety at C1, which together form a distinct hydrophobic surface. Perturbation of that surface, especially by the introduction of highly polar groups at C1, significantly reduces binding. However, the site is not sterically hindered since nonpolar additions such as ethyl and propyl groups at the C1 position reduce binding only moderately (Peterson *et al.*, 1977). Hydrogen bonding, which involves electrophilic residues in the binding site, potentially anchors the hormone at the epoxide and C1 positions and may be more important for interaction with JHIII than JHI. Support for this idea comes from the studies of Prestwich and Wawrzęńczyk (1985), who found that the interaction between the naturally occurring JHI enantiomer and hJHBP was only several fold greater than its antipode, suggesting that the binding site could not only accommodate the 10*R*,11*S* enantiomer but the 10*S*,11*R* enantiomer as well. In contrast, the significant binding energy derived from the (10*R*)-JHIII epoxide-hJHBP interaction is probably disrupted in the (10*S*) configuration. Thus, the reduction in

the hydrophobic “face” of JH III places considerably more importance on the hydrogen bonding between the epoxide and methyl ester of JH III and the hJHBP.

Using photoaffinity labeling, Touhara and Prestwich (1992) attempted to map the binding site of the *M. sexta* hJHBP. On the basis of labeling patterns, they proposed that the region from Ala184 to Asn226, a region predicted to be a hydrophobic domain, is involved in the recognition of the lipophilic backbone of JH. Another region, Asp1 to Glu34, also appears to be involved, leading these investigators to propose a model in which the two regions are connected by disulfide bridges to form a two-sided hormone binding pocket. More recently, Tesch and Goodman (unpublished data) discovered that trypsin treatment of hormone-saturated (holo-hJHBP) and hormone-free hJHBP (apo-hJHBP) from *M. sexta* yields strikingly different results, raising questions about the Touhara and Prestwich model. When holo-hJHBP is treated with trypsin, the C-terminus of hJHBP from Lys180 onward is rapidly removed. The N-terminus (first 180 residues) of the molecule remains intact for approximately 1 h, despite the fact that it contains at least 10 well-documented tryptic cleavage sites. The trypsin-truncated hJHBP is still capable of binding JH, indicating that the C-terminus (last 46 residues) of the protein, despite its elevated level of hydrophobicity, is not directly involved in binding. Conversely, apo-hJHBP, when treated with trypsin, is completely destroyed within a few minutes. Similar findings were observed in *G. mellonella* (Wieczorek and Kochman, 1991). These results suggest that the binding site is not located in the C-terminus as proposed by the Touhara and Prestwich model but rather lies upstream, closer to the N-terminus. Rodriguez-Parkitna *et al.* (2002) hypothesize that the C-terminus may serve as a “barrel cover” that changes its position upon hormone binding. Thus, while the C-terminus may not be directly involved in hormone binding, it may have other important roles, such as target cell docking functions or hormone transfer to the cell.

Hormone-induced conformational changes have been observed in certain of the vertebrate serum steroid hormone binding proteins (Grishkovskaya *et al.*, 2002), and there is good evidence indicating that hJHBP undergoes a conformational change upon interaction with JH, but the degree of change appears to vary with the species. While the hormone appears to induce conformational changes that mask certain regions, no differences were detected between apo- and holo-hJHBP in sedimentation rate or electrophoretic migration studies in *M. sexta*

(Tesch and Goodman, unpublished data). The studies of Touhara *et al.* (1993) on recombinant *M. sexta* hJHBP did not reveal differences in circular dichroism spectra of apo- and holo-hJHBP, indicating that the hormone does not induce changes in secondary structure. In contrast to these studies, Wieczorek and Kochman (1991) demonstrated a shift in the sedimentation coefficient between the apo-hJHBP (2.30S) and holo-hJHBP (2.71S) forms of the hJHBP from *G. mellonella*, as well as slight changes in electrophoretic mobility between these two forms. More recently, the Kochman group used circular dichroism analyses to confirm their earlier discovery that hormone binding leads to changes in the secondary structure of hJHBP in *G. mellonella* (Krzyzanowska *et al.*, 1998). It is unclear why these changes in secondary structure were not detected in the *M. sexta* hJHBP. It may well be that small but significant differences between the primary and secondary structures of the *M. sexta* and *G. mellonella* hJHBPs are responsible. These initial studies on ligand-induced conformational change are highly intriguing but unfortunately offer only a glimpse into the structure and dynamic conformational changes. Crystallographic studies, such as those currently under way with the *G. mellonella* hJHBP (Kolodziejczyk *et al.*, 2003) will provide the desired information on the nature of the JH binding site and ligand-induced conformational change in the protein.

8.8.2.2. Protein structures The high affinity, low molecular weight hJHBPs that have been sequenced to date display a reasonable degree of identity in alignment (~25%), but as noted by Rodriguez-Parkitna *et al.* (2002), no one region shows a higher than average homology, thus precluding the identification of a putative JH-binding domain (Figure 14). These investigators examined other JH-associating proteins for potential sequence similarities and concluded that there was little similarity among the hJHBPs and ligand binding domains of potential nuclear receptors, insect transferrins, or the high affinity, high molecular weight JHBPs. From this analysis, it may be inferred that the composition and sequence(s) of amino acid residues making up the hydrophobic JH binding domain are not under extreme selective pressure, as long as the site is lined with hydrophobic residues that permit interaction with the aliphatic side chains of the hormone. However, the site must contain the hydrophilic residues to permit interaction with the epoxide moiety.

As more protein sequences enter the data bases, a picture is emerging that suggests the lepidopteran

two closely spaced ECR response elements (see Chapter 7) approximately 5 kb upstream from the start site. The JHs are presumed to act on the ecdysteroid signaling pathway (Riddiford, 1994), regulating gene expression by modulating the transcriptional activity of the ecdysteroid receptor and its heterodimeric partner, Ultraspiracle (Jones and Sharp, 1997) (see Section 8.12.2). Considering that both the protein concentration (Hidayat and Goodman, 1994) and the *hJHBP* gene expression (Orth *et al.*, 1999) undergo a significant decline during a larval-to-larval molting period, it is reasonable to speculate that ecdysteroids, acting via these ecdysteroid response elements, may be involved in the regulation of *hJHBP* expression and may indirectly modulate JH titers.

Recent studies reveal that strain-related polymorphisms in the *hJHBP* nucleotide sequence, outside the open reading frame, lead to significant differences in the titers of this protein. A comparison of hJHBP levels in two different strains of *M. sexta* revealed that one strain, the Seattle wild-type (*Swt*), has levels that are nearly 60% lower than those of the Madison wild-type strain (*Mwt*) (Young *et al.*, 2003). The *Swt* strain exhibits an *hJHBP* allele, *Snb*, that differs significantly from the previously described *Md* allele found in the *Mwt* strain (Orth *et al.*, 2003a) by the presence of a novel insertion in intron 2. The relatively small size of the insert (<500 bp), its position between direct repeats, its integration into an A/T rich region, and its lack of an open reading frame all suggest that the element is similar to a small, integrated nuclear element (Adams *et al.*, 1986; Robertson and Lampe, 1995; Jurka and Klonowski, 1996). This element, termed *Ms1*, is abundant in the genome of *Mwt* (Young *et al.*, 2003); however, the *Md* gene locus of the strain does not contain *Ms1*. While lepidopteran insertion elements are not particularly novel, the effect of *Ms1* on *Swt* expression of *hJHBP* is significant. A potential mechanism by which *Snb* may depress hJHBP titers can be deduced from the sequence of *Ms1*. Zhou and Liu (2001) report that sequences within the *B. mori* repetitive insertion element *Bm1*, termed matrix-associating regions (MARs), interact differently with the nonchromatin scaffolding than do insertion elements lacking MARs. The insertion of MARs into a gene or locus may have significant effects on gene expression by hindering DNA unwinding during transcription (Walter *et al.*, 1998; Carey and Smale, 2000); thus, expression of hJHBP by the *Swt* strain may be downregulated by this unique insertion element. From the physiological standpoint, the reduced levels of hJHBP in individuals expressing the *Snb*

gene locus appear to have negligible effects on JH titers at hour 50 of the fourth stadium, the only time point examined in this study. Due to the high levels of hJHBP in wild-type animals (Hidayat and Goodman, 1994) and the high affinity of hJHBP for JH (Park *et al.*, 1993), it may well be that the insect can withstand a loss of as much as 60% of the circulating hJHBP and still maintain levels of the hormone sufficient to prevent metamorphosis. The discovery of *Ms1* serves as a warning that laboratory strains of the same species, held in reproductive isolation for long periods of time, are rapidly diverging from their common ancestor. The belief that all wild-type strains of the same species will respond to physiological challenges in an identical fashion may be a naive assumption.

8.8.2.4. Regulation The discovery of the high affinity JHBP naturally leads to the question of whether these proteins have a direct influence on JH titers. In most insects, the exceedingly high ratio of hJHBP to JH makes it unlikely that even significant shifts in hJHBP levels would play a direct role in hormone regulation (Hidayat and Goodman, 1994). As noted above, a 60% reduction in hJHBP titers in a strain of *M. sexta* does not markedly affect JH titer. Nevertheless, the hJHBP titers fluctuate significantly during development (Goodman, 1990; Hidayat and Goodman, 1994; Rodriguez-Parkitna *et al.*, 2002), and these changes do not mirror the changes in total hemolymph protein levels. Assessment of bound and unbound hormone levels indicates that virtually all of the hormone is bound at any given time and that the level of holo-hJHBP is a tiny fraction of apo-hJHBP. Why should the fat body, the site of synthesis of hJHBP (Nowock *et al.*, 1975), be engaged in excessive synthesis of this protein, and why should its levels be so precisely controlled? The presence of excess hJHBP remains a mystery, but one intriguing hypothesis suggests that the protein acts as a high affinity scavenger to sequester the hormone in the hemolymph where it can be metabolized by hemolymph enzymes (Touhara *et al.*, 1996). This scavenger hypothesis has also been proposed for the role of hJHBP in the embryo, where maternal JH may need to be compartmentalized to prevent interference with embryonic development (Orth *et al.*, 2003b). While this hypothesis is provocative, it assumes that cellular degradation of the hormone is minimal, and as noted below (see Section 8.9.1), this may not be the case.

Although it is unclear why the levels of hJHBP need to be controlled so precisely during development, a picture of the elements controlling the

expression of its gene is beginning to emerge. As previously noted, the presence of ecdysteroid response elements in the 5' flanking region of *hJHBP* suggests ecdysteroids may be involved in the regulation of *hJHBP* expression. Conversely, there is reasonably good evidence that JH itself has a role in regulating the expression of this gene. It has been observed that an inverse relationship exists between JH and hJHBP titers in *M. sexta* (Hidayat and Goodman, 1994). Hormone titers at the beginning of the penultimate stadium are high, but hJHBP titers are low. When JH titers drop during the late intermolt period, levels of hJHBP rise (Hidayat and Goodman, 1994). Topical application of JH during the late intermolt period leads to a striking fivefold increase in hJHBP mRNA, beginning approximately 2 h after hormone application and persisting for approximately 18 h (Orth *et al.*, 1999). The response is both dose-dependent and saturable, with higher levels of JH (>1 ng) inducing a downregulation of hJHBP mRNA abundance. Curiously, hormone application at other times during the stadium does not elicit this response, which has led to the suggestion that JH-induced transcription of *hJHBP* is already maximally stimulated at these times and further elevation of hormone levels by topical application has a dampening response. Thus, the control of hJHBP levels appears to be tied to fluctuating JH titers during the intermolt period; elevated titers of the hormone downregulate hJHBP mRNA and protein, while reduced JH titers elevate both message and protein. During the molting period, the elevated levels of ecdysteroids required for molting reduce expression of the *hJHBP* gene. If the sole function of hJHBP is hormone delivery, then the developmental pattern and excess titer remain a mystery, but as with the vertebrate serum hormone binding proteins, the hJHBPs may be more than transport macromolecules.

8.8.3. Functions

Facilitating the transport and dispersal of JH to distant target sites is the most obvious function of the hJHBPs, yet other equally important roles for the binding protein have been hypothesized or experimentally determined (Westphal, 1986; Goodman, 1990). These functions include: (1) reducing the levels of promiscuous binding to nontarget tissues; (2) reducing enzymatic degradation of the hormone (Hammock *et al.*, 1975); (3) acting as a scavenger to enhance JH metabolism in larval hemolymph (Touhara *et al.*, 1996) or the developing embryo (Orth *et al.*, 2003b); (4) providing a peripheral reservoir of hormone that is readily available at the

target tissue; (5) enhancing JH synthesis in the CA by acting as a sink; and (6) aiding hormone movement from the hemolymph into the target cell. The first four functions are either well established or have at least gained a level of scientific credibility.

Preliminary evidence now suggests that hJHBP may promote the synthesis of JH by dispersing the hormone from the immediate vicinity of the CA. Using the RCA to assess the effect of several JH-interacting proteins (bovine serum albumin, JH-directed antiserum, and *M. sexta* hJHBP) on JH biosynthesis *in vitro*, it was observed that only hJHBP significantly increased the incorporation of radiolabeled tracer into JH (Granger and Goodman, unpublished data). *Manduca sexta* CA synthesized ~60% more JH when incubated in medium containing physiological levels of hJHBP. While the results are preliminary, they do suggest that the binding proteins aid the transit of JH from the CA and thus could prevent hormone from accumulating nonspecifically in the vicinity of the glands. The binding proteins, in essence, create a longer feedback loop and thus block short-circuiting of JH biosynthesis.

The most intriguing unexplored aspect of the hJHBP functions is that of its action at the surface of a target cell. A considerable body of evidence has convinced most vertebrate endocrinologists that hormone binding proteins release their ligands into the circulatory system, thus allowing them to enter the cell in the unbound state (Westphal, 1986; Mendel, 1989; Grasberger *et al.*, 2002). For some hormones, this is a reasonable conclusion; however, several protein-bound hormones and vitamins have specific cell surface receptors for their respective transport proteins (review: Kahn *et al.*, 2002). Retinol binding protein (RBP), for example, binds to a plasma membrane receptor that participates in transfer of the ligand from the serum transport protein to the cytoplasmic retinol binding protein (Sundaram *et al.*, 1998, 2002). As levels of apo-RBP far exceed those of the holo-RBP, a conformational change in the protein must occur so that cell surface receptors are not interacting with ligand-free RBP (Chau *et al.*, 1999).

Even more striking are the roles of sex hormone binding globulin (SHBG), a vertebrate serum protein that binds and transports the sex steroids. As is the case for RBP, target tissues requiring the sex steroids display a plasma membrane receptor for SHBG. Unlike the RBP receptor, the SHBG receptor binds only the unloaded form of the transport protein; the hormone-loaded SHBG complex does not bind (Kahn *et al.*, 2002). Sex steroids then bind to the receptor-bound SHBG on the cell surface which, in turn, activates adenyl cyclase. The role of the

activated second messenger cascade remains unknown. The extracellular transport protein is more than just a vehicle for hormone dispersal; indeed, it may need to be anchored at the cell surface to facilitate uptake. Moreover, the receptor–protein complex may have its own intrinsic signaling ability. Whether this complex cascade of hormone transfer, cell surface receptors, and activation of second messengers occurs in insects remains to be investigated.

8.9. Catabolism of the Juvenile Hormones

The maintenance of effective hormone titers at the target site is a delicate balancing act among various processes: synthesis, delivery, metabolism, and cellular uptake. Endocrine signals, by their very nature, must be transitory to effect their exquisite control of the target response. Soon after the chemical structures of JHs were elucidated in the late 1960s, a search for hormone-inactivating enzymes began that has continued unabated to the present, with better than 350 articles describing some aspect of JH metabolism. This prodigious number of publications reflects the agricultural interest in targeting catabolism as a means of insect pest control, since it is assumed that dramatically increasing or decreasing catabolism of JH may lead to developmental derailment (Bonning and Hammock, 1996). While this novel means of pest control has yet to be commercially exploited, the biological information gleaned from studies on JH metabolism is of considerable importance in understanding hormone titer regulation. For earlier reviews of JH catabolism, the reader is referred to Hammock (1985), Roe and Venkatesh (1990), deKort and Granger (1996), and Gilbert *et al.* (2000).

The initial step in JH catabolism may occur in the hemolymph or in target cells, and results in different products; however, the end point is the same: biological inactivation of the hormone. Hemolymph inactivation of JH is the result of enzymatic activity of several different esterases capable of hydrolyzing the hormone at the C1 position to form the metabolite, JH acid (Hammock, 1985; Roe and Venkatesh, 1990) (Figure 2). Although cellular inactivation of JH is primarily the result of epoxide hydrolases that hydrate the epoxide at the C10, C11 position to form the JH diol (Figure 2), cells also possess esterase activity that yields JH acid (Lassiter *et al.*, 1995; Roe and Venkatesh, 1990). Our examination of JH catabolism begins with the hemolymph where esterases represent the primary mechanism of hormone inactivation.

8.9.1. Juvenile Hormone Esterases

The hemolymph of a number of species contains esterases that are capable of hydrolyzing the ester at the C1 position to form JH acid. While the early literature suggested that there are two classes of esterases responsible for JH hydrolysis, JH-specific esterase (JHE) and nonspecific or general esterases (Sanburg *et al.*, 1975), recent work has been less clear about the role of the nonspecific enzyme(s) (Gilbert *et al.*, 2000). General esterases were originally defined as those enzymes that could metabolize both a general substrate, α -naphthyl acetate, and JH (Sanburg *et al.*, 1975) and were inhibited by the inhibitor *O,O*-diisopropyl phosphorofluoridate (DFP). More recent evidence suggests that the general esterases are less important in JH metabolism than first thought (Roe and Venkatesh, 1990; Gilbert *et al.*, 2000).

Because conversion to JH acid can be carried out by a number of enzymes, Hammock (1985) proposed a working definition for a JHE. From a biochemical standpoint, such an enzyme should have a low apparent K_m for JH and it should therefore hydrolyze JH with a high $k_{cat}:K_m$ ratio (see Section 8.9.1.2). Furthermore, the enzyme must be able to hydrolyze JH in the presence or absence of a JH binding protein. From a biological standpoint, the JHE must have activity that correlates with a decline in JH titer. The premise that these enzymes are vital to hormone titer regulation has thus received considerable attention.

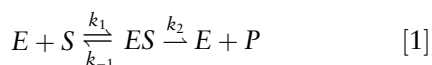
8.9.1.1. Physical properties JH-specific esterases (EC 3.1.1.1) are members of the carboxylesterase family and have been studied in at least six orders of insects, including Thysanura, Orthoptera, Hemiptera, Coleoptera, Diptera, and Hymenoptera (Hammock, 1985); as molecular cloning becomes more widely used, more insect orders will undoubtedly be added to this list. To date, the best characterized of the JHEs are those from the hemolymph of Lepidoptera.

The JHEs of Lepidoptera appear to be composed of a single polypeptide chain with a relative molecular mass of approximately 63–67 kDa (Roe and Venkatesh, 1990; Hinton and Hammock, 2003). The hemolymph of the coleopteran *L. decemlineata*, contains a JHE with a native Mr of 120 kDa; it appears to be composed of two polypeptide chains of approximately 57 kDa each (Vermunt *et al.*, 1997a). A similar situation exists in the orthopteran *Gryllus assimilis*, where the native JHEs displays a Mr of 98 kDa and is composed of two identical subunits of 52 kDa (Zera *et al.*, 2002). The

isoelectric points of all the JHEs studied are acidic, falling into the range of 5.2 to 6.1 (Abdel-Aal and Hammock, 1986; Hinton and Hammock, 2003). Isoelectric focusing of apparently pure JHE from several species yields two to four distinct proteins, suggesting heterogeneity in posttranslational modifications such as glycosylation. The JHE of the lepidopteran *T. ni* is glycosylated (Hanzlik and Hammock, 1987); however, the JHE of *M. sexta* lacks glycosylation, even though computational analysis of its sequence indicates three potential glycosylation sites (Kamita *et al.*, 2003). It should be noted that even a baculovirus expression system containing a single JHE construct still yielded several isoforms that could not be explained by limited proteolysis or differential deglycosylation (Hinton and Hammock, 2003).

8.9.1.2. Kinetic parameters and specificity JHE kinetics and substrate specificity have long been a central focus of JHE studies, owing to the obvious agricultural potential of this information. Elucidation of JHE kinetic constants also permits predictions of the rate limits of JH metabolism, as well as provide valuable insight into to how peripheral JH levels are regulated. A number of reports from the 1980s indicate that among lepidopterans, the apparent Michaelis constant (K_m) for the naturally occurring JHs ranged from 10^{-8} to 10^{-6} M (Roe and Venkatesh, 1990). JH titers in the species tested are at least 10–100 times lower than the estimated K_m concentration, indicating that the enzyme is very sensitive to changes in JH concentration, but suggesting that its catalytic potential is wasted. Since the K_m values seem unduly high with regard to substrate concentration, investigators have examined the individual rate components of the enzymatic reaction to better explain the observed data (Sparks and Rose, 1983; Hammock, 1985; Abdel-Aal and Hammock, 1986).

In the simplest of terms, the velocity of JHE or any enzymatic reaction can be viewed as the sum of various rate components and the concentration of the reactants, and can thus be defined by the familiar equation:



where E represents enzyme, S represents substrate, ES represent the Michaelis complex, and P the product. Under physiological conditions, where the concentration of JH is several orders of magnitude lower than the K_m , the rate at which JH acid is formed is a function of JH interaction with JHE (k_1 and k_{-1}) and the rate of product formation

(k_2). Several important lessons can be drawn from the kinetic data for JHE. First, JHE has a very high affinity for JH. Second, it has a relatively low turnover number or k_{cat} , where k_{cat} is the maximum number of substrate molecules (JH) converted to product (JH acid) per active site per unit time. These factors make the enzyme a very effective scavenger that can “find” JH at physiological concentrations and convert it to JH acid (Abdel-Aal and Hammock, 1986).

Substrate specificity of JHE represents another characteristic that seems counterintuitive. The K_m and V_{max} that the JHEs display towards the JH homologs is surprisingly low when compared to other substrates, such as α -naphthyl acetate. For example, recombinant *M. sexta* JHE displays a K_m (410 μ M) and V_{max} (21 μ mol min^{-1} mg protein $^{-1}$) for α -naphthyl acetate that is considerably higher than for its natural substrate, JHIII ($K_m = 0.052$ μ M, $V_{max} = 1.4$ μ mol min^{-1} mg protein $^{-1}$) (Hinton and Hammock, 2003). As noted by Fersht (1985), when specificity is used to discriminate between two competing compounds, it should be determined by the k_{cat}/K_m ratios, and not K_m alone. The k_{cat}/K_m ratio for JHIII and α -naphthyl acetate are 27 and 0.04, respectively, underscoring the high degree of specificity of JHE for JHIII (Hinton and Hammock, 2003). While these kinetic parameters suggest that the enzyme has a high degree of specificity, it appears that JHEs from several species also hydrolyze methyl and ethyl esters of JHI and III at very similar rates (Grieneisen *et al.*, 1997). Moreover, even *n*-propyl and *n*-butyl esters of these homologs can serve effectively as substrates, albeit at a lower rate of hydrolysis. As might be expected, the unnatural (2*Z*,6*E*)-JHI ethyl ester isomer is not metabolized by JHEs from *M. sexta* or *H. virescens*.

Since hJHBPs have a significant effect on substrate availability (Peter *et al.*, 1979; Peter, 1990; King and Tobe, 1993), studies on JHE specificity must be performed with relatively pure preparations of the enzyme. In those few studies using highly purified or recombinant *T. ni* JHE, it was determined that the JHE hydrolyzed JHI more rapidly than JHII or III and that it displayed a faster catalytic rate for the naturally occurring enantiomer, (1*R*,11*S*)-JHII, than for the (1*S*,11*R*)-JHII form (Hanzlik and Hammock, 1987). However, a comparison of the k_{cat}/K_m ratios for the enantiomers indicated that hydrolysis of the two forms was equivalent (Hanzlik and Hammock, 1987). The same holds true for the gypsy moth, *Lymantria dispar*, in which the JHE shows no enantiomeric selectivity and appears unable to discriminate between

homologs (Valaitis, 1991). One of the most unexpected groups of JHE substrates is found in the naphthyl and *p*-nitrophenyl series. While it was originally concluded that hemolymph JHE from some species, such as *M. sexta*, could not use α -naphthyl acetate as a substrate (Coudron, 1981), the JHEs of other species can recognize members of the naphthyl series (Rudnicka and Kochman, 1984; Hanzlik and Hammock, 1987). More recently it was demonstrated that recombinant JHE from *M. sexta*, *H. virescens*, and *T. molitor* can hydrolyze naphthyl compounds with chain derivatives eight carbons long, thus dispelling the long-held belief that JHE from *M. sexta* is unable to use naphthyl derivatives as substrates (Kamita *et al.*, 2003).

Another counterintuitive observation is that while the major role of JHE is the conversion of JH to JH acid, both native and recombinant JHEs from several species can, under the appropriate conditions, transesterify JH to form the higher ester homologs, i.e., JH ethyl, JH *n*-propyl, and JH *n*-butyl esters (Grieneisen *et al.*, 1997). Although JHE-mediated JH transesterification may be a curiosity limited to the test tube, Debernard *et al.* (1995) demonstrated that when JHIII, dissolved in ethanol (10 μ l), was injected into *L. migratoria*, it was both converted to JHIII acid and transesterified to the JHIII ethyl ester. With regard to this particular study, it should be noted that care must be taken to avoid artifacts when alcohols are used as carrier solvents for the hormone in JHE assays. Nevertheless, while JHE in a biological milieu clearly serves as an esterase, these new findings imply that the enzyme may have other physiological roles (see Anspaugh *et al.* (1995) for other possible roles for JHE).

8.9.1.3. Protein structure and catalytic site

While the catalytic action of JHE is probably similar to that of other esterases, there is some uncertainty about the molecular mechanism by which it operates and some question as to how it can effectively hydrolyze a chemically stable conjugated ester (Hinton and Hammock, 2003). A putative three-dimensional structure of the JHE of *H. zea* has been generated using homology modeling, a structure-building process that uses computer algorithms, based on the assumption that proteins with homologous sequences have similar three-dimensional structures (Thomas *et al.*, 1999). Using the structures of two well-defined carboxylesterases (sequence identity \sim 28%) as models, these investigators demonstrated that JHE belongs to the α/β hydrolase fold family. The *H. zea* JHE (GenBank Accession no AF037196) possesses a putative catalytic triad of amino acids, including the nucleophilic Ser224,

as part of the characteristic motif, G-X-S-X-G (X represents any amino acid residue). The second and third components to the triad are a base, His465, which is part of the G-X-X-H-X-X-D/E motif, and an acid, Glu353, that lie in a deep cleft of approximately 23 Å. The importance of these residues to JH catalysis was confirmed through site-directed mutagenesis (Ward *et al.*, 1992). In addition to the catalytic site, the cleft is lined with a number of hydrophobic amino acids, a situation that might be expected, considering the hydrophobic nature of JH.

One might reasonably expect the catalytic site of JHEs of other species to show some sequence similarity and, not surprisingly, they do. A comparison of sequences among the known lepidopteran JHEs, those of *B. mori* (Hirai *et al.*, 2002), *Choristoneura fumiferana* (Feng *et al.*, 1999), *H. virescens* (Hanzlik *et al.*, 1989), and *M. sexta*, (Hinton and Hammock, 2001), reveals better than 30% identity. The residues associated with the catalytic site are in complete agreement and display the appropriate alignment. Even in species outside the order, such as *D. melanogaster* (Campbell *et al.*, 1992) and *T. molitor* (Hinton and Hammock, 2003), the catalytic sites align with those in Lepidoptera (Feng *et al.*, 1999; Kamita *et al.*, 2003). The only species that does not show alignment of the putative catalytic site is *L. decemlineata* (Vermunt *et al.*, 1997b), which is surprising given the consensus displayed by another beetle, *T. molitor*. With the exception the putative catalytic site residues and a sequence surrounding Ser224, the JHEs as a family are not highly conserved. This might be expected, however, since only the cleft into which the substrate fits needs to be lined with hydrophobic residues (Kamita *et al.*, 2003).

8.9.1.4. Juvenile hormone esterase inhibitors

The development of effective JHE inhibitors has been an ongoing process since the first studies more than 25 years ago. While JHE inhibitors have yet not been commercialized for agricultural use, they have, nevertheless, been important tools in the discovery of the role of JH catabolism *in vivo*. Two important series of JHE inhibitors have emerged from these studies, the trifluoromethyl ketones (TFKs) and the phosphoramidothiolates (Roe and Venkatesh, 1990). The TFKs, such as 1,1,1-trifluorotetradecan-2-one (TFT), were initially suspected to act as transitional state analogs, mimicking the α,β saturation of JH (Hammock *et al.*, 1982) (Figure 15). The transitional state, where the enzyme and substrate form a complex, represents a transitory period in which the chemical bonds of the substrate

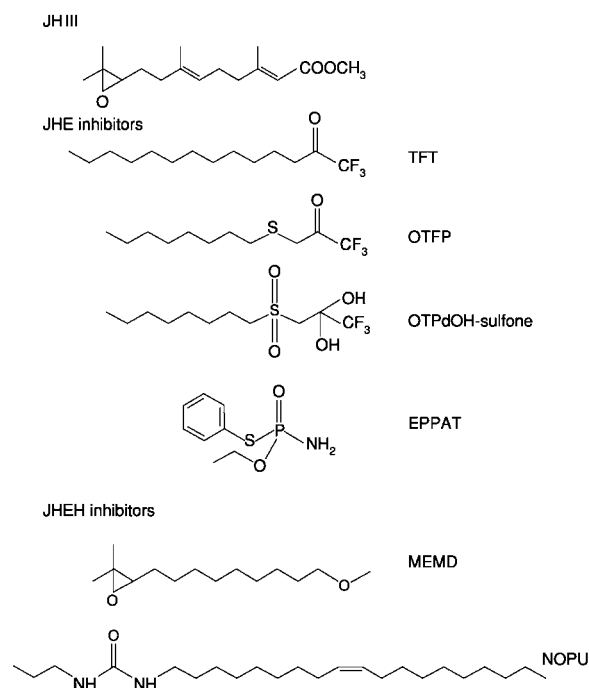


Figure 15 Chemical structures of JH III, JH esterase and JH epoxide hydrolase inhibitors. TFT, 1,1,1-trifluorotetradecan-2-one; OTFP, 3-octylthio-1,1,1-trifluoro-2-propanone; OTPdOH-sulfone, 1-octyl[1-(3,3,3-trifluoropropan-2,2-dihydroxy)] sulfone; EPPAT, O-ethyl S-phenyl phosphoramidothiolate; MEMD, methyl 10,11-epoxy-11-methyldodecanoate; NOPU, N-[(Z)-9-octadecenyl]-N'-propyl urea.

are in the process of being broken and made. Thus, a transitional state analog has the properties of the unstable intermediate. In the catalytic site, the electron-withdrawing inhibitor increases the electrophilicity of the carbonyl group, enhancing its susceptibility to nucleophilic attack by the reactive serine (Hammock *et al.*, 1982). While it was thought that mimicking the JH backbone was important to recognition, further modifications of TFT to resemble JH structure did not improve JHE inhibition. However, introducing a thioether beta to the carbonyl 3-octylthio-1,1,1-trifluoro-2-propanone (OTFP) (Figure 15) increased the potency of inhibition at least 100-fold (Abdel-Aal and Hammock, 1985). More recently, it has been demonstrated that 1-octyl[1-(3,3,3-trifluoropropan-2,2-dihydroxy)] sulfone (OTPdOH-sulfone) is a better inhibitor of JHE than OTFP (Roe *et al.*, 1997). The increased inhibition displayed by OTPd-sulfone stems from the increased hydration (Roe *et al.*, 1997; Wheelock *et al.*, 2001). The attractiveness of the TFK-containing inhibitors lies in their reversible binding of the enzyme, which permits their use in affinity-matrix purification of JHEs. Moreover, the TFKs are useful for *in vivo* studies

since they are highly specific and appear to have little effect on nonspecific esterases (Roe *et al.*, 1997).

In contrast to the TFKs, the organophosphate inhibitors of JHE, most importantly O-ethyl S-phenyl phosphoramidothiolate (EPPAT) (Figure 15), bind irreversibly to JHE (Hammock, 1985). Like the TFKs, EPPAT appears to be highly selective for JHE and can be used *in vivo*. While EPPAT is only a moderately effective inhibitor of JHE, it has a much longer life *in vitro* than the TFKs.

8.9.1.5. Genomic structures At the present time, the genomic structures of only two *JHEs* are completely known, those of *H. virescens* (GenBank Accession no. J04955) (Hanzlik *et al.*, 1989; Harshman *et al.*, 1994) and *D. melanogaster* (GenBank Accession no. AF304352) (Campbell *et al.*, 2001). The *JHE* locus of *H. virescens* is 8 kb in length and is composed of five exons that give rise to an open reading frame of 1.8 kb (Harshman *et al.*, 1994). Unfortunately, nothing is known about the 5' upstream region in this gene, although the core promoter region for *JHE* has been elucidated in another noctuid, *T. ni* (Jones *et al.*, 1998). In *D. melanogaster*, the *JHE* is localized on the second chromosome and covers approximately 2.5 kb in region 52F1-2 (FlyBase). The gene is composed of five exons (note: Flybase incorrectly indicates six exons) that contain an open reading frame of 1.7 kb. In the 5' position immediately adjacent to the *JHE* gene is another carboxylesterase gene (Flybase CG 8424) that displays 42% identity with *JHE* and contains the putative catalytic sites (Kamita *et al.*, 2003). This gene is nearly identical in length and is suggested to be a gene duplication (Campbell *et al.*, 2001). Just downstream from *JHE* is the gene *spin*, which is reported to be implicated in female behavior (FlyBase CG8428). Since gene expression may be coordinately regulated by domain control elements (Spitz *et al.*, 2003), a knowledge of neighboring genes may provide new insights into the regulation of *JHE* and potential molecular cascades in which the gene is involved.

8.9.1.6. Regulation The importance of JHE in regulating JH titers has prompted a number of investigators to examine its physiological and genetic regulation (review: Gilbert *et al.*, 2000). The primary site of synthesis for the hemolymph JHEs, the fat body (Whitmore *et al.*, 1974; Hammock *et al.*, 1975; Wing *et al.*, 1981), has been the primary focus of many studies that have been reviewed previously in some detail (Roe and Venkatesh, 1990; Roe *et al.*, 1993; deKort and Granger, 1996; Gilbert *et al.*, 2000). Tissues other than the fat body can also

express the enzyme (see Roe and Venkatesh, 1990 for a review of the different tissues; Feng *et al.*, 1999). Even the CA have been found to have JHE activity in a number of species (Sparagana *et al.*, 1984; Wisniewski *et al.*, 1986; Meyer and Lanzrein, 1989a, 1989b), and in one case, the glands secrete measurable, and developmentally fluctuating, amounts of the enzyme into incubation medium (Sparks *et al.*, 1989; Janzen *et al.*, 1991). Most studies conclude that the JHEs of different tissues and at different developmental time points are similar, if not identical, to the hemolymph form of JHE; however, their regulation may be different (Jesudason *et al.*, 1992). Sparks *et al.* (1989) made the novel suggestion that the multiple isoforms of hemolymph JHE may be the result of its production and release into the hemolymph from a variety of tissues.

To best understand JHE regulation, a brief description of hemolymph JHE levels is in order. In *M. sexta*, a peak in hemolymph JHE is observed prior to ecdysis in larval stadia two through five (Roe and Venkatesh, 1990), with activity in each stadium increasing in direct proportion to larval weight (Roe *et al.*, 1993). Although levels of JHE activity during the earlier stadia are lower, the precise timing and appearance of JHE and its developmental profile in relation to total hemolymph protein suggests specific regulation. However, the role of the JHE in the early instars is unclear, since hemolymph JH titers remain relatively high even in the presence of enzyme (Hidayat and Goodman, 1994).

In contrast to the earlier stadia, the developmental profiles of JHE activity and of JH titers during the last stadium of *M. sexta* reveal two peaks (Vince and Gilbert, 1977; Baker *et al.*, 1987; Roe and Venkatesh, 1990). Titers of JH are highest in the hours immediately following the molt from the fourth to the fifth stadium (Figure 16). They then drop dramatically, becoming undetectable by 48 h after ecdysis. JH titers rise a second time to peak at 156 h and then decline prior to metamorphosis. JHE activity begins to climb midway through the feeding period and peaks shortly before wandering. A smaller rise in JHE activity appears after the second peak of JH but drops at pupation (Baker *et al.*, 1987). Similar developmental profiles are seen in the last stadium of *S. littoralis* (Zimowska *et al.*, 1989) and *L. decemlineata* (deKort, 1990). Most studies have pinpointed the fat body as the primary source of JHE in the hemolymph (Wing *et al.*, 1981; Wroblewski *et al.*, 1990). However, Jesudason *et al.* (1992) suggest that the first peak of JHE, which occurs during the feeding stage, is of fat body origin, while the second peak, during the prepupal phase, is not. Alternatively, it could be that in this study, the preparation of prepupal fat body samples in the absence of protease inhibitors led to the destruction of JHE at this stage. While the JHE profile in hemolymph appears to be similar for most lepidopterans, it appears to differ outside of this order: some species have a single burst of JHE activity that declines either midway through the last stadium or at the very end of larval life (review: Roe and Venkatesh, 1990).

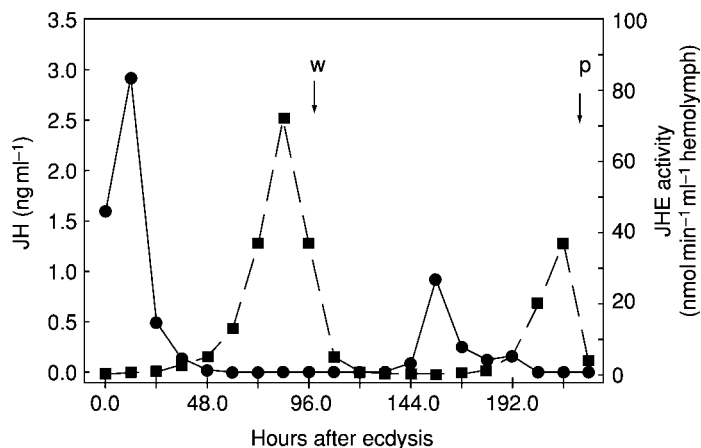


Figure 16 Hemolymph JH titer and JHE activity during the fifth stadium of *M. sexta* larval development. Closed circles, total titer for all homologs detected (JH 0, I, II, III). JH acids are not included. JH titers were combined for both females and males and expressed as the average. Closed squares, JHE activity. JHE activities were combined for both females and males and expressed as the average. W, onset of wandering behavior; P, time of pupal ecdysis. (Adapted from Baker, F.C., Tsai, L.W., Reuter, C.C., Schooley, D.A., 1987. *In vivo* fluctuation of JH, JH acid, and ecdysteroid titer, and JH esterase activity, during development of fifth stadium *Manduca sexta*. *Insect Biochem.* 17, 989–996.)

As can be seen in Figure 16, the rise in JHE activity after the JH titer drops does not support the concept that the enzyme is directly responsible for the decline in JH titers. Nevertheless, the enzyme displays exceptional scavenging properties, and it may be that the increase in enzyme activity occurs to ensure that no detectable JH is present during commitment to pupation. More problematic is the observation that JH is not totally eliminated when JHE is present. For example, a comparison of JH levels in the penultimate stadium of *M. sexta* (Hidayat and Goodman, 1994) with JHE levels during this same period (Roe and Venkatesh, 1990) reveals measurable JH in the presence of JHE. The results of similar studies of the fifth stadium in this species strongly suggest that the pre-wandering peak of JHE has no functional relationship to metamorphosis (Browder *et al.*, 2001). While not stated explicitly, the same conclusion can be drawn from studies in *T. ni* (Sparks *et al.*, 1979; Hanzlik and Hammock, 1988; Jones *et al.*, 1990b), and in still another case, JH levels appear refractory to JHE in diapausing larvae of the European cornborer, *Ostrinia nubilalis* (Bean *et al.*, 1982). Although hemolymph JHE levels follow a developmental pattern that suggests the enzyme may be directly involved in JH catabolism, there remains a degree of uncertainty about the assumed role of JHE in eliminating the hormone from the circulatory system.

That JHE levels do not follow a developmental pattern typical of other hemolymph proteins prompted a search for possible regulatory mechanisms modulating its activity. A logical candidate for JHE regulation is JH itself, but regulation of JHE by JH in lepidopteran larvae, especially during the last larval stadium, depends on whether the insect is in the pre-wandering or post-wandering phase. In the pre-wandering phase of the last larval stadium, JH regulation of hemolymph JHE levels is ambiguous. The brain appears to play an inhibitory role in control of JHE activity, and there are other factors, such as tissue competence, that seem to be involved as well (Venkatesh and Roe, 1988; Jesudason *et al.*, 1992; Roe *et al.*, 1993). In contrast, manipulation of JH titers by surgical and chemical means during the post-wandering period of the last stadium has a direct impact on JHE activity (Roe *et al.*, 1993; review: Gilbert *et al.*, 2000). Treatment of larvae with JH or JH analogs leads to an increase in JHE activity in the hemolymph, while allatectomy leads to a decline in JHE that can be reversed by supplying exogenous hormone. At the molecular level, nuclear run-on experiments using the fat body of *T. ni* have confirmed that exogenous JH or JH analogs stimulate *JHE* expression and that the response can be

detected within 3 h of treatment (Venkataraman *et al.*, 1994). Using a cell line derived from the midgut of *C. fumiferana*, Feng *et al.* (1999) demonstrated that JH can increase the abundance of JHE mRNA within 1 h, suggesting a potential upregulation of *JHE* expression by JH. However, these investigators rightfully caution that the apparent rise in message could also be due to stabilization of JHE mRNA. Curiously, the potent protein synthesis inhibitor, cyclohexamide, mimicked the action of JH, prompting the suggestion that an inhibition of protein synthesis leads to an increase in the accumulation of JHE message or, alternatively, stabilization of the message. In contrast to the apparent stimulation of expression levels by JH, the presence of 20E in the medium leads to a decrease in JHE mRNA, in a dose-dependent manner (Feng *et al.*, 1999). This discovery indicates that regulation of *JHE* expression is more complex than first thought and that ecdysteroids must be considered in the overall regulatory system.

The endocrine system is an important bridge between the target cell and the environment, and it is axiomatic that environmental factors are major players in regulation of the endocrine system. Factors such as stress (Gruntenko *et al.*, 2000), circadian cues that induce diapause (Bean *et al.*, 1982, 1983), nutrition (Cymborowski *et al.*, 1982; Sparks *et al.*, 1983; Venkatesh and Roe, 1988; Roe and Venkatesh, 1990), and parasitism (Hayakawa, 1990; see Edwards *et al.*, 2001 and references therein) are all thought to play a role in regulating JHE levels and are undoubtedly transduced by the nervous system.

8.9.1.7. Catabolism of juvenile hormone esterase

After a rapid increase midway through the feeding period of the last stadium in Lepidoptera, JHE levels drop dramatically immediately prior to the onset of wandering. Experimental evidence corroborates this observation: the half-life of recombinant JHE when injected into second instars of *M. sexta* is approximately 20 min, while other exogenously supplied proteins of similar molecular mass display half-lives measured in days (Ichinose *et al.*, 1992). Since no cleavage products of JHE can be detected in the hemolymph, Ichinose *et al.* (1992) suggested that the enzyme is removed from the circulatory system by cellular uptake. It was discovered that the pericardial cells were responsible for uptake and destruction of the hemolymph JHE (Ichinose *et al.*, 1992). Pericardial cells are a collection of cells that surround the insect heart in various configurations, depending upon the order (Wigglesworth, 1972; Crossley, 1985). In *M. sexta*,

the pericardial mass is punctuated with numerous labyrinthine channels that increase exposure of the cells to the hemolymph (Brockhouse *et al.*, 1999), and it is thought that this tissue mass is responsible for the removal of hemolymph proteins and small colloidal particles from the hemolymph (Crossley, 1985).

It is speculated that the rapid removal of JHE from the hemolymph by the pericardial cells occurs via receptor-mediated endocytosis, followed by transport to lysosomes for destruction (Bonning *et al.*, 1997). During the passage from the endosome to the lysosome, JHE interacts with putative heat shock cognate proteins with relative molecular masses of 100 and 140 kDa. These pericardial cell-specific proteins cross-react with a universal antiserum directed towards heat shock protein 70, hence the designation as cognate (Shanmugavelu *et al.*, 2000). In addition to the heat shock proteins, a novel 29 kDa protein, termed P29, appears to bind and target JHE for lysosomal destruction. This protein has been detected in fat body and pericardial cells of insects from all five larval stadia in *M. sexta*. The P29 gene has been identified using a phage display library and has been sequenced (GenBank Accession no. AF233526).

8.9.2. Juvenile Hormone Epoxide Hydrolases

The preponderance of literature on JH catabolism has focused on the role of JHE in metamorphosis, yet the less well-studied pathway, i.e., epoxide hydration via JH epoxide hydrolase (JHEH), may actually be more biologically relevant in some species (Gilbert *et al.*, 2000). For example, when JHI is injected into early fifth instars of *M. sexta*, the major metabolite is not JH acid or JH acid-diol, but a JH diol phosphate conjugate (Halarankar *et al.*, 1993). Further evidence that JHEH plays a significant role in the reduction of JH titers at critical times can be found in the mosquito *Culex quinquefasciatus*, in which JHEH activity is two to four times higher than JHE activity throughout most of the life cycle (Lassiter *et al.*, 1994). Moreover, two peaks of JHEH are seen during the last stadium, suggesting that the enzyme is involved in JH metabolism during critical developmental periods (Lassiter *et al.*, 1995). Even in *T. ni*, with which so many of the JHE studies have been done, JH diol is a major metabolite or intermediate (Kallapur *et al.*, 1996). It appears that the contribution of JHEH to the overall catabolism of JH varies extensively, even within the same order (Hammock, 1985).

8.9.2.1. Physical properties The JHEHs (EC.3.3.2.3) belong to a large family of proteins

that display the $\alpha\beta$ hydroxylase fold (Debernard *et al.*, 1998) and they are responsible for hydration of the JH epoxide to a diol. Since hydrolases are involved in a wide range of enzymatic reactions and utilize multiple substrates, it has been justifiably cautioned that JHEHs may not be as specific as they are portrayed (Harris *et al.*, 1999). Nevertheless, the term JHEH will be employed as it is commonly used in the literature.

A number of species have been catalogued as having JHEH activity (Hammock, 1985), but in only a few species have the JHEHs been well characterized. Because a single species may have more than one form of JHEH (Harshman *et al.*, 1991; Keiser *et al.*, 2002), it has been suggested that the different JHEHs represent tissue-specific enzymes (Harshman *et al.*, 1991). Epoxide hydrolases are present in both soluble (cytoplasmic) and insoluble (microsomal) forms, but the EHs responsible for hydration of the epoxide are found associated mainly with the microsomal fraction (Wisniewski *et al.*, 1986; Touhara and Prestwich, 1993; Wojtasek and Prestwich, 1996; Harris *et al.*, 1999; Keiser *et al.*, 2002). JHEH appears to be a monomer, with a relative molecular mass ranging from 46 to 53 kDa (Harshman *et al.*, 1991; Touhara and Prestwich, 1993; Harris *et al.*, 1999; Keiser *et al.*, 2002). In general, the pH range for JHEH activity is broad, extending from approximately pH 5 to 9, probably reflecting the involvement of the reactive histidine (pK_a 6.5 for the imidazole group) in the catalytic site (Debernard *et al.*, 1998). The JHEHs of *D. melanogaster* are heat- and organic solvent-tolerant, withstanding incubation at 55 °C and concentrations of ethanol exceeding 40% (Harshman *et al.*, 1991). Moreover, the recombinant enzyme from *M. sexta* retains full activity in the presence of low levels of the reducing agent dithiothreitol and a sulphhydryl modifying reagent, iodoacetamide (Debernard *et al.*, 1998).

8.9.2.2. Kinetic parameters, specificity, and the catalytic site Determinations of the kinetic parameters of the JHEH from the microsomal fraction of *M. sexta* eggs revealed K_m values for JHI, II, and III of 0.61, 0.55, and 0.28 μ M, respectively (Touhara and Prestwich, 1993). Interestingly, the $V_{max}:K_m$ ratio for JH III, the least abundant egg homolog (Bergot *et al.*, 1981a), was 25 times greater than that for JHI, suggesting that the enzyme displays considerably more specificity for JH III than the higher homologs (Touhara and Prestwich, 1993). This observation was confirmed by Debernard *et al.* (1998), using recombinant JHEH from *M. sexta*; while the enzyme could hydrolyze

JHI, II, and several synthetic substrates, JHIII was the most favored substrate. JHIII is also a better substrate for JHEH than JH acid (Touhara and Prestwich, 1993; Kallapur *et al.*, 1996), and in *D. melanogaster*, JHIII is a more suitable substrate than JHB₃ (Casas *et al.*, 1991). If JHIII is the optimal substrate for JHEH, then JHEH may indeed be the enzyme responsible for intracellular JH catabolism, especially since with the exception of JHB₃, JHIII is the only or predominant JH in all insect orders but Lepidoptera (see Section 8.2.1). However, as noted by Harris *et al.* (1999), more direct evidence, such as a correlation between inhibition of the enzyme *in vivo* and a decrease in JH metabolism, is required before assuming the enzyme is JHIII-specific.

As noted by several groups, the high degree of conservation of the catalytic site suggests that the mechanism of epoxide hydration is similar to that found in mammals (Roe *et al.*, 1996; Wojtasek and Prestwich, 1996; Debernard *et al.*, 1998). Using the JHEH of *M. sexta* as a model, Debernard *et al.* (1998) proposed a two-step catalytic process. The first step involves a nucleophilic attack of the epoxide at the least hindered position, C10, by Asp227. This, in turn, opens the ring, leading to the formation of an hydroxyl-alkyl enzyme. The neighboring Trp228 is thought to activate the epoxide for a nucleophilic attack (Harris *et al.*, 1999). The second step involves the hydrolysis of this covalent intermediate by a water molecule that is activated by His428 and Asp350. The histidine residue activates water with an Asp or Glu residue acting as the proton scavenger from the histidine to reactivate the enzyme. The resulting product is (10*S*,11*S*)-JH diol. A variation on this putative reactive site was found by Linderman *et al.* (2000), in which the site uses Glu403 instead of Asp350 as the charge relay partner with the histidine residue. There is remarkable sequence conservation of the catalytic triad and its surrounding residues among both insects and mammals. All JHEHs, like their mammalian counterparts, contain a tryptophan residue at positions 150–155, forming part of the oxyanion hole that may stabilize the hydroxyl-alkyl intermediate (Lacourciere and Armstrong, 1993; Wojtasek and Prestwich, 1996; Keiser *et al.*, 2002).

8.9.2.3. Juvenile hormone epoxide hydrolase inhibitors In contrast to the early and successful discoveries of highly effective JHE inhibitors, the search for JHEH inhibitors has been less productive until recently (Hammock, 1985; Casas *et al.*, 1991; Harshman *et al.*, 1991; Roe *et al.*, 1996). Investigations have taken one of two directions, examining

either compounds that mimic the JH backbone (Roe *et al.*, 1996; Linderman *et al.*, 2000) or compounds based on urea and amide pharmacophores that are not subject to metabolism through epoxide degradation (Severson *et al.*, 2002).

Roe *et al.* (1996) demonstrated that methyl 10,11-epoxy-11-methyldodecanoate (MEMD), a long-chain aliphatic epoxide (Figure 15) displays an I_{50} (molar concentration needed to inhibit 50% of enzyme activity) in the low nanomolar range. A promising group of MEMD analogs was investigated by Linderman *et al.* (2000), who re-examined the structure–activity relationship of MEMD epoxide substitution and enantioselectivity. Two classes of MEMD analogs were synthesized, a glycidol-ester series and an epoxy-ester series. As a group, the glycidol-esters were more potent inhibitors than the corresponding epoxy-esters, by an order of magnitude. The inhibitory activity in both classes was found to be dependent upon the absolute configuration of the epoxide at C10, with the *R* configuration displaying the higher degree of inhibition. The inhibitory activity of the most potent compound of the series ($I_{50} = 1.2 \times 10^{-8}$ M) is thought to be due to the hydroxyl group in the active site, which forms an additional hydrogen bond. This bond may stabilize the enzyme–inhibitor complex by inducing a conformational change, or it could reduce the rate at which the diol product dissociates from the enzyme's active site.

The other class of JHEH inhibitors is the analogs of the ureas and amide pharmacophores that have been demonstrated to be potent inhibitors of mammalian soluble and microsomal epoxide hydrolases in mammals (Severson *et al.*, 2002). To date, none of the nearly 60 compounds tested are as active as the glycidol-esters in the inhibition of recombinant *M. sexta* JHEH (Figure 15), a surprising result, given their action on mammalian enzymes. The most potent of the series, *N*-[(*Z*)-9-octadecenyl]-*N'*-propyl urea (NOPU), has an $I_{50} = 8.0 \times 10^{-8}$ M. Severson *et al.* (2002) suggest that when the inhibitor enters the catalytic site, the carbonyl group of the urea interacts with two tyrosine residues in the oxyanion hole. Since the inhibitor lacks an epoxide, it does not covalently bind to the reactive Asp227 residue, but it does block the catalytic site from further activity.

8.9.2.4. Genomic structures At the present time, the genomic structure of only one insect JHEH gene is known, that of *D. melanogaster*. The gene cluster, approximately 8.6 kb, is located on chromosome 2 in region 55F7-8, near the *JHE* locus at 52F1. As suggested by Harshman *et al.* (1991) and confirmed

through genomic studies (FlyBase), there are multiple forms of *JHEH* in *D. melanogaster*. Annotation of the *JHEH* locus identifies three putative genes that display approximately 37% identity in their amino acid sequence. The genomic region containing *JHEH1* (GenBank Accession no. NM_137541; CG15101) is approximately 1.8 kb and contains four exons. Approximately 0.7 kb downstream from *JHEH1* lies *JHEH2* (GenBank Accession no. NM_137542; CG15102), which is 2.2 kb in length and has two potential transcripts. One transcript has four exons (GenBank Accession no. NM_137524; CG15102) while the other has three (GenBank Accession no. NM_176233; CG15102). At 0.3 kb downstream from *JHEH2* lies the gene *Bari* which encodes a transposable element. *JHEH3* (GenBank Accession no. NM_137543; CG15106) lies about 0.3 kb downstream from the transposable element and is composed of three exons. No genes of obvious relation to JH action or catabolism lie close to the *JHEH* locus. *JHEH1* and 2 are more related to each other than to *JHEH3*; all three putative genes display the residues of the catalytic triad in the correct sequence, and all are of about the same length. Computational analysis using CLUSTALW indicates that *JHEH1* and 3 are most divergent in their first 70 residues, which might be expected if the N-terminal is needed for attachment to cell membranes. Since *JHEH* activity has been demonstrated in other cell fractions besides microsomes (Casas *et al.*, 1991; Harshman *et al.*, 1991), it may well be that the first exons of *JHEH1* and 3 code for sequences that target these enzymes for different locations within the cell.

Recent evidence suggests that the N-terminal of EHs may play another role. The soluble human EH, EPXH2, also possesses phosphatase activity, so the enzyme can not only transform epoxy fatty acids to their corresponding diols but also dephosphorylate dihydroxy lipid phosphates (Newman *et al.*, 2003). The phosphatase activity localized to the N-terminal domain is unaffected by a number of classic phosphatase inhibitors. Alignment of EPXH2 with several of the insect *JHEHs* shows less than 25% amino acid sequence similarity under nonstringent conditions, and regions of similarity are limited to the C-terminal domain. Nevertheless, the possibility of an insect *JHEH* with phosphatase activity presents an interesting twist in the metabolic pathway for JH catabolism (see Section 8.9.3).

8.9.3. Secondary Metabolism of Juvenile Hormone: Juvenile Hormone Diol Kinase

Secondary metabolism of JH has been examined in a number of investigations (Roe and Venkatesh, 1990;

Halarnkar *et al.*, 1993; Grieneisen *et al.*, 1995), but as noted by Halarnkar *et al.* (1993), the results of these studies may be misleading, since the enzymes used may have contained multiple hydrolytic activities. In only one instance has an actual JH conjugate been unequivocally identified. The conjugate, (10*S*,11*S*)-JH diol phosphate (Figure 1), is the product of a two-step enzymatic process: conversion of JH to JH diol and then addition of a phosphate group to C10 (Halarnkar *et al.*, 1993). The enzyme responsible for the phosphorylation of JH diol is JH diol kinase (JHDK), which has been characterized from the Malpighian tubules of early fifth instars of *M. sexta* (Grieneisen *et al.*, 1995; Maxwell *et al.*, 2002a, 2000b). The discovery of JHDK (EC 2.1.7.3) was made when an analysis of JHI metabolites *in vivo* yielded, in addition to JH diol and JH acid, a very polar JH I conjugate that was subsequently identified as JHI diol phosphate (Halarnkar and Schooley, 1990).

8.9.3.1. Physical properties JHDK from *M. sexta* Malpighian tubules is a cytosolic protein composed of two identical subunits of 20 kDa, as determined by mass spectrometry (Maxwell *et al.*, 2002a). Gel filtration studies indicate it has a molecular mass of approximately 43 kDa, and it has been suggested that the native form of the enzyme is homodimeric. JHDK displays a K_m in the nanomolar range for JHI diol, which is appropriate for an enzyme responsible for clearance of a hormone whose titers rarely exceeds 10 nM. Most significantly, the developmental profile of catalytic activity for JHDK parallels that for *JHEH*, a requisite if JH diol phosphate is a legitimate terminal metabolite. Analysis of the k_{cat}/K_m parameter for the diols of JHI, II, and III indicates that JHI diol is the preferred substrate, suggesting a preference for an ethyl group at the C7 position. JHDK requires both Mg^{2+} and ATP for activity (Grieneisen *et al.*, 1995; Maxwell *et al.*, 2002a), although excess Mg^{2+} or Ca^{2+} inhibits its activity (Maxwell *et al.*, 2002a). When stored in the appropriate buffers, the enzyme is reasonably stable but it is very sensitive to various metal ions.

The specificity of JHDK for JHI diol is relatively high, considering the multitude of potential phosphate acceptor groups present in a cell. The enzyme does not recognize methyl geranoate diol (one isoprenyl unit shorter than JH) nor methyl geranylgeranoate diol (one isoprenyl group longer than JH), yet it does recognize JHI ethyl ester diol. It also recognizes both JH diol enantiomers, indicating that absolute stereospecificity of the hydroxyl groups is of minor importance.

Most surprising is the enzyme's inability to recognize JH acid diols. If JH acid diol cannot be phosphorylated by JHDK, the fact that JH diol phosphate is a significant metabolite (Halarnkar *et al.*, 1993) must be explained otherwise. JH acid diol could undergo further catabolism by cytochrome P-450s (Sutherland *et al.*, 1998). However, the role of cellular JHE becomes problematic if the pathway catalyzed by JHEH and JHDK is the major pathway for JH catabolism in the cell. There may yet be undiscovered enzymes in cellular JH catabolism.

8.9.3.2. Genomic structures The sequence and hypothetical structure of the *M. sexta* and *D. melanogaster* JHDK genes have recently been examined by Maxwell *et al.* (2002b). A partial characterization of JHDK from whole body homogenates of *D. melanogaster* indicates that it is similar to the enzyme in *M. sexta*, with the exception of its subunit structure. The active *D. melanogaster* JHDK is composed of a single monomer of ~20 kDa, while the active *M. sexta* JHDK is composed of two identical 20 kDa subunits. This latter gene (GenBank Accession no. AJ430670) has been sequenced and found to code for an enzyme that has 59% sequence identity and >80% similarity to sarcoplasmic calcium-binding protein 2 (dSCP2) of *D. melanogaster* (GenBank Accession no. AF093240; CG14904). Similarities in chromatographic properties, isoelectric point, and enzyme activity led Maxwell *et al.* (2002b) to conclude that dSCP2 is the probable *D. melanogaster* homolog of *M. sexta* JHDK.

Maxwell *et al.* (2002b) used computer modeling and docking programs to generate a three-dimensional model of JHDK. They capitalized on two facts: (1) the catalytic site of JHDK must contain both a purine binding site (MgATP or MgGTP) and a hydrophobic pocket for JH diol; (2) the scaffolding for another sarcoplasmic calcium-binding protein is known. Both the *M. sexta* and *D. melanogaster* JHDKs contain the three conserved nucleotide-binding elements common to nucleotide binding proteins, surrounding the putative substrate-binding site. The model further demonstrates that the protein contains four domains that form two pairs of a helix-loop-helix motif (EF-hand) (Branden and Tooze, 1999). The model's charge interactions in the hydrophobic binding pocket, as well as its depth (19 Å), are complementary to the extended conformation of the diol. Moreover, the hydrophobic nature of the binding pocket complements the C1 ester of the substrate and supports the

observation that JH diol is the only substrate for this enzyme (Maxwell *et al.*, 2002a).

8.9.4. Juvenile Hormone Catabolism and New Directions

The field of JH catabolism is changing with the application of sophisticated analytical tools and the use of metabolism studies *in vivo* to uncover potential catabolic pathways. The combination of the two approaches has led to the surprising discovery that JH diol phosphate conjugates are major JH catabolites, thus emphasizing the role of JHEHs in clearing JH from the body (Halarnkar *et al.*, 1993; Gilbert *et al.*, 2000). That discovery has revealed a significant problem in the JHE phase-separation assay. While this assay is a powerful tool, it also detects polar metabolites resulting from enzymatic activity other than that of JHE. In addition, it is critical that the correct controls be employed. This point is driven home by the fact that JHEs from several species can, under the appropriate conditions, transesterify JH (Debernard *et al.*, 1995; Grieneisen *et al.*, 1997). Thus, the JHE phase separation assay should be employed only as the first step in the search for new JH catabolites. The assay should be followed by the use of the advanced chromatographic tools and detection systems now available for identifying trace polar metabolites. The need to demonstrate biological relevance applies not only to the newly discovered JH-like molecules (see Section 8.3.1), but also to the enzymes involved in JH catabolism. It may well be that some of the enzymes are really not JH-directed, but will, under experimental conditions, generate metabolites not seen under *in vivo* conditions.

Analysis of JHE activity is further complicated by the existence of both hemolymph and tissue JH binding proteins that preferentially bind certain homologs (Goodman *et al.*, 1976; Park *et al.*, 1993) and enantiomers (Schooley *et al.*, 1978b). These proteins have a significant influence on JHE activity (Hammock *et al.*, 1975) due to preferential binding of one enantiomer (Peter, 1990) or homolog (Halarnkar *et al.*, 1993) over another (see Section 8.8.2.1). The recent development of chromatographic methods to separate racemic preparations of JH should alleviate some of these problems (Cusson *et al.*, 1997).

Finally, in the drive to discover a unifying theory to explain JH catabolism, the diversity of the class Insecta is often overlooked. While synthesis of the results of different studies is unavoidable, to assume that all insect species use a single common pathway for JH catabolism would be a gross

oversimplification of the real situation. One need only compare JH metabolism in the flea *Ctenocephalides felis*, which utilizes JHE (Keiser *et al.*, 2002), *D. melanogaster*, which utilizes JHEH (Casas *et al.*, 1991), and the moth *T. ni*, which utilizes both pathways (Kallapur *et al.*, 1996), to understand that multiple pathways for JH catabolism have evolved in this diverse class.

8.10. Juvenile Hormones in Embryological Development

The role of JHs in embryonic development remains an enigma despite the discovery of the hormones in the eggs of *H. cecropia* more than 40 years ago (Gilbert and Schneiderman, 1961). However, the paradigm that so elegantly describes the developmental action of JH during larval to pupal metamorphosis has not, until recently, seemed applicable to the embryonic development of insects. The observation that JH titers rise at different times during embryogenesis of hemimetabolous and holometabolous insects has led Truman and Riddiford (1999) to speculate that JH may have played a significant role in the evolutionary divergence of these two groups. As the evidence presented below indicates, the role of JH during embryonic development warrants further consideration.

8.10.1. Juvenile Hormone Homologs and Precursors Present during Embryogenesis

The JH homologs from embryos of only a few species have been identified thus far (Table 1), giving us a very incomplete picture. What does emerge from these limited data is confirmation that JH III in nonlepidopteran insects is the predominant

embryonic hormone, and following the pattern observed in other stages (Schooley *et al.*, 1984). Furthermore, JH homologs and precursors not detected in larvae or adults exist in eggs, including JH 0, 4-methyl JHI, and the JH precursor, MF. Even more remarkable are the measurable titers of these homologs and precursors in whole-body extracts of embryos. While it is difficult to compare titers in whole-body extracts with those in hemolymph, the levels of JH in the embryo do appear significantly higher than those in hemolymph at any time during larval development (Edwards *et al.*, 2001).

8.10.2. Role of Methyl Farnesoate during Embryogenesis

One of the more interesting JH-like molecules found in embryos is MF, the final precursor in the pathway to JH III biosynthesis. While MF possesses the farnesyl backbone of the JHs, it lacks the terminal epoxide at the C10 position (Figure 1) and exhibits relatively low activity in bioassays when compared to JH III (Sláma *et al.*, 1974). Several investigators have reported high levels of JH III and MF in *N. cinerea* midway through embryonic development (Baker *et al.*, 1984; Lanzrein *et al.*, 1984; Brüning *et al.*, 1985). Using the RCA, Bürgin and Lanzrein (1988) demonstrated that the embryonic CA are indeed synthesizing JH III and MF. The CA of *N. cinerea* become differentiated just before dorsal closure of the embryo, and synthesis of MF and JH III begins shortly thereafter (Bürgin and Lanzrein, 1988). MF levels rise first, followed by JH III about a day later. Both MF and JH III levels remain elevated for several days, exceeding 800 ng g^{-1} , and then decline during the later stages of embryonic development with MF decreasing to undetectable levels (Brüning *et al.*, 1985).

Table 1 Juvenile hormone homologs present during insect embryogenesis

Species	Hormones	Reference
<i>Thermobia domestica</i>	JH III	Baker <i>et al.</i> (1984)
<i>Blatella orientalis</i>	JH III	Short and Edwards (1992)
<i>Nauphoeta cinerea</i>	JH III, methyl farnesoate	Baker <i>et al.</i> (1984), Brüning <i>et al.</i> (1985)
<i>Locusta migratoria</i>	JH III	Temin <i>et al.</i> (1986), Pener <i>et al.</i> (1986)
<i>Telogyllus commodus</i>	JH III	Loher <i>et al.</i> (1983)
<i>Oncopeltus fasciatus</i>	JH III	Bergot <i>et al.</i> (1981a)
<i>Leptinotarsa decemlineata</i>	JH III	deKort <i>et al.</i> (1982)
<i>Melolontha melolontha</i>	JH III	Trautmann <i>et al.</i> (1974)
<i>Heliiothis virescens</i>	JH 0, I, II	Bergot <i>et al.</i> (1981a)
<i>Spodoptera littoralis</i>	JH I, II, III	Steiner <i>et al.</i> (1999)
<i>Manduca sexta</i>	JH 0, I, II, 4-Me JHI	Bergot <i>et al.</i> (1981a)
<i>Hyalophora cecropia</i>	JH 0, I, II	Bergot <i>et al.</i> (1981a)
<i>Bombyx mori</i>	JH I, II, III	Gharib <i>et al.</i> (1983)
<i>Apis mellifera</i>	JH III	Rembold <i>et al.</i> (1992)

Recent studies on another cockroach, *D. punctata*, support the hypothesis that MF is a major product of the embryonic CA (Stay *et al.*, 2002). Using the RCA to monitor rates of MF and JHIII synthesis, these investigators demonstrated that production of MF increases approximately a day before that of JHIII and, as in the case of *N. cinerea*, synthesis of both compounds begin shortly after dorsal closure. Ultrastructural studies of the CA confirm the synthetic activity of the glands after dorsal closure (Lee and Chiang, 1997). In contrast to *N. cinerea*, where MF and JHIII are found in equivalent amounts, the biosynthetic rate of JH in *D. punctata* exceeds that of MF by 30-fold.

Stay *et al.* (2002), examining the role of *D. punctata* allatostatin 7 (see Section 8.7.2) in regulating MF and JH biosynthesis in *D. punctata* embryos, found the process complex. This potent inhibitor of JHIII biosynthesis in adults also inhibits synthesis of MF and JH after dorsal closure. However, prior to dorsal closure, the allatostatin has the reverse effect, stimulating MF biosynthesis. Prior to innervation by neurons transporting the allatostatin, the CA produce MF and can be stimulated by Dippu-AST to produce MF in a dose-dependent manner. Following innervation by Dippu-AST-containing neurons, the glands begin to synthesize both MF and JH, and exogenous Dippu-AST downregulates synthesis of both molecules.

A study with *N. cinerea* embryos is a reminder that MF and JHIII cannot be viewed as equivalent or interchangeable (Brüning and Lanzrein, 1987). Treatment of *N. cinerea* embryos with ethoxy-precocene eliminates JHIII titers but has little effect on MF levels; yet, the investigators were able to rescue ethoxy-precocene treated embryos with JHIII. Interestingly, although ethoxy-precocene is known to induce destruction of the CA in certain insect species, it does not chemically allatectomize this species. Histological studies revealed that as late as 10 days after ethoxy-precocene treatment, the CA were similar to control CA in size and cell number but the nuclei appeared pycnotic. It remains to be seen whether MF is important to embryonic development in *N. cinerea* and *D. punctata*, or in any other species.

8.10.3. Juvenile Hormone Titers during Embryogenesis: Correlation with Developmental Events

As noted previously (see Section 8.10.1), the action of JH during embryogenesis is unclear and may be different from its role of maintaining the status quo role during the larval stage. Associating changes in JH titers to events in organogenesis could provide

clues to the role(s) of JH during embryogenesis. Short and Edwards (1992) measured JH titers in embryos of the cockroach *Blattella orientalis*, and demonstrated that JHIII is present at very low levels at oviposition and in the period preceding dorsal closure. Midway through embryogenesis, JHIII titers rise dramatically, reaching better than 600 ng g^{-1} of tissue before decreasing. Unfortunately, the investigators did not associate the JH titers with specific embryological events, thus making it difficult to link the peak with any specific developmental process. By contrast, Brüning *et al.* (1985) were able to demonstrate that JHIII appears in the embryo of *N. cinerea* only after dorsal closure; no hormone was detected prior to dorsal closure. As in the case of *B. orientalis*, JHIII in embryos of *N. cinerea* rises to a very high level (800 ng g^{-1} of tissue). The titer remains high for about 8 days and then falls to undetectable levels prior to emergence.

Temin *et al.* (1986) conducted a closely timed study of JH titers during embryonic development of *L. migratoria*. During the first 60 h after oviposition, JHIII levels remain reasonably constant at about 15 pg per egg. Blastokinesis, a movement of the embryo in relation to the yolk mass that consists of two phases (anatrepsis and katatrepsis) (Johannsen and Butt, 1941), occurs from 60 to 108 h after oviposition. During this time, JH is undetectable; however, at dorsal closure, which occurs at approximately 120 h, JH titers begin to rise and reach their highest level at 180 h. Unlike the massive peak in the JH titer observed in *B. orientalis* and *N. cinerea*, the peak titer in *L. migratoria* is approximately 45–50 pg per egg. Levels then fall slowly and are undetectable at the time of nymphal emergence. It is interesting that no JH is detected during the period between the initiation of blastokinesis and dorsal closure, a time that corresponds to the most active period of organogenesis (Temin *et al.*, 1986). However, as noted below, exposure to JH during this period can have profound effects on the development of the embryo and larva.

Dorn (1975), using a bioassay to titer embryonic JH in *Oncopeltus fasciatus*, found that newly oviposited eggs contain low, but detectable, levels of JH. He suggests that the hormone found at oviposition is maternal in origin. The titers remain stable during the first 2 days of embryonic development, but then rise rapidly during the third day. This period in *O. fasciatus* is marked by the completion of CA development and katatrepsis. Levels of JH continue to rise during the fourth day of embryonic development, a period in which final dorsal closure occurs, and peak during the fifth day at levels nearly

15 times that found at oviposition. JH levels drop at the time of hatching.

Titers of JH in the embryos of Lepidoptera present a more confusing picture. Using a bioassay, Gilbert and Schneiderman (1961) were the first to demonstrate that unfertilized eggs of *H. cecropia* contained JH. Bergot *et al.* (1981a) subsequently used GC-MS to detect low levels of JHI at 108 h after fertilization, a period following dorsal closure in this species (Riddiford, 1970). After dorsal closure, JH titers rise to a peak at 156 h ($520 \text{ pg tissue g}^{-1}$) and then slowly decline to $50 \text{ pg tissue g}^{-1}$ at the time of emergence, approximately 250 h after oviposition.

In *M. sexta* embryos, organogenesis has been extensively studied and correlated to JH titers. JH titers are undetectable by GC-MS prior to provisional dorsal closure (~ 54 h after oviposition) (Bergot *et al.*, 1981a; Dorn *et al.*, 1987). The titers rise to their highest level between 60 and 72 h after oviposition, when a true larval cuticle appears. Interestingly, this peak in JH is composed of several homologs, including JH0 and 4-methyl JHI, that are unique to the embryonic stage of Lepidoptera. The titers drop to less than 50 pg g^{-1} total JH at the time of hatching, approximately 120 h after oviposition.

Using an HPLC/bioassay method to measure JH levels in the embryo of the noctuid *T. ni*, Grossniklaus-Bürgin and Lanzrein (1990) showed that JH titers rise during the period of early segmentation, prior to dorsal closure, and remain detectable throughout the period of early organogenesis. As has been observed in other species, the JH titer increases to its highest level shortly after dorsal closure and then declines at hatching. The extensive embryological landmarks available for *T. ni* permit a direct correlation of the peak in the JH titer with the formation of the first larval cuticle.

A similar JH profile is observed in another noctuid, *S. littoralis* (Steiner *et al.*, 1999). GC-MS quantitation was used to demonstrate that low levels of JHII are detectable prior to dorsal closure, during a period in which segmentation and limb growth is extensive. JH titers are at their highest after dorsal closure and more precisely, highest at the time the true larval cuticle is being formed. Titers drop as the embryo develops, but in contrast to other species where the hormone declines to undetectable levels at hatching, *S. littoralis* maintains elevated titers of JH through to emergence.

Despite the extensive body of work concerning JH and caste differentiation in *A. mellifera* (Huang *et al.*, 1991; Schulz *et al.*, 2002), there is little information linking embryological markers with JH titers in this species. Rembold *et al.* (1992)

found that JHIII levels are undetectable by GC-MS until very late in embryogenic development (66 h after oviposition). The late peak in the JH titer (72–76 h) may reflect the fact that the CA are not fully developed until 62–64 h after oviposition (Nelson, 1915). Moreover, dorsal closure occurs late in this species, (62–66 h) which is in keeping with the established developmental correlation between dorsal closure and the peak in the embryonic JH titer. It should be noted that the *A. mellifera* embryo does not undergo blastokinesis, thus making it difficult to relate changes in the JH titer with events surrounding and within this process.

As previously noted, the very early presence of JH in the egg of some species has led several investigators to suggest the origin of the hormone at this stage is maternal. In other words, hemolymph JH from the adult female is sequestered by the embryo during the process of vitellogenesis. Gilbert and Schneiderman (1961), in their pioneering work on JH, demonstrated that newly oviposited eggs contain low levels of JH; however, allatectomy of adult gravid females leads to eggs devoid of JH. They, as well as later investigators (Temin *et al.*, 1986; Steiner *et al.*, 1999), proposed that JH is taken up passively by the embryo. Indeed, presupposing that all JH is derived from the CA, this premise would be true, but the possibility remains that extra-allatal sites are involved in JH biosynthesis. Hartmann *et al.* (1987) demonstrated that the serosa of *L. migratoria* is capable of methylation of JHIII acid to form JHIII. Whether this process can yield sufficient JH to account for the amount of JH detected before dorsal closure is uncertain, nor is it clear whether other insects utilize this unique tissue for biosynthesis of JH. A summary of JH titers during embryogenesis is seen in Table 2.

Do these profiles indicate that JH is requisite at key times during embryonic development? Unfortunately, the markers that denote embryological development vary widely among species and make comparisons difficult. For example, blastokinesis varies radically among species (Johannsen and Butt, 1941) and does not provide an adequate morphological marker to make generalizations. Moreover, while the movements of blastokinesis may appear similar in many species, this process may not necessarily signify a developmentally defining homologous event (Heming, 2003). By contrast, dorsal closure is an anatomical reference point that signifies a specific developmental event. However, an embryonic cell layer, more commonly referred to as a membrane, is known to create a provisional dorsal closure that occurs earlier than the final

Table 2 JH levels during insect embryogenesis^a

Order and species	Oviposition	Blastokinesis	Dorsal closure or larval cuticulogenesis	Reference
<i>Hemimetabolous</i>				
Orthoptera				
<i>Locusta migratoria</i>	Low ^b	None	High ^d	Temin <i>et al.</i> (1986)
Dictyoptera				
<i>Nauphoeta cinerea</i>	None	None	High	Brüning <i>et al.</i> (1985)
<i>Blatta orientalis</i>	Low	Low	High	Short and Edwards (1992)
Hemiptera				
<i>Oncopeltus fasciatus</i>	Low	Medium ^c	High	Dorn (1975)
<i>Holometabolous</i>				
Lepidoptera				
<i>Manduca sexta</i>	None	None	High	Bergot <i>et al.</i> (1981a)
<i>Spodoptera littoralis</i>	Low	Not determined	High	Steiner <i>et al.</i> (1999)
<i>Trichoplusia ni</i>	None	Low	High	Grossniklaus-Bürgin and Lanzrein (1990)
<i>Hyalophora cecropia</i>	Low	Low	High	Gilbert and Schneiderman (1961), Bergot <i>et al.</i> (1981)
Hymenoptera				
<i>Apis mellifera</i>	None	None ^e	High	Rembold <i>et al.</i> (1992)
Diptera				
<i>Drosophila melanogaster</i>	None	None ^e	None	Bownes and Rembold (1987)

^aJH titers were determined by GC/MS or HPLC/bioassay.

^bLow JH titers are those that are less than one-third of the highest level reported.

^cMedium JH levels are those that fall between one-third and two-thirds of the highest level reported.

^dHigh JH levels are those that are greater than two-thirds of the highest level reported.

^eBlastokinesis is not observed in these species; in these species, JH titers are reported for the period corresponding to approximately half way between oviposition and dorsal closure.

dorsal closure. An example of the confusion this can cause is readily seen in studies of the embryo of *M. sexta*, where a provisional dorsal closure is evident at about 50 h after oviposition (Broadie *et al.*, 1991), while the final dorsal closure becomes apparent only at 102 h after oviposition (Dorn *et al.*, 1987). While knowledge of JH titers offers correlative information when linked to a key point in embryogenesis, it can provide only indirect evidence for the role(s) the hormone plays during this period.

8.10.4. Roles of Juvenile Hormone during Embryogenesis

To better understand the embryological roles of JH, two experimental approaches have been taken: chemical allatectomy using precocene and the application of exogenous JH or a JH agonist. Application of JHI or II to eggs of *Thermobia domestica*, an ametabolous insect, any time from oviposition to 2 days before hatching, leads to highly deformed embryos and poor emergence rates (Rohdendorf and Sehnal, 1973). Injeyan *et al.* (1979) applied JHIII to *S. gregaria* embryos and found a different pattern of JH-induced disruption. JH applied shortly after oviposition has little effect on embryogenesis, but when applied from

days 3 to 9 postoviposition has a profound effect on development, including disruption of blastokinesis, inhibition of postblastokinesis development, and failure of the vermiform larva either to initiate or to complete ecdysis. JH has no effect on embryonic development when applied between day 10 and day 16 (hatching). Disruption of development is accompanied by increased pigmentation of the embryos, and it was discovered that the pigmented regions of treated insects have a much thinner procuticle. In contrast to these results, Sbrenna-Micciarelli (1977) demonstrated that high doses of the analog farnesyl methyl ether applied to the embryo just before blastokinesis induces premature development of the cuticle. Although the results are conflicting, both authors conclude that JH may have a role in cuticulogenesis.

Application of precocene to *N. cinerea* embryos following dorsal closure results in abnormal midgut development, which appears to stem from the failure of ectodermal cells to migrate from the developing stomodaeum and proctodaeum into the midgut rudiments to form the gut epithelium (Brüning *et al.*, 1985). In addition, the absence of JH after dorsal closure leads to disintegration of the fat body and abnormalities in the cuticle. *Oncopeltus fasciatus*

embryos treated with precocene at blastokinesis fail to undergo final dorsal closure, suggesting that JH is involved in development of ectodermal tissue (Dorn, 1982). This developmental aberration can be rescued by topical application of JH. Enslee and Riddiford (1977) demonstrated that blastokinesis in *P. apterus* was vulnerable to exogenous JH, which interfered with dorsal closure and induced truncated appendage development and early pigmentation of developing eye and abdominal structures. These authors suggested that the extraembryonic “membranes” might be the target of JH. Day-old embryos of *R. prolixus*, when treated with high levels of fenoxycarb, do not develop past katatrepsis, and patterns of protein synthesis, as determined by two-dimensional gel electrophoresis, are significantly altered (Kelly and Huebner, 1987). In yet another hemipteran, *Aphis fabae*, precocene inhibits embryogenesis, but the process can be rescued by JH (Hardie, 1987). Interestingly, JH appears to accelerate embryonic development via a mechanism independent of the parthenogenic reproductive strategy employed by aphids.

The effect of JH or analogs on the embryogenesis of holometabolous insects is similar to that seen in hemimetabolous insects. Unfortunately fewer studies have been carried out and all of them utilize the addition of exogenous JH to determine an effect. In Lepidoptera, JH injected into the female prior to oviposition halts embryogenesis at formation of the blastoderm, while JH applied directly to the egg shortly after oviposition has no effect on blastoderm formation, but blocks blastokinesis (Riddiford, 1970). Even without blastokinesis, embryos continue larval differentiation and form recognizable but incomplete first instars, as in *P. apterus* (Enslee and Riddiford, 1977). In another lepidopteran embryo, that of *Plodia interpunctella*, JH agonists induce aberrant dorsal closure and a loss of trachea (Dyby and Silhacek, 1997). A very different situation exists in *D. melanogaster*, a species in which no JH is detected during embryogenesis (Bownes and Rembold, 1987). While JH analogs can disrupt development if applied very early, large doses of JHI do not have an effect on embryogenesis (Smith and Arking, 1975).

The underlying molecular role of JH during embryogenesis is even more obscure. In an early study, Rao and Krishnakumaran (1974) attempted to elucidate the role of JH at the molecular level. When these investigators examined morphological alterations and changes in DNA synthesis in embryos of *A. domesticus* challenged with JHI, they found a qualitative drop in incorporation of thymidine into DNA, as monitored by autoradiography.

Embryonic epidermal tissue is the first to show reduced incorporation of thymidine in the presence of exogenous JH; however, some tissues appear refractory to the hormone. Interestingly, JH-treated *A. domesticus* embryos develop legs and antennae that are short and stumpy, which led Rao and Krishnakumaran (1974) to suggest that JH may suppress DNA synthesis and cell division in the embryonic appendages.

Despite the paucity of information, it is tempting to speculate that embryonic ectodermal tissue is a major target of JH. The effects of JH on cuticular morphology, dorsal closure, and pigmentation of embryonic epidermal structures support this idea, and are in keeping with the function of JH during larval development. The fact that JH acts on midgut and fat body development in at least some species indicates there also may be a wider role for the hormone.

8.10.5. Juvenile Hormone Binding Proteins of the Embryo

The JHBPs have long been known to modulate the catabolism of hemolymph JH during the larval period (Hammock *et al.*, 1975; Roe and Venkatesh, 1990; deKort and Granger, 1996) (see Section 8.9). It is generally assumed that JHBP has a unique hormone-binding domain whose steric arrangement hinders the access of catabolic enzymes to JH (Goodman, 1990). While the roles of the hJHBP in larval development are well defined (see Section 8.8.3), the role of embryonic JHBP is less clear. It is now clear that significant levels of JHBP are present throughout embryogenesis even though the circulatory system is not yet fully functional until late in development, and JH levels, as noted above, do not rise until the insect is midway through embryonic development.

In *L. migratoria*, a JHBP similar to that found in nymphal hemolymph is present in both the embryo and serosa (Hartmann *et al.*, 1987), prior to the development of the CA and a functional circulatory system. These investigators demonstrated that the serosa has a methyl transferase capable of converting JHIII acid to JHIII, and surmised that the embryonic JHBP serves either to distribute the hormone or to act as a buffer, protecting the embryo from excess maternal JH. The hypothesis of a protective function for embryonic JHBP is supported by the fact that JH levels in the gravid female are at least an order of magnitude higher than in the eggs (Temin *et al.*, 1986). Thus, the JHBP could act as a “sponge”, keeping the JH accessible to catabolic enzymes that can readily inactivate the hormone.

In *M. sexta*, a hJHBP-like molecule is present at oviposition and remains at detectable levels throughout embryogenesis (Touhara and Prestwich, 1994; Touhara *et al.*, 1994). A re-examination of the characteristics and titers of the embryonic JHBP demonstrated that the embryonic JHBP is identical in binding characteristics (Touhara and Prestwich, 1994; Touhara *et al.*, 1994), molecular mass, and immunochemical properties to the larval hemolymph form (Orth *et al.*, 2003a). Furthermore, the nucleotide sequence of the embryonic *JHBP* gene is identical to that of the larval gene (Orth *et al.*, 2003b); thus, it can be concluded that the embryonic JHBP is identical to hJHBP. Expression of the *JHBP* gene begins in both the embryo and the serosa approximately 15 h after oviposition, peaks between 24 and 36 h, and then declines to undetectable levels at 72 h. The burst of *JHBP* expression occurs before emergence of functional CA, fat body, or circulatory system. In contrast to its gene expression, titers of JHBP are at their highest during the first half of embryogenesis (Figure 17). The titer declines rapidly between 36 and 60 h after oviposition and then slowly drops to its lowest level at the time of emergence. The peak of gene expression does not appear to enhance the already high levels of JHBP and the reason for this temporal discordance between gene expression and the JHBP titer is unclear. As in the

case of *L. migratoria*, it appears that both the serosa and the embryo of *M. sexta* are capable of expressing *JHBP*. Curiously, the expression of *JHBP* in *M. sexta* begins about 15 h after oviposition, corresponding to the time at which the serosa becomes active in secretion of the first of several embryonic “membranes” (Lamer and Dorn, 2001).

Most of the embryonic JHBP in *M. sexta*, especially in the early hours following oviposition, is maternally derived and is assumed to be sequestered from hemolymph during the process of vitellogenesis. The presence of relatively high levels of the protein prior to development of the CA or circulatory system suggests that, as in *L. migratoria*, high levels of JHBP in the early embryo of *M. sexta* ensure low levels of unbound JH, thus protecting the embryo from excess JH (Orth *et al.*, 2003b). JH bioassay data indicate that hemolymph JH titers in adult female *M. sexta* are as high as those found in the early fourth stadium larvae (Judy, personal communication). Thus, during the early part of embryogenesis, the maternally derived JHBP acts as an effective buffer, constantly binding and releasing the hormone in accordance with equilibrium conditions. Embryonic JHE displays a developmental profile similar to that of JHBP (Share *et al.*, 1988) and together, these proteins regulate bioavailability of the hormone to the developing embryo.

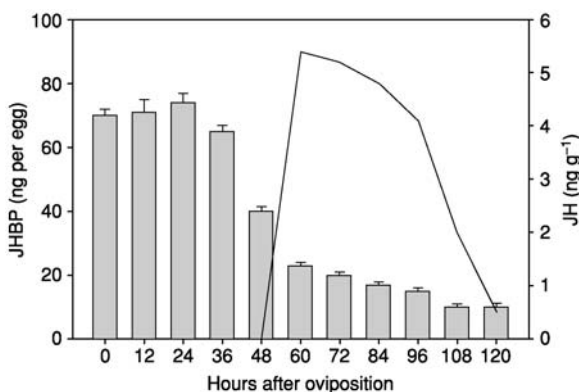


Figure 17 Titers of JH and juvenile hormone binding protein during embryological development of *M. sexta*. Bars, JHBP mean titers (\pm SD) (JHBP adapted from Orth, A.P., Tauchman, S.J., Doll, S.C., Goodman, W.G., 2003b. Embryonic expression of juvenile hormone binding protein and its relationship to the toxic effects of juvenile hormone in *Manduca sexta*. *Insect Biochem. Mol. Biol.* 33, 1275–1284. Line (—), total JH titer data adapted from Bergot, B.J., Baker, F.C., Cerf, D.C., Jamieson, G., Schooley, D.A., 1981a. Qualitative and quantitative aspects of juvenile hormone titers in developing embryos of several insect species: discovery of a new JH-like substance extracted from eggs of *Manduca sexta*. In: Pratt, G.E., Brooks, G.T. (Eds.), *Juvenile Hormone Biochemistry*. Elsevier/North-Holland, Amsterdam, pp. 33–45.

8.10.5.1. Juvenile hormone catabolism during embryogenesis: methyl ester hydrolysis Embryonic titers of JH are the result of both biosynthesis and catabolism. While this area has received considerable attention in the larval stage (see Section 8.9), scant attention has been focused on these catabolic enzymes in the egg. Roe *et al.* (1987a, 1987b) examined the metabolism of JH in the eggs of the cricket *A. domesticus*. Their studies found that the major route of metabolism is the conversion of JH to JH acid. They reported that general esterase activity, as determined using α -naphthyl acetate as a substrate, remains relatively constant throughout embryonic development. In contrast, JHE activity is high in unfertilized eggs and remains high until the time of dorsal closure, when it drops to its lowest level and remains relatively low until nymphal emergence.

In a very thorough analysis of JH titers (Brüning *et al.*, 1985), JH biosynthetic rates, and JHE activity in *N. cinerea* embryos, Lanzrein and her colleagues demonstrated that JHE activity is very low following dorsal closure (20 days after oviposition) (Bürgin and Lanzrein, 1988). Figure 18 shows that the massive peak representing both JHIII and MF begins to decline very rapidly when JHE activity

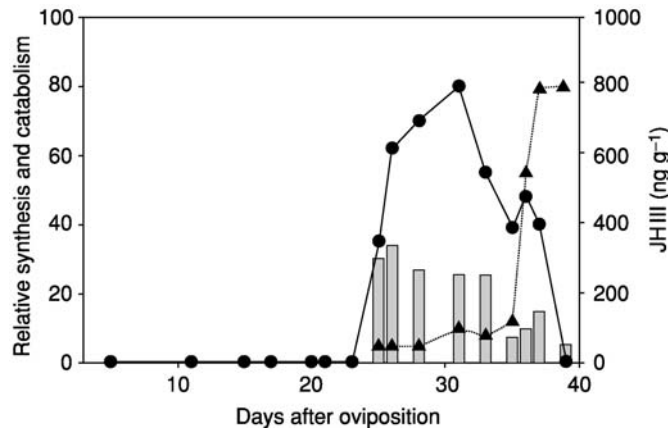


Figure 18 JH titers, JH biosynthetic rates, and JHE activity from *Nauphoeta cinerea* embryos. Closed circles, JH III titers (Brüning *et al.*, 1985); bars, relative JH III biosynthesis as determined by the radiochemical assay (Bürgin and Lanzrein, 1988); closed triangles, JHE activity (Bürgin and Lanzrein, 1988). Dorsal closure occurs at day 20.

rises at day 35. According to these investigators, the rise in JHE corresponds to the period of chorion breakdown; as a result, the increase in available oxygen significantly changes the physiology of the rapidly developing embryo. Interestingly, JHE activity does not appear to be localized to the hemolymph, but rather is distributed generally throughout the entire embryo (Bürgin and Lanzrein, 1988). Since JH III titers in the first instar nymphs are low (1 ng g^{-1}), the dramatic rise in JHE activity may be necessary to clear both JH and MF prior to nymphal emergence (Brüning *et al.*, 1985).

The rapid rise in JHE activity observed at the end of embryonic development in *N. cinerea* contrasts with that observed in *M. sexta*. In this insect, JHE activity is high in preovipositional eggs, but declines well before provisional dorsal closure (Share *et al.*, 1988). JHE activity remains relatively constant throughout the rest of embryonic development and does not show the dramatic increase in activity that is observed in *N. cinerea* (Bürgin and Lanzrein, 1988). Despite the presence of JHE activity, JH titers increase dramatically in the period surrounding larval cuticulogenesis (Bergot *et al.*, 1981a). The rise in the JH titer, in the presence of significant JHE activity, contradicts the generally held view that JHE is involved in inactivation of the hormone (see Section 8.9.1). It may well be that JH and JHE are sufficiently compartmentalized at the onset of cuticulogenesis that catabolism of the hormone is limited. Once the larval circulatory system is fully functional, approximately 24 h later as determined by vigorous dorsal vessel contraction (Broadie *et al.*, 1991), contact between the hormone and the catabolic enzymes result in a reduced JH titer as the enzyme carries out its anticipated function.

8.10.5.2. Juvenile hormone catabolism during embryogenesis: epoxide hydrolysis While most studies on JH catabolism have been focused on the JHEs, a handful of investigators have examined the action of the JHEH (see Section 8.9.2). Share *et al.* (1988), in their studies on JH metabolism during embryogenesis in *M. sexta*, demonstrated that JHEH activity is responsible for only a small amount of the total catabolic activity. They found that this enzyme hydrolyzes JH III about six times better than JH I, but they did not take into account the fact that JH I binds to the JHBP with a significantly higher affinity and thus protects the hormone from degradation (Park *et al.*, 1993).

The first characterization of a specific JHEH early in development was carried out by Touhara and Prestwich (1993) in a study of JH metabolism in eggs of *M. sexta*. Using the JH photoaffinity label [³H]epoxyfarnesyl diazoacetate, these investigators demonstrated that the radiolabeled analog covalently attaches to two proteins in the egg, the juvenile hormone binding protein and a 50 kDa protein that was identified as a JHEH. The majority of the JHEH activity is found in the microsomal fraction of the egg homogenate. The purified JHEH displays an apparent K_m of $0.61 \mu\text{M}$ for JH I and $0.28 \mu\text{M}$ for JH III, confirming the earlier observation by Share *et al.* (1988) that JH III appears to be the preferred substrate. A subsequent characterization of recombinant JHEH from *M. sexta* reconfirmed this substrate specificity (Debernard *et al.*, 1998). This study also found that the hJHBP protects the hormone from epoxide hydration, but whether this occurs *in vivo* is uncertain since the binding protein is present in the hemolymph (with the exception of the fat body), while the JHEH is localized to the cellular compartment.

8.10.6. Juvenile Hormones and the Evolution of Insect Metamorphosis

Truman and Riddiford (1999, 2002) have proposed that the temporal appearance of JH during embryonic development may have played an important role in the evolution of holometabolous insects. Their hypothesis is based, in part, on the idea that ancestral insects displayed three stages after embryogenesis: the pronymph, the nymph, and the adult. The pronymphal stage represents a specialized hatching stage in ametabolous and hemimetabolous insects that is (1) nonfeeding, (2) often mobile, (3) often surrounded by an embryonic cuticle, and (4) different in body proportions from that of the first instar. Depending upon the species, the pronymphal stage is initiated after completion of blastokinesis and may end prior to hatching or persist for several days after hatching. The Truman and Riddiford hypothesis states that the pronymphal and nymphal stages of this insect are developmentally equivalent to the larval and pupal stages, respectively, of the holometabolous insect. Moreover, there is a requirement for a JH-free period in ametabolous and hemimetabolous species that allows nymphal development to proceed towards adult stage.

Can experimental manipulation mimic the evolutionary processes that led to the transition to the holometabolous state? As noted earlier, JH can induce certain tissues in hemimetabolous species to undergo apparent premature differentiation before those tissues are completely patterned. This curious phenomenon has led Truman and Riddiford (1999, 2002) to suggest that the early appearance of JH during embryogenesis, either experimentally or naturally, modifies the developmental trajectory of the holometabolous insect to the adult stage, giving rise to a protolarval stage. From an evolutionary standpoint, advancing the time at which JH appears during embryogenesis may have been crucial to the transition from the pronymphal stage of the hemimetabolous insect to the protolarval stage of holometabolous species. While this hypothesis deserves much merit for simplicity and elegance, it is not without criticism (Heming, 2003). As demonstrated in **Table 2**, JH titers have been rigorously determined in only a handful of species, thus leaving any generalizations about the endocrine-driven rise of holometabolous development open to question. Moreover, working definitions of key embryogenic events, such as blastokinesis and dorsal closure, may be interpreted differently, depending on the investigator. The problem is further compounded by contradictory data on the time at which JH titers rise in relation to the key embryonic events. Truman and

Riddiford (2002) suggest that JH titers in the embryo of the lepidopteran *M. sexta* are highest at the time of katatrepsis, yet Ziese and Dorn (2003) place katatrepsis at a time when JH titers (Bergot *et al.*, 1981a) are undetectable. Assigning an embryological role to JH when the levels are low raises yet another issue: low JH titers at oviposition and at blastokinesis in some species contradict the hypothesis that a JH-free period is required for further normal development (**Table 2**). While the Truman and Riddiford hypothesis about the rise of holometabolous insects presents an intriguing model that deserves further critical study, the role that JH may have had in this process is still open.

8.11. Juvenile Hormones in Premetamorphic Development

8.11.1. Juvenile Hormone Titrers

The premetamorphic titers of JH appear to vary greatly among the insect orders, with some orders, such as Dictyoptera, displaying levels 100-fold greater than Lepidoptera (Gilbert *et al.*, 2000). Yet one pattern has remained constant since the discovery of JH in the 1930s: JH titers, on average, are high while the larva is growing and feeding but drop at a well-defined point to permit metamorphosis. This pattern can be further refined to distinguish between hemimetabolous and holometabolous insects (Riddiford, 1994). In hemimetabolous insects, JH titers are low to undetectable during the final stadium. In holometabolous insects, JH titers are relatively high at the beginning of the final larval stadium but decline to undetectable levels prior to the cessation of feeding. The absence of JH permits the release of the prothoracicotropic hormone (PTTH) from specific cerebral neurosecretory cells (Rountree and Bollenbacher, 1986), and PTTH then induces ecdysteroid synthesis by the prothoracic glands, initiating a small rise in hemolymph ecdysteroid levels (Bollenbacher *et al.*, 1981) (see **Chapter 6**). This rise in the ecdysteroid titer in the absence of JH initiates metamorphosis. In contrast to the hemimetabolous insects, a second increase in the hemolymph JH titer is typically observed after the insect has found a suitable pupation site. This peak in the titer is thought to prevent precocious adult differentiation of imaginal discs and other imaginal precursors (Riddiford, 1994).

8.11.2. Potential Problems with Titer Determinations

One of the major impediments to better understanding the role(s) of JH is the lack of precise titers

during biologically relevant periods. Virtually all hormones studied in vertebrates display daily oscillations in their titer and release and are, in many cases, linked to critical homeostatic events (Czeisler and Klerman, 1999). Early studies demonstrated a daily, bimodal, rhythmic fluctuation in the size of the nuclei in CA of adult *D. melanogaster* (Rensing, 1964). While size of the CA cells does not necessarily indicate a gland active in JH biosynthesis (Tobe and Stay, 1985), there is ample evidence that indicates enlarged glands are biosynthetically active (Chiang *et al.*, 1989, 1991). Thus, fluctuations in CA cell size suggests circadian fluctuations in JH synthesis.

The importance of examining titer fluctuations in detail can be seen in the elegant work from Steel's laboratory, which demonstrates a circadian rhythmicity of ecdysteroid titers in nymphal *R. prolixus* (Steel and Ampleford, 1984; Vafopoulou and Steel, 2001) (see Chapter 9). A single-point JH determination every 24 h may not be a serious problem for the student of metamorphosis, where JH-regulated events stretch over a period of several days. However, it does make a difference when relatively rapid changes in JH titer regulate critical cellular events that occur during a short time period. Recent studies demonstrating that JH titers fluctuate rapidly in adult insects supports the premise that detailed studies of JH titers during the premetamorphic stages are needed. Elekonich *et al.* (2001) found that honeybee foragers show a diurnal increase in hemolymph JH titers, from about 100 ng ml⁻¹ in the late morning to over 350 ng ml⁻¹ by late evening, a period spanning less than 12 h. A similar change is seen in the fourth stadium of *M. sexta*, where titers initially rise and then drop significantly over an 8 h period and may have some influence on levels of the transport protein, hJHBP (Fain and Riddiford, 1975; Orth *et al.*, 1999). This is not to infer that JH titers must be absolutely accurate; the important information is knowledge of changes in the relative titers at well-defined times.

However, even if precise staging is possible, the measurement of JH in samples from species or stages displaying low levels of hormone, or from smaller sized species, requires that tissue collections be pooled from a number of individuals. Valuable information about population variability is thus lost. Ideally, a single individual should be sampled sequentially over time, but this process leads to wound-induced changes (Caveney, 1970) that may significantly alter JH titers. In our hands, wounding has rapid and significant effects on hJHBP mRNA expression, which potentially modulates JH titers (Orth and Goodman, unpublished data). Even

moderate handling prior to sample collection may have an effect on JH titers (Varjas *et al.*, 1992).

8.11.3. Premetamorphic Roles of the Juvenile Hormones

The roles of JH in the stages preceding metamorphosis to the adult are ubiquitous, affecting behavior, organs and tissues, cellular organelles, and biochemical pathways. Several excellent reviews have covered these areas (Nijhout, 1994; Riddiford, 1994, 1996), and the focus here is on research since that time.

8.11.3.1. Behavioral and neuronal responses There are a number of behavioral phenomena ascribed to JH during the adult stage, including pheromone production and calling (review: Cusson *et al.*, 1994), migration (review: Dingle and Winchell, 1997), phonotaxis (Stout *et al.*, 1992, 1998; Bronsert *et al.*, 2003), and caste determination and anatomical changes in the brain of honeybees (reviews: Robinson and Vargo, 1997; Elekonich and Robinson, 2000). However, little is known about the effect of JH on behavior during the premetamorphic period, and most of these studies have been done in social or migratory insects.

Late in the third stadium, honeybee larvae destined to become queens have JH titers five times higher than larvae destined to become workers (Rachinsky and Hartfelder, 1991; Rembold *et al.*, 1992). In another hymenopteran, the ant *Phidole bicarinata*, large doses of methoprene, if applied during a critical period in the last stadium, induce worker-destined larvae to become soldiers (Wheeler and Nijhout, 1981). Higher JH titers during the last stadium of certain migratory species appear to elicit a stationary adult stage, rather than migration (Yagi and Kuramochi, 1976; Nijhout and Wheeler, 1982). Yin *et al.* (1987) demonstrated that methoprene can affect the circadian system in the lepidopteran *Diatraea grandiosella*, inducing a dose-dependent phase shift in adult eclosion.

At the neuronal level, most endocrine studies have focused on the effect of ecdysteroids on the neural circuitry during metamorphosis (see Chapter 4), although JH appears to play a role as well. Truman and Reiss (1988) demonstrated that reorganization of neurons during metamorphosis of *M. sexta* is, in part, under the control of JH, but the regulatory elements involved are still undefined. Recent work suggests that JH affects larval neurons innervating the prothoracic gland of the cockroach *P. americana* (Richter and Gronert, 1999). Exposure of the insect to exogenous JH III and methoprene both *in vivo* and *in vitro* induces a short-term depression of spike

activity in neurons innervating the prothoracic gland, but has no effect on the nervous connectives of the stomatogastric nervous system. This response to JHIII is very rapid, occurring within 3 min of treatment and reaching a low (75% reduction) within 15 min. Curiously, fenoxycarb, a potent JH analog, was only half as active as JHIII and methoprene; methyl laurate, a control lipid, had no effect. The authors speculate that JH is acting directly on membrane receptors to elicit the reduced neurotropic activity; this observation relates to an earlier hypothesis that JH can influence inhibitory neurotransmitter receptors such as γ -aminobutyric acid (GABA) (Stout *et al.*, 1992).

8.11.3.2. Epidermal responses It has long been known that JH has a marked influence on epidermal and cuticular structure. The early work of Williams (1952) demonstrated that JH-containing extracts, when applied at a developmentally sensitive time during the pupal period, can induce a second pupal cuticle. This discovery prompted Williams to suggest that JH is a “status quo” hormone, a label that is still in frequent use (Willis, 1996). The integument of the insect is composed of an outer layer of secreted proteins, lipids, pigments, and complex carbohydrates termed the cuticle. The single layer of cells immediately beneath the cuticle, the epidermis, is responsible for synthesis and secretion of most, but not all, of the proteins found in the cuticle (Willis, 1996). The epidermis itself contains another subset of proteins, pigment-associating proteins, the expression and position of which in the cells appear to be regulated by JH. The radical changes in morphology at metamorphosis led Wigglesworth (1959) to predict that each stage of development had its own set of unique genes. While there does appear to be a limited group of stage-specific cuticular proteins, especially in the case of the higher flies, current evidence demonstrates that many cuticular proteins can be found in more than one stage of the life cycle (review: Willis, 1996).

Hormonal regulation of epidermal gene expression has been examined in a number of insect species, but the most extensive studies, by Riddiford and her colleagues, have been carried out on the epidermal genes of *M. sexta* (Riddiford, 1994, 1996; Riddiford *et al.*, 2001, 2003). These include the larval cuticular protein 14 (LCP14) (GenBank Accession no. 813279) (Rebers and Riddiford, 1988), LCP14.6 (GenBank Acc. No. U65902) (Rebers *et al.*, 1997), LCP16/17 (GenBank Accession no. M25486) (Horodyski and Riddiford, 1989), the biliverdin-associating proteins, insecticyanin a and b (GenBank Accession nos. 864714

and 864715) (Li and Riddiford, 1992), and dopa decarboxylase (DDC) (GenBank Accession no. U03909) (Hiruma and Riddiford, 1988; Hiruma *et al.*, 1995). Studies using the epidermis are particularly compelling, since the results of *in vitro* manipulations mirror those observed *in vivo*, and importantly, the genomic structure and flanking regions of their genes have been determined.

LCP14 is a larval-specific gene that encodes for a 14 kDa protein expressed during the feeding period of each stadium (Riddiford, 1994). At the onset of a larval molt, mRNA for LCP14 rapidly becomes undetectable and remains so until the insect molts to the next stadium, at which time the expression levels rise again and the process repeats itself. In the last stadium, levels of LCP14 message rise during the first several days, but fall sharply prior to wandering and are no longer detectable. *In vitro* manipulation indicates that 20E suppresses the expression of this gene. If JH is present when 20E titers rise, suppression by 20E is only transient; if JH is absent, the gene is permanently silenced. While it is uncertain how JH acts at the molecular level to maintain the expression of *LCP14*, its function may involve the transcription factor β FTZ-F1. It has been shown that there are three potential binding sites for β FTZ-F1 in the *LCP14* gene, one approximately 2 kb upstream from the start site and two in the first intron (Lan, personal communication). The expression of β FTZ-F1 begins about 16 h before molting, peaks at about 9 h before molting, and then ceases approximately 3 h before molting (Weller *et al.*, 2001). Functional analysis of the putative *LCP14* promoter indicates that the β FTZ-F1 response elements in the first intron are involved in downregulating *LCP14* expression (Lan, personal communication). The decline in β FTZ-F1 expression coincides with the rise in JH at the very beginning of the fifth stadium and with it, the increase in *LCP14* expression. Whether JH acts directly on the gene, or is involved in modulating the ecdysteroid effect, remains unclear.

LCP14.6 is another hormonally controlled cuticular gene, which, like *LCP14*, is downregulated by 20E, but in contrast to *LCP14*, is suppressed *in vitro* by large doses of methoprene (Riddiford, 1986). Its expression is temporally and spatially complex and is not stage-specific, since it occurs in the larval, pupal, and adult stages. A comparison of its expression pattern with the JH titer profile suggests that, *in vivo*, *LCP14.6* expression may be suppressed by the hormone. *LCP16/17* encodes a multigene family of three proteins that appear midway through the feeding period of the fifth stadium (Horodyski and Riddiford, 1989). The developmental appearance of

these proteins correlates with a thinning of cuticular lamellae and a corresponding increase in stiffness. Like *LCP14.6*, expression of the *LCP16/17* is suppressed by large doses of methoprene.

One of the more striking effects elicited by JH is its action on larval pigmentation (Nijhout, 1994; Applebaum *et al.*, 1997). Nowhere is this more evident than in larval *M. sexta*, where two mutants have been discovered with anomalous pigmentation and aberrant JH titers. In the *bl* mutant (Safranek and Riddiford, 1975), JH titers are low at certain critical developmental points and the larvae are highly melanized; in the white mutant, the opposite is the case (Panchapakesan *et al.*, 1994). To maintain the normal wild-type phenotype following a molt, i.e., a transparent cuticle and an epidermis with intracellular vesicles containing the biliverdin-associating protein, insecticyanin (Riley *et al.*, 1984; Goodman *et al.*, 1985), JH titers must be sufficiently high at the time of head capsule slippage (Hiruma and Riddiford, 1988; Riddiford, 1994). If JH titers are too high at the time of head capsule slippage, the resulting cuticle will be transparent, and the underlying epidermal cells will lack insecticyanin vesicles, causing the insect to appear white (Panchapakesan *et al.*, 1994). Conversely, if JH titers are too low at the time of head capsule slippage, the resulting cuticle will be highly melanized and the epidermis devoid of insecticyanin vesicles, causing the insect to appear black (Goodman *et al.*, 1987).

Insecticyanin (Ins) is a 21 kDa protein that associates with biliverdin IX to yield an intensely blue protein important to larval camouflage (Goodman *et al.*, 1985). Ins is found in epidermal cells, where it is sequestered in 1 μm vesicles (Figure 19). These vesicles, together with other epidermal and cuticular pigments, give the wild-type insect its distinctive blue-green hue during the feeding period (Riddiford *et al.*, 1990). Analysis of epidermal ultrastructure indicates that the densely packed vesicles appear to be held in position by a web of microtubules (Goodman, unpublished data). A typical wild-type epidermal cell contains approximately 100 of the membrane-coated vesicles, localized in the apical region. The epidermal cell also secretes significant quantities of Ins into the hemolymph, making it one of the more prominent hemolymph proteins (Riddiford *et al.*, 1990). At commitment to pupation, when JH is no longer present and ecdysteroid levels rise, the entire population of Ins-containing vesicles is secreted from the epidermal cell (Sedlak *et al.*, 1983) and the insect ceases production of the protein (Goodman *et al.*, 1987; Riddiford *et al.*, 1990). Interestingly, the *bl* mutant, which has a low JH titer at head capsule slippage, lacks the

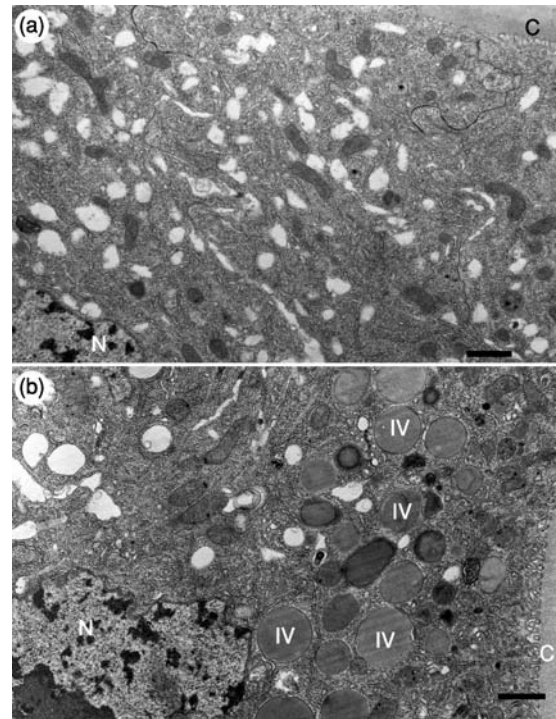


Figure 19 Electron micrographs of the epidermis of the *bl* strain of *M. sexta*. Fourth instars, just prior to head capsule slippage, were treated with either acetone (a) or 50 ng JH I dissolved in acetone (b). Tissue was collected approximately 30 h later, at the time of the fifth larval ecdysis, and prepared for microscopy. C, cuticle; IV, insecticyanin vesicle; N, nucleus. Scale bars represent 1 μm . (Goodman, unpublished data.)

epidermal Ins-containing vesicles, thus mimicking the ultrastructure of the wandering fifth stadium epidermis. Topical application of JH to *bl* mutants, just prior to head capsule slippage, will induce the appearance of the Ins vesicles in the next stadium, similar in number and position to those in the wild-type (Goodman *et al.*, 1987) (Figure 19). Moreover, JH, when applied at head capsule slippage, prevents cuticular melanization in the next stadium (Safranek and Riddiford, 1975). The role of JH in this process is still unclear but ultrastructural data suggests that the cytoskeleton may be involved in retaining the Ins vesicles in the apical portion of the epidermal cell.

In addition to the presence of Ins mRNA in the epidermis of wild-type larvae, Ins mRNA has also been found in the fat body (Li and Riddiford, 1994; Li, 1996); however, the protein could not be detected immunologically unless pericardial cells were included in the preparation (Goodman *et al.*, 1987). Curiously, JH appears to upregulate epidermal Ins mRNA abundance, while it downregulates this mRNA in the fat body (Li, 1996). As in the case of *LCP14*, 20E downregulates the epidermal Ins mRNA (Riddiford *et al.*, 1990) during the molting period.

Cloning of the *Ins* gene revealed a pair of duplicated genes termed *Ins a* and *Ins b* (Li and Riddiford, 1992) that are expressed in both the epidermis and fat body. Only the product encoded by the *Ins b* gene is found in the hemolymph (Riddiford, 1994). The difference in expression patterns between *Ins a* and *Ins b* led Li (1996) to investigate the structure of the genes, to determine whether different promoters were involved. Each of the genes displays a unique sequence in the 3' noncoding region, suggesting differential expression. Preliminary computational analysis of the 5' upstream region of *Ins a* indicated no known response elements (Lan, personal communication).

DDC is the enzyme responsible for catalyzing the conversion of dopa to dopamine (Hiruma and Riddiford, 1984) while granular phenoloxidase (PO) catalyzes the oxidative dehydrogenation of diphenols to quinones (Hopkins and Kramer, 1992). For cuticular melanization to occur, vesicles containing dopamine, the granular proenzyme form of PO, and other enzymes necessary for melanin synthesis are secreted into the new endocuticle following head capsule slippage. If JH titers are low or absent at the time of head capsule slippage, the proenzyme form of PO will be synthesized by the epidermis and deposited in the endocuticle for a period of approximately 12–14 h. Approximately 3 h before ecdysis, PO is activated, possibly by proteolytic activation (Jiang *et al.*, 2003), and melanin begins to appear in the new cuticle. Topical application of JH to *bl* larvae, or to wild-type larvae that have been neck-ligated at head capsule slippage, will block phenoloxidase activity, and a new transparent cuticle will be formed (Riddiford *et al.*, 2003). While JH suppresses cuticular melanization in a striking fashion, it remains unclear whether the hormone is acting on transcription or translational events that regulate PO activity.

Hormonal regulation of epidermal DDC (EC 4.1.1.28) in *D. melanogaster* and *M. sexta* has been extensively studied by the Hodgett (Chen *et al.*, 2002a, 2002b) and Riddiford laboratories (Riddiford, 1994; Riddiford *et al.*, 2003). In *M. sexta*, 20E works directly on epidermis to block DDC synthesis; however, as the ecdysteroid titers decline during the head capsule slippage period, levels of DDC begin to rise and they peak near the time of the larval molt. A study of the 5' upstream region of the *DDC* gene indicates that there are several transcription factor binding sites, including a β FTZ-F1 response element between the TATA box and the transcriptional start site (Hiruma *et al.*, 1995). Recently, Beckstead *et al.* (2001) demonstrated that the *D. melanogaster* gene *bonus*

(*bon*) encodes a homolog of the vertebrate TIF1 transcriptional cofactors. Among the many phenotypes associated with this gene is pigmentation (Beckstead *et al.*, 2000). *Bon* binds to the AF-2 activation domain present in the ligand-binding domain of β FTZ-F1 and behaves as a transcriptional inhibitor *in vivo*. A transcription factor implicated in regulation of melanization as well as metamorphosis is the Broad-complex (Br-C). In the metamorphosis of *D. melanogaster*, Br-C is a required mediator in the *DDC* response to ecdysteroids. At pupariation and at eclosion, Br-C uncouples *DDC* from an active silencing mechanism that functions through two distinct *cis*-acting regions of the *DDC* locus (Chen *et al.*, 2002a, 2002b). While much is known about *DDC* and its regulation via ecdysteroids, the role of JH in its control remains unclear (Hiruma *et al.*, 1995; Chen *et al.*, 2002a, 2002b; Riddiford *et al.*, 2003). It has been demonstrated that the epidermis of allatectomized *M. sexta* larvae displays approximately 50% more DDC activity than epidermis from sham-operated insects, and that activity of the enzyme can be suppressed by moderate levels of JHI. While this observation is certainly provocative, it remains to be seen whether the JH effect on *DDC* expression is direct, or as some speculate, indirect (Hiruma, personal communication).

8.11.3.3. Fat body responses The premetamorphic fat body is responsible for metabolism of nutrients, synthesis of most hemolymph proteins, and detoxification of xenobiotics (Locke, 1984; Sondergaard, 1993; Haunerland and Shirk, 1995). Given the central roles this tissue plays, it is not surprising that an extensive body of literature has focused on the role of JH in regulating cellular and molecular events occurring in the fat body.

Numerous studies involving hormonal regulation of ultrastructural changes in adult fat body, especially those regarding vitellogenesis, have been conducted, but few have focused on the ultrastructural changes induced by JH in the larval fat body. Such experiments are problematic since JH titers are already relatively high during most of larval life, and challenging the fat body with additional JH may result in pharmacological responses that mask the actual underlying events. Thus, the most comprehensive studies are developmental, correlating JH titers with ultrastructural changes occurring during the premetamorphic and metamorphic periods (Locke, 1984; Dean *et al.*, 1985). In one series of studies, it was found that during the last stadium of both hemimetabolous and holometabolous insects, when JH

titers are dropping, the fat body synthesizes and secretes several hexameric proteins collectively termed storage proteins (Levenbook, 1985). These proteins are retrieved from the hemolymph by the fat body and stored in relatively large vesicles for later use during the pupal–adult transformation (Locke, 1984; Dean *et al.*, 1985). It has also been demonstrated that a JH analog affects fat body nuclei. Treatment of nymphal *L. migratoria* with high levels of methoprene leads to an increase in the size of fat body nuclei, which could be linked to either increased transcription rates or to increased ploidy levels (Cotton and Anstee, 1991). The latter option seems likely, since Jensen and Brasch (1985) demonstrated that allatectomy of adult *L. migratoria* prevents polyploidization, while methoprene restores the process.

At the molecular level, the role of JH on fat body gene expression has been studied by a number of investigators in several different insect species. Certain of these gene targets are discussed below (see Section 8.12), and a comprehensive discussion of the various fat body genes thought to be under the control of JH has been presented by Riddiford (1994). One of the most well-studied groups of premetamorphic fat body genes and proteins is the hexamerins (Burmester *et al.*, 1998; Burmester, 2002). The insect hexamerins encompass at least five different subgroups based on amino acid sequence, including: the coleopteran and dictyopteran JH-suppressible arylphorins, the lepidopteran JH-suppressible hexamerins, lepidopteran aromatic amino acid-rich arylphorins, the lepidopteran methionine-rich hexamerins, and the dipteran arylphorins (Beintema *et al.*, 1994). The products encoded by the hexamerin genes are expressed predominantly during late larval life, and thus appear to be negatively regulated by JH. Another group of proteins in the hexamerin superfamily are the hemolymph cyanoproteins (Miura *et al.*, 1998) and the orthopteran hemolymph JH binding proteins (Koopmanschap and deKort, 1988; Braun and Wyatt, 1996). As first demonstrated by Jones *et al.* (1988, 1990a), the expression patterns of certain fat body genes are downregulated by JH and JH analogs; however, exceedingly high doses of JH analogs were used to elicit the responses. More recently, Hwang *et al.* (2001) and Cheon *et al.* (2002) have isolated two genes encoding hexamerins that are JH-suppressible at low doses of JH analog. Expression of these genes is not dose-dependent, but can be downregulated within a 6 h period, suggesting that JH is acting directly on transcription. Thus, a general pattern is emerging that indicates fat body hexamerin expression is suppressed in the presence of JH.

While few insect endocrinologists would argue with the idea that the fat body is a target for JH, there remain technical difficulties in working with the fat body. Its cellular heterogeneity, massive tracheal penetration, and apparent sensitivity to wounding or to culture *in vitro* make experimental manipulation of the premetamorphic fat body difficult. More troubling is the fact that many of the studies lack a dose–response curve, and data are based on a single large dose of hormone or analog. As demonstrated by Orth *et al.* (1999) in their study on JH regulation of *bJHBP* expression, the effect of increasing doses of JH on the fat body is stimulatory only within a limited range; after peak stimulation is reached, further increases in JH result in a negative response. In this case, a single large dose of hormone would have obscured very important data. Another issue with many of the studies is the lack of authentic controls, especially for experiments *in vitro*. Large doses of JH can form a monolayer at the surface of the medium, hindering gaseous exchange and compromising the physiology of the fat body in culture. Unless a comparable lipid is used as a control, the results may be meaningless. It is anticipated that technological advances, particularly in molecular analyses, coupled with a more physiological approach to hormone treatment, should ultimately reveal how JH functions with respect to the fat body.

8.11.3.4. Muscle responses Myogenesis occurs during two developmental periods: the embryonic period in which larval muscles are developed and the period of larval–pupal metamorphosis, in which adult muscles are formed. In addition to myogenesis, existing larval muscles can undergo cell death, or become restructured and/or reoriented for a new role in the adult (Riddiford, 1994; Roy and Vijay Raghavan, 1999). At the present time there is little evidence to suggest that JH has a direct role on embryonic myogenesis; however, JH involvement in the regulation of muscle development and muscle fate during metamorphosis is well documented (Schwartz, 1992; Riddiford, 1994; Hegstrom *et al.*, 1998; Roy and Vijay Raghavan, 1999; Buszczak and Segraves, 2000; Cascone and Schwartz, 2001; Lee *et al.*, 2002). The fate of larval muscles is determined by a finely tuned interplay between JH and ecdysteroids, an example of which is found in the process of proleg retraction in *M. sexta* (Weeks and Truman, 1986a, 1986b). After the larva wanders in the last larval stadium, the prolegs are no longer required, and muscles that move these structures begin to degenerate. The signal for this process is the commitment peak in the ecdysteroid titer, which

occurs in the absence of JH. Under these conditions, the retractor muscles become committed to undergo apoptosis (programmed cell death) (see Chapter 5), with the actual process of degradation elicited by the large prepupal peak of ecdysteroids that occurs several days later. Despite its concurrent presence at the time of the prepupal peak in the ecdysteroid titer, JH can no longer inhibit apoptosis of the retractor muscles.

Adult-specific muscles are formed from specific myoblasts that proliferate and differentiate during the late larval or early pupal stages (Roy and VijayRaghavan, 1999). In those muscles that make up the larval ventral diaphragm of *M. sexta*, proliferation and differentiation are controlled by ecdysteroids, but these two events have different hormonal requirements (Champlin *et al.*, 1999). Moderate levels of ecdysteroids induce proliferation of the ventral diaphragm myoblasts, while high or low doses arrest cellular proliferation in the G₂ phase of the cell cycle in these cells. High doses of ecdysteroids, such as those observed at the prepupal peak, induce the myoblasts to exit the cell cycle and express muscle myosin. The ability of critical titers of ecdysteroids to initiate either proliferation or differentiation is modified in the presence of methoprene. For example, high levels of ecdysteroids induce myoblast proliferation, but are unable to induce myoblast differentiation, in the presence of methoprene. These experimental observations correlate well with myoblast development *in vivo*. The prepupal peak of ecdysteroids, which is sufficient to induce differentiation, is accompanied by a concomitant rise in JH that prevents the myoblasts from differentiating. The myoblasts continue to proliferate until day 7 of the pupal stage when, in the presence of the pupal ecdysteroid peak but in the absence of JH, they then begin to differentiate. While both the prepupal and pupal surges of ecdysteroids are sufficient to cause differentiation, the pupal surge is not accompanied by a rise in JH levels, thus allowing the myoblasts to differentiate.

A similar interplay between JH and ecdysteroids is observed in the development of flight muscle in the desert locust, *S. gregaria*. Flight muscle development is initiated at the beginning of the last nymphal stadium and completed shortly after adult metamorphosis (Wang *et al.*, 1993). In addition to its fully differentiated myofibrils, functional flight muscle contains a high level of a fatty acid binding protein that is involved in sequestering lipids for energy. Flight muscle development begins during the fifth nymphal stadium, when JH is absent and ecdysteroid titers are high. When a large dose of methoprene is applied to the last-stadium nymph, the insect

undergoes another nymphal molt, and flight muscles that should have formed working myofibrils remain undifferentiated and lack fatty acid binding protein. Thus, elevated JH titers in the last nymphal stadium can lead to either retention of nymphal muscle or delay in the development of adult muscles.

In the adult stage, JH can have the opposite effect on muscles, inducing them to undergo degradation to yield precursors for vitellogenin production (Rose *et al.*, 2001). Treating the alate or winged form of the aphid *Acyrtosiphon pisum* with a JH analog initiates histolysis of indirect flight muscles via the ubiquitin-dependent pathway for apoptosis (Kobayashi and Ishikawa, 1994). Conversely, treatment with precocene II, a compound that reduces JH biosynthesis in this species, prevents flight muscle breakdown. A similar situation is found in another aphid species, *A. fabae* (Hardie *et al.*, 1990). The development of flight muscle in Orthoptera appears to follow this same pattern, i.e., JH either induces and/or is involved in the histolysis of these muscles. Application of JHIII or methoprene to the cricket *Modicogryllus confirmatus* induces the degeneration of flight muscles within 3 days (Tanaka, 1994). In addition to JH, there may be neural factors that are required for the degeneration of specific flight muscles (Shiga *et al.*, 2002).

8.11.3.5. Prothoracic gland responses It has long been known that the prothoracic glands, the site of synthesis of ecdysteroids, are regulated by ecdysone itself, via short positive and negative feedback loops (Williams, 1952; Siew and Gilbert, 1971; Sakurai and Williams, 1989). Given the close relationship between JH and ecdysteroids in regulation of metamorphosis, the effect of JH on the prothoracic glands has also been examined (Gilbert, 1962; Siew and Gilbert, 1971). JH has two separate and distinct effects: prevention of precocious degeneration of the glands and regulation of ecdysteroid synthesis/secretion during the larval stage. In *M. sexta*, the prothoracic glands undergo apoptosis between 5 and 6 days after pupation, at the time of the pupal peak in the ecdysteroid titer. Dai and Gilbert (1999) demonstrated that early pupal glands, in the presence of ecdysteroids *in vitro*, will also undergo apoptosis. Glandular degeneration can be delayed for up to a week, if a newly molted pupa is injected with a large dose of JHII. Moreover, the glands remain capable of synthesizing ecdysone (Dai and Gilbert, 1998). The underlying mechanisms by which JH prevents this important developmental event is still unclear.

Nijhout (1994), in his elegant review of JH effects on ecdysteroid synthesis, came to the conclusion

that JH does not act directly on the prothoracic glands to modulate ecdysone biosynthesis. His model suggests that elevated levels of JH during the early part of the last larval stadium act on the brain (or other intermediary tissue) to suppress synthesis or release of PTTH, the hormone responsible for stimulating ecdysteroidogenesis in the prothoracic glands. It is only when the JH titers drop that PTTH can be released to initiate commitment to pupation by stimulating synthesis of the commitment peak in the ecdysteroid titer (Nijhout and Williams, 1974; Rountree and Bollenbacher, 1986). The idea that the brain is the major target is supported by the work of Gruetzmacher *et al.* (1984), who demonstrated that although PTTH could stimulate *M. sexta* prothoracic gland activity *in vitro*, none of the JH homologs or methoprene, even at a number of different doses, were able to do so. Evidence presented by Sakurai *et al.* (1989) suggests that JH not only acts on the brain but also on the glands themselves, to directly suppress the acquisition of competence to respond to PTTH. Thus, under normal physiological conditions in the last larval stadium, the JH-regulated inhibition of PTTH secretion and suppression of gland competence probably evolved as a safety mechanism to prevent the premature onset of the metamorphic molt (Nijhout, 1994).

While it is accepted that high levels of JH inhibit PTTH production or secretion during the feeding period of the last larval stadium, this does not appear to be the case either in the earlier stadia or after commitment to pupation. In the earlier stadia, JH titers are high to maintain the larval state. Presumably, these high levels of JH should inhibit PTTH secretion, yet the insects undergo normal molts. In early studies, the implantation of extra CA into penultimate and last instars of *G. mellonella* invariably led to perfect supernumerary molts, often more than one (Granger and Sehnal, 1974; Sehnal and Granger, 1975). In more recent studies, supplementing normal JH titers with an analog, fenoxycarb, did not block third and fourth instars of *B. mori* from undergoing normal molts to fourth and fifth instars; however, an extra molt to a perfect sixth instar was noted (Kamimura and Kiuichi, 2002). An identical result was seen in *M. sexta* using methoprene (Lonard *et al.*, 1996). An *in vitro* study provides a basis for the results of these *in vivo* experiments. Addition of fenoxycarb to incubations *in vitro* of *B. mori* prothoracic glands taken throughout the last stadium results in an extreme suppression of ecdysteroidogenesis (Dedos and Fugo, 1996). Although a recent report has shown that JHI, applied in large doses early in the last

larval stadium, can transiently elevate PTTH levels in the hemolymph of *B. mori* (Mizoguchi, 2001), the majority of the studies done so far indicate that in contrast to the model for JH–ecdysteroid interaction during larval–pupal metamorphosis, JH is capable of acting directly on the prothoracic glands and does so by suppressing ecdysteroid synthesis.

Biochemical evidence supports this contention: hydroprene, a JH analog, and JHI significantly delay the ability of the prothoracic glands to respond to PTTH or MIX, an inhibitor of cyclic nucleotide phosphodiesterase (Gu *et al.*, 1997). Since PTTH activates an intracellular second messenger system involving cyclic nucleotides (Gilbert *et al.*, 2000) (see Chapter 6), MIX can serve as a surrogate for PTTH by inhibiting their catabolism. The fact that neither PTTH nor MIX can activate ecdysteroidogenesis in the presence of JH strongly reinforces the idea that JH can act directly on the glands.

Several groups have demonstrated that the effect of JH on the prothoracic glands changes after commitment to pupation. Insects that have been debrained or neck-ligated to remove the source of PTTH respond to JH or analog treatment with increased ecdysteroid synthesis (Hiruma *et al.*, 1978; Safranek *et al.*, 1980; Gruetzmacher *et al.*, 1984; Dedos and Fugo, 1996; Dai and Gilbert, 1998). Thus, a reversal in hormone action appears to have taken place at, and possibly as a result of, commitment. As we have seen with other tissues, the response to JH can vary depending upon the developmental period. In the case of the prothoracic glands, the regulatory processes that control ecdysteroidogenesis and involve JH are very complex.

8.12. Molecular Mode of Action of the Juvenile Hormones

No sooner had the chemical structure of JHI been reported by Röller *et al.* (1967), one of the first of many reviews on JH action at the molecular level appeared in print (Kroeger, 1968). Since that time, there have been a number of major reviews focusing on the topic, with at least nine appearing during the last dozen years (Kumaran, 1990; Riddiford, 1994, 1996; Jones, 1995; Wyatt and Davey, 1996; Gade *et al.*, 1997; Gilbert *et al.*, 2000; Lafont, 2000; Wheeler and Nijhout, 2003).

Despite the volumes written about the subject, the molecular action of JH remains very much a mystery. This has led some to suggest that JH may have multiple roles in cellular and molecular mechanisms (Jones, 1995; Wheeler and Nijhout, 2003). Our assessment of the literature suggests that some of the problems in deciphering the molecular action

of JH lie not in unfathomable molecular mechanisms, but rather in the quality of the hormone, choice of experimental model, and development of experimental design.

A major experimental pitfall is the quality of the hormone, especially commercially available JHIII from a US supplier. The supplier acknowledges that the hormone is racemic and the level of contamination is significant. Yet there appears to be a tacit assumption among investigators that the unnatural racemate and contaminants have no effect on the experimental procedure, an assumption that is unwarranted until proven. Long-term storage of the hormone and its metabolites may also be problematic and a means of repurifying the compounds must be available. A similar criticism can be leveled at the use of commercial-grade JH agonists that may contain significant amounts of incipients with unknown biological functions.

Metamorphosis is undoubtedly one of the most striking phenomena in the biological world, yet it has not yielded its molecular secrets easily, particularly with regard to JH action. While major strides have been made in the last few years, using a paradigm that depends on the absence of JH makes dissecting the molecular action of the hormone challenging. This problem is further compounded by the fact that the metamorphic actions of JH on cellular and molecular events often require lengthy incubation periods. These long periods make separating key regulatory events from secondary events difficult. It may well be that the extreme complexity of metamorphosis does not lend itself well to isolating the actions of JH, and, as Jones (1995) and Wheeler and Nijhout (2003) note, the hormone may act on a number of molecular pathways in the same cell. As our knowledge of larval physiology and molecular biology expands, nonmetamorphic events regulated by JH, such as pigmentation and metabolic processes, may provide more tractable experimental models with which to study the molecular actions of JH at the genomic level.

8.12.1. Juvenile Hormone Interaction with Ultraspiracle

One of the more intriguing discoveries since the last major reviews is that presented by Jones and Sharp (1997), who demonstrated that the nuclear receptor Ultraspiracle (USP) can bind JHIII (see Chapter 7). USP, an orphan receptor for which no endogenous ligand has been unambiguously established (Billas *et al.*, 2001), is a heterodimeric partner that interacts with ECR to form the functional ECR complex (Thomas *et al.*, 1993; Yao *et al.*, 1993) or with

DHR38, the *D. melanogaster* ortholog of the mammalian NGFI-B subfamily of orphan nuclear receptors (Baker *et al.*, 2003) (see Chapter 7). USP is an ortholog of the vertebrate retinoid X receptor (RXR) (Oro *et al.*, 1990), which is responsive to very high levels of methoprene (Harmon *et al.*, 1995).

Using a fluorescence assay that detects changes in protein conformation induced by ligand binding, Jones and Sharp (1997) demonstrated that JHIII and JHIII acid change the conformation of recombinant *D. melanogaster* USP, while farnesol and 20E do not. The USP–JH III acid interaction generates anomalous spectra, suggesting that it may not trigger a conformational change that is identical to that obtained with JH III. These investigators estimate the dissociation constant of the USP: JH III complex to be approximately 1 μ M, a value considerably higher than might be expected for a nuclear receptor that must sequester hormone directly from the circulatory system. Further refinement of the original studies indicated that methoprene competes with JH III for a binding site on USP (Jones *et al.*, 2001). It should be cautioned that these studies used only partially purified (75%) racemic JHIII, yet the investigators chose to express ligand concentrations as “concentrations of the natural active isomer in the respective binding reaction” (Jones *et al.*, 2001). Arbitrarily overlooking the impurities in the hormone preparation and using an estimated 10R JH III concentration may present problems in the interpretation of the binding constants.

Nevertheless, evidence from functional transcription assays supports this model. Using modified USP response elements coupled to a JHE core promoter (–61 to +28 relative to the start site) and a reporter gene, Jones *et al.* (2001) demonstrated that construct expression could be induced by JHIII in a dose-dependent fashion. The promoter construct was only weakly responsive to all *trans*-retinoic acid and did not respond to triiodo-L-thyronine (Jones *et al.*, 2001). Functional transcriptional assays also demonstrate that point mutations in the putative JH III binding site of USP, which abolish JH binding, cause the mutant receptor to act as a dominant negative and suppress JHIII activation (Xu *et al.*, 2002).

Jones *et al.* (2001) suggested that high levels of JH III induce a conformational change in structure to stabilize dimeric/oligomeric forms of USP. This interaction may be important in dimerization of USP with its ecdysteroid receptor partner, but how it occurs and the role of USP–DNA binding in stabilization of the complex is not yet understood. It is well known that ecdysteroids induce enhanced

heterodimer formation even when the binding of the heterodimers to cognate DNA response elements does not occur (Lezzi *et al.*, 2002).

The initial crystallographic analyses of the USP ligand binding domains from *H. virescens* (Billas *et al.*, 2001) and *D. melanogaster* (Clayton *et al.*, 2001) indicated that the putative ligand binding site is locked in an antagonist conformation. More recently, Sasorith *et al.* (2002) reinvestigated the site and found that recombinant USP could bind ligands and attempted, using computer algorithms, to dock putative ligands in the binding site. Their studies indicate that the ligand binding site is relatively large and predominantly lined with hydrophobic residues. Interestingly, the binding site of the recombinant USP contains a phospholipid inserted by the expression host, and it would be of considerable interest to know how this lipid can affect JH binding.

Computation docking of JH and analogs in the binding site of recombinant *H. virescens* USP suggests that JH may fit the putative site; however, the percentage of occupancy of the ligand binding sites by these ligands lies in the bottom range of values for classical nuclear receptors, thus raising concern about the validity of USP as the juvenile hormone receptor (Sasorith *et al.*, 2002).

USP, with its large ligand binding site and a low level of occupancy, behaves more like a sensor than a classical high affinity receptor. Indeed, using the sensor model (Chawla *et al.*, 2001), one might envision an entirely different role for USP, based on nutritional levels in the premetamorphic larva. When sufficient levels of a key dietary lipid(s) have been attained, the USP binding site becomes saturated with that nutritional ligand, which, in turn, alters the conformation of the protein. Ligand-activated USP might then enhance ecdysteroid binding by its partner, ECR. Ligand activation of the appropriate ecdysteroid receptor isoforms leads to changes in neuronal activity (Hewes and Truman, 1994; Truman, 1996) that may trigger activity in the prothoracicotropes or dendritic fields enervating them. While this hypothesis is simplistic, it could account for the very early signaling events that initiate ecdysis. It now seems clear that USP binds JH, albeit with low affinity, as well as other lipids. It may be that refinement of experiments presented by the Jones laboratory will shed new light on how the hormone acts at the molecular level.

8.12.2. Juvenile Hormone Interaction with the Methoprene-Tolerant Gene

Using mutagenesis studies to dissect the molecular action of JH, Wilson and his group have examined a

locus in *D. melanogaster*, the *Methoprene-tolerant* (*Met*) locus, that encodes for a protein similar in sequence to proteins in the basic-helix-loop-helix-PAS (bHLH-PAS) family of transcriptional regulators (Wilson and Ashok, 1998). Treating wild-type flies with high levels of methoprene at certain times during larval development leads to a distinct array of developmental abnormalities, including aberrations in the central nervous system, salivary glands, and muscles (Restifo and Wilson, 1998). In contrast to wild-types, flies carrying the *Met* mutation are resistant to high levels of methoprene and appear to develop and reproduce normally (Wilson and Fabian, 1986).

The *Met* gene (GenBank Accession no. AF034859) encodes for a 716-residue protein which in wild-type flies displays a dissociation constant of 6 nM for JH III (Shemshedini *et al.*, 1990). Competitive displacement studies comparing the homologs indicate that JH I, JH II, JH III acid, and methoprene are weak competitors when compared with JH III; methoprene binding is 100-fold weaker than that of JH III. Flies carrying the *Met* mutation exhibited a significantly reduced (sixfold) binding affinity for JH III (38 nM). The reduced affinity of the 85 kDa *Met* protein for methoprene translates into a 50–100-fold increase in resistance to both its toxic and morphogenetic effects. Flies carrying the *Met* mutation are also resistant to toxic levels of the naturally occurring hormones JH III and JHB₃, but not to various other classes of insecticides. This important distinction demonstrates that *Met* is not a general insecticide-resistance gene, but is specific for JH and JH analogs (Wilson *et al.*, 2003).

Analysis of the *Met* sequence indicates it has three regions displaying similarity to the bHLH-PAS family of transcriptional regulators. It is instructive to note that the bHLH-PAS gene family was named for three important members of the group: the *Period* gene, the *Aryl hydrocarbon receptor* gene, and the *Single-minded* gene. Of particular importance is the similarity of *Met* to the *Aryl hydrocarbon receptor* (*Ahr*) gene that encodes for a xenobiotic binding protein (Ashok *et al.*, 1998). The fact that methoprene interacts with a member of the AHR family of receptors may explain why so many compounds structurally distant from JH have JH-like activity (Stahl, 1975). With its similarity to AHRs, *Met* could bind hydrophobic ligands that marginally resemble JH and regulate certain JH-responsive genes (Ashok *et al.*, 1998). Equally interesting is the fact that the vertebrate AHR partners with another protein, the aryl hydrocarbon nuclear translocator, to form an active transcriptional regulator that activates genes responsible for mediating the

response to xenobiotics. While no partner has yet been found for Met, the potential for such an interaction has been noted (Restifo and Wilson, 1998).

Immunolocalization studies suggest that Met is limited to nuclei (Pursley *et al.*, 2000), thus ruling out the earlier suggestion that Met acts as a cytosolic “sink” (Shemshedini *et al.*, 1990). Developmentally, it is present in all cells of the *Drosophila* embryo from the 256 cell stage until early gastrulation, when the signal begins to decline. In second and third stadium larvae Met is found in salivary glands, fat body, imaginal discs, and gut primordium. Met is also present in pupal histoblasts and in adult reproductive tissues, including the female ovarian follicle cells and spermatheca and the male accessory glands (Pursley *et al.*, 2000).

To date, over a dozen alleles of Met have been recovered in genetic screens (Wilson *et al.*, 2003), but one in particular, *Met*²⁷, poses a perplexing question about the role of Met in JH action. When examined by Northern analysis and RT-PCR, flies homozygous for *Met*²⁷ lack Met transcripts, yet these insects survive and develop into adults. The lack of Met expression becomes evident only in the adult female, in which there is an 80% reduction in oogenesis (Wilson and Ashok, 1998). If flies carrying a null mutation in Met can undergo seemingly normal development and limited oogenesis, both events being under JH control, the role of Met in JH action is open to question. Pursley *et al.* (2000) suggest that “genetic redundancy” (Krakauer and Plotkin, 2002), the equivalent of back-up systems for biological phenomena, may be involved in this situation. Given the apparent lack of Met functions during the larval stages, but its significant role in both male and female reproduction (Wilson and Ashok, 1998; Wilson *et al.*, 2003), it may be that JH utilizes several different and distinct molecular pathways to control development and reproduction.

8.12.3. Juvenile Hormone and Metamorphosis: The Role of the Broad-Complex

A growing body of evidence has implicated the transcription factor Broad (FlyBase identification no. FBgn0010011) as a key component in the initiation of metamorphosis (Riddiford *et al.*, 2003). Mutations in *Broad*, a member of the Broad-Tramtrack-Bric-a-Brac family, allow normal larval development to occur but prevent pupation (Kiss *et al.*, 1988; Bayer *et al.*, 1996; Riddiford *et al.*, 2003). Less severe Broad phenotypes have wings that are shorter and wider than wild-types. The *Broad* gene is large (100 kb) and in *Drosophila* encodes for at least six isoforms of the protein,

which all share a common N-terminal of approximately 425 amino acids (Bayer *et al.*, 1996). Through alternative splicing, the C-terminal can be represented by any one of four pairs of C₂H₂-type zinc finger domains, Z1, Z2, Z3, and Z4. The N- and C-terminals are connected by linkage domains of varying lengths. The Z4 transcript encodes a protein of 877 amino acids.

After the onset of metamorphosis, the nuclei of all larval and imaginal cells display Broad; however, each tissue appears to have its own specific constellation of isoforms that appear in a temporally unique sequence (Mugat *et al.*, 2000). For example, Mugat *et al.* (2000), using an isoform-specific antibody in their studies on *D. melanogaster* fat body, followed a shift from Z2 to the other isoforms as metamorphic events proceed. Bayer *et al.* (1996) demonstrated that Z1 is the predominant isoform during pupal cuticle formation in the abdominal epidermis and imaginal discs.

Ingestion of the JH analog pyriproxifen by first instar *D. melanogaster* prolongs the third stadium by several days (Riddiford *et al.*, 2003) and results in a 12 h delay in the appearance of Broad in abdominal epidermis. By the time of pupation, however, Broad protein levels were similar to controls, indicating that this potent JH analog cannot prevent the appearance of this protein. The adult abdominal epidermis, formed from nests of tissue called histoblasts, begins to proliferate and spread over the abdomen after puparium formation. These new cells displace the existing larval cells. Broad is found in larval cells that are destined to die, but cannot be found in imaginal cells once they have spread across the abdomen. Interestingly, JH treatment delays the loss of Broad in the imaginal cells and causes these cells to produce proteins indicative of a pupal-like cuticle. Increasing Z1 levels through misexpression at the onset of adult cuticle formation mimics the effects of JH, causing the reappearance of mRNAs encoding pupal cuticular proteins and the suppression of mRNAs encoding adult cuticular proteins (Zhou and Riddiford, 2002). These investigators suggest that JH prolongs the expression of the Z1 isoform, and suppresses the onset of adult cuticular formation.

In *M. sexta*, only three Broad isoforms have been discovered, Z2, Z3, and Z4, with Z4 being most prominent in epidermal tissue involved in pupal cuticle formation (Zhou *et al.*, 1998; Zhou and Liu 2001). As observed in *D. melanogaster*, the protein's appearance is well-defined spatially and temporally, following the pattern of pupal commitment, as defined by the loss of sensitivity to JH. Most significantly, within 6 h of 20E treatment, epidermal

preparations *in vitro* display a marked rise in Broad mRNA (Zhou *et al.*, 1998; Zhou and Riddiford, 2002). This rise signals the onset of commitment to pupation. In cells that are not yet committed, JHI prevents the induction of Broad mRNA synthesis by 20E (Zhou *et al.*, 1998). Once Broad appears in the cells, JH can no longer suppress the accumulation of Broad mRNA, and its levels remain elevated, even during the rise in JH titers later in the stadium. *Broad* expression eventually disappears during the early pupal stage coinciding with the onset of adult cuticular synthesis. The role of Broad was further defined by applying sufficient JH to pupae to elicit the formation of a second pupal cuticle; in this case, Broad mRNA and protein rose after exposure to 20E, then dropped after the second pupal cuticle was formed (Zhou and Riddiford, 2002). Thus, Broad's presence in the cell appears to inhibit larval cuticular synthesis, while permitting pupal cuticular synthesis to proceed. Moreover, Broad appears to suppress the synthesis of the adult cuticle. Exogenous JH can delay but not prevent the expression of Broad, but the molecular actions of the hormone remain unclear.

8.12.4. Juvenile Hormone Regulation of Cytosolic Malate Dehydrogenase

JH has long been implicated in the regulation of cellular energy production and of biochemical and second messenger pathways. This long history includes research on such diverse mechanisms as modification of oxidative phosphorylation (Clarke and Baldwin, 1960; Chefurka, 1978); shifts in cellular potassium levels (Kroeger, 1968); membrane perturbation (Baumann, 1969; Barber *et al.*, 1981); second messenger regulation (Everson and Feir, 1976; Kensler *et al.*, 1978; Yamamoto *et al.*, 1988); changes in cytoskeletal structure (Capella and Hartfelder, 2002); and the development of asymmetric organs (Adam *et al.*, 2003). Interest in certain of these areas has been rekindled by a series of studies carried out by Farkas and his associates on the regulation of cytosolic malate dehydrogenase in *D. melanogaster* (Farkas and Knopp, 1997, 1998; Farkas and Sutakova, 2001; Farkas *et al.*, 2002).

Cytosolic malate dehydrogenase (EC 1.1.1.40), also known as NADP-malic enzyme (ME), is involved in production of cytoplasmic NADPH. The enzyme is particularly prevalent in tissues that are involved in fatty acid synthesis and it is a target for various hormones in vertebrates (Farkas *et al.*, 2002). In a series of papers, Farkas and his group have demonstrated that ME, a product of the *Men* gene (CG 10120; FBgn 0002719), is under the

regulation of JH during the mid-third stadium of *D. melanogaster*. A dose of 5 pg of JHIII will elicit half-maximal ME activity and while this level is higher than physiological levels (0.02 pg per larva) (Sliter *et al.*, 1987), it is still lower than the dose typically used to induce morphological aberrations (Riddiford and Ashburner, 1991).

The response to JHIII occurs in two phases, an early phase occurring 3 h after treatment, in which ME activity doubles, and a late phase occurring 12 h after treatment, in which a threefold increase over the control is observed. The transcriptional inhibitor actinomycin D does not block the early ME rise, but does block the late rise, suggesting that JH can increase ME activity without new protein synthesis. That JH added directly to the cytosol can not induce the ME rise indicates that the hormone may be acting through a pre-existing pathway that is immediately affected, such as that of a second messenger cascade (Farkas *et al.*, 2002). In contrast to the early rise, the late rise in ME activity is sensitive to actinomycin D, indicating that this rise is dependent on *de novo* transcription of ME. The authors have yet to examine the levels of ME mRNA that would confirm the inhibitor studies.

Using developmentally staged, wild-type larvae and the temperature-sensitive mutants *ecd¹* and *su(f)^{ts67g}*, Farkas *et al.* (2002) demonstrated that ecdysteroids are also involved in the JH-regulated ME rise. Wild-type flies respond to JH only near the time of pupariation, when ecdysteroid titers begin to rise. By contrast, in temperature-sensitive, ecdysone-deficient mutants, JH is unable to initiate the expected ME rise at elevated temperatures. These observations suggest that a complex synergistic regulatory mechanism is acting to control ME levels. A low level of ecdysteroid must be present before JH can induce activation of ME, yet high levels of ecdysteroid, such as those seen at pupariation, downregulate the activity of ME. The physiological role of JH in upregulating this event cannot be well understood unless placed in the context of lipogenesis. Farkas *et al.* (2002) suggest that the ME–JH interaction may play a role in increasing the levels of unsaturated fatty acids. JH and certain JH analogs have been shown to induce a rise in unsaturated fatty acids (Schneider *et al.*, 1995) and phospholipid levels (Della-Cioppa and Engelmann, 1984) in adult *S. gregaria* and *L. maderae*. In *D. melanogaster*, the ecdysteroid-induced onset of metamorphosis leads to the cessation of feeding; it may be that high levels of JH during the earlier stadia and the early part of the last stadia ensure availability of lipid resources during metamorphosis. While the pathways are still not clear, this paradigm of JH

action at the molecular level holds much promise for better understanding how the hormone may regulate homeostatic mechanisms that are necessary for insect growth and development.

References

- Abdel-Aal, Y.A.I., Hammock, B.D., 1985. 3-Octylthio-1,1,1-trifluoro-2-propanone, a high affinity and slow binding inhibitor of juvenile hormone esterase from *Trichoplusia ni*. *Insect Biochem. Mol. Biol.* 15, 111–122.
- Abdel-Aal, Y.A.I., Hammock, B.D., 1986. Transition state analogs as ligands for affinity purification of juvenile hormone esterase. *Science* 233, 1073–1076.
- Abdel-Latif, M., Meyering-Vos, M., Hoffmann, K.H., 2003. Molecular characterization of cDNAs from the fall armyworm *Spodoptera frugiperda* encoding *Manduca sexta* allatotropin and allatostatin. *Insect Biochem. Mol. Biol.* 33, 467–476.
- Abdu, U., Takac, P., Laufer, H., Sagi, A., 1998. Effect of methyl farnesoate on late larval development and metamorphosis in the prawn *Macrobrachium rosenbergii*: a juvenoid-like effect? *Biol. Bull.* 195, 112–119.
- Adam, G., Perrimon, N., Noselli, S., 2003. The retinoic-like juvenile hormone controls the looping of left-right asymmetric organs in *Drosophila*. *Development* 130, 2397–2405.
- Adams, D.S., Eickbush, T.H., Herrera, R.J., Lizardi, P.M., 1986. A highly reiterated family of transcribed oligo(A)-terminated, interspersed DNA elements in the genome of *Bombyx mori*. *J. Mol. Biol.* 187, 465–478.
- Allen, C.U., Janzen, W.P., Granger, N.A., 1992a. Manipulation of intracellular calcium affects *in vitro* juvenile hormone synthesis by larval corpora allata of *Manduca sexta*. *Mol. Cell. Endocrinol.* 84, 227–241.
- Allen, C.U., Herman, B., Granger, N.A., 1992b. Fura-2 measurement of cytosolic free Ca^{2+} concentration in corpus allatum cells of larval *Manduca sexta*. *J. Exp. Biol.* 166, 253–266.
- Anspaugh, D.D., Kennedy, G.G., Roe, R.M., 1995. Purification and characterization of a resistance-associated esterase from the Colorado potato beetle, *Leptinotarsa decemlineata*. *Pestic. Biochem. Physiol.* 53, 84–96.
- Applebaum, S.W., Avisar, E., Heifetz, Y., 1997. Juvenile hormone and locust phase. *Arch. Insect Biochem. Physiol.* 35, 375–391.
- Ashok, M., Turner, C., Wilson, T.G., 1998. Insect juvenile hormone resistance gene homology with the bHLH-PAS family of transcriptional regulators. *Proc. Natl Acad. Sci. USA* 95, 2761–2766.
- Aucoin, R.R., Rankin, S.M., Stay, B., Tobe, S.S., 1987. Calcium and cyclic AMP involvement in the regulation of juvenile hormone biosynthesis in *Diptera punctata*. *Insect Biochem.* 17, 965–969.
- Audsley, N., Weaver, R.J., Edwards, J.P., 1998. Enzyme linked immunosorbent assay for *Manduca sexta* allatostatin (Mas-AS), isolation and measurement of Mas-AS immunoreactive peptide in *Lacanobia oleracea*. *Insect Biochem. Mol. Biol.* 28, 775–784.
- Audsley, N., Weaver, R.J., Edwards, J.P., 1999a. Juvenile hormone synthesis by corpora allata of tomato moth, *Lacanobia oleracea*, and the effects of allatostatin and allatotropin *in vitro*. *Eur. J. Entomol.* 96, 287–297.
- Audsley, N., Weaver, R.J., Edwards, J.P., 1999b. Significance of *Manduca sexta* allatostatin in the tomato moth *Lacanobia oleracea*. In: Sandman, C.A., Strand, F.L., Beckman, B., Cronwall, B.M., Flynn, F.W., Nachman, F.W. (Eds.), *Neuropeptides, Structure and Function in Biology and Behavior*, vol. 897. New York Academy of Science, New York, pp. 330–341.
- Audsley, N., Weaver, R.J., Edwards, J.P., 2000. Juvenile hormone biosynthesis by corpora allata of larval tomato moth, *Lacanobia oleracea*, and regulation by *Manduca sexta* allatostatin and allatotropin. *Insect Biochem. Mol. Biol.* 30, 681–689.
- Audsley, N., Weaver, R.J., Edwards, J.P., 2002. Degradation of *Manduca sexta* allatostatin and allatotropin by proteases associated with the foregut of *Lacanobia oleracea* larvae. *Peptides* 23, 2015–2023.
- Auerswald, L., Birgul, N., Gade, G., Kreienkamp, H.J., Richter, D., 2001. Structural, functional, and evolutionary characterization of novel members of the allatostatin receptor family from insects. *Biochem. Biophys. Res. Commun.* 282, 904–909.
- Baehr, J.-C., Caruelle, J.P., Poras, M., 1986. The activity of denervated corpora allata in a diapausing strain of *Locusta migratoria*: *in vivo* and *in vitro* studies. *Int. J. Invertebr. Reprod. Devel.* 10, 143–150.
- Baehr, J.-C., Porcheron, P., Papillon, M., Dray, F., 1979. Haemolymph levels of juvenile hormone, ecdysteroids and protein during the last two larval instars of *Locusta migratoria*. *J. Insect Physiol.* 25, 371–460.
- Baehr, J.-C., Pradelles, P., Lebreux, C., Cassier, P., Dray, F., 1976. A simple and sensitive radioimmunoassay of insect juvenile hormone using an iodinated tracer. *FEBS Lett.* 69, 123–128.
- Baker, F.C., 1990. Techniques for identification and quantification of juvenile hormones and related compounds in arthropods. In: Gupta, A.P. (Ed.), *Morphogenetic Hormones of Arthropods*. Rutgers University Press, New Brunswick, NJ, pp. 389–444.
- Baker, F.C., Lanzrein, B., Miller, C.A., Tsai, L.W., Jamieson, G.C., et al., 1984. Detection of only JHIII in several life-stages of *Nauphoeta cinerea* and *Thermobia domestica*. *Life Sci.* 35, 1553–1560.
- Baker, K.D., Shewchuk, L.M., Kozlova, T., Makishima, M., Hassell, A., et al., 2003. The *Drosophila* orphan nuclear receptor DHR38 mediates an atypical ecdysteroid signaling pathway. *Cell* 113, 731–742.
- Baker, F.C., Tsai, L.W., Reuter, C.C., Schooley, D.A., 1987. *In vivo* fluctuation of JH, JH acid, and ecdysteroid titer, and JH esterase activity, during development of fifth stadium *Manduca sexta*. *Insect Biochem.* 17, 989–996.
- Baker, F.C., Tsai, L.W., Reuter, C.C., Schooley, D.A., 1988. The absence of significant levels of the known

- juvenile hormones and related compounds in the milkweed bug, *Oncopeltus fasciatus*. *Insect Biochem.* 18, 453–462.
- Barber, R.F., Downer, R.G.H., Thompson, R.G.H., 1981. Perturbation of phospholipid membranes by juvenile hormone. *Biochim. Biophys. Acta* 643, 593–600.
- Barker, J.F., Davey, K.G., 1983. A polypeptide from the brain and corpus cardiacum of male *Rhodnius prolixus* which stimulates *in vitro* protein synthesis in the transparent accessory reproductive gland. *Insect Biochem.* 13, 7–10.
- Baumann, G., 1969. Juvenile hormone: effects on bimolecular lipid membranes. *Nature* 223, 316–317.
- Bayer, C., von, Kalm L., Fristrom, J.W., 1996. Gene regulation in imaginal discs and salivary gland development during metamorphosis. In: Gilbert, L.I., Tata, J.R., Atkinson, B.G. (Eds.), *Metamorphosis*. Academic Press, London, pp. 321–361.
- Bean, D.W., Beck, S.D., Goodman, W.G., 1982. Juvenile hormone esterases in diapause and nondiapause larvae of the European corn borer, *Ostrinia nubilalis*. *J. Insect Physiol.* 6, 485–492.
- Bean, D.W., Goodman, W.G., Beck, S.D., 1983. Regulation of juvenile hormone esterase activity in the European corn borer, *Ostrinia nubilalis*. *J. Insect Physiol.* 29, 877–883.
- Beckstead, R., Ortiz, J.A., Sanchez, C., Prokopenko, S.N., Chambon, P., *et al.*, 2001. *Bonus*, a *Drosophila* homolog of TIF1 proteins, interacts with nuclear receptors and can inhibit beta *FTZ-F1*-dependent transcription. *Mol. Cell* 7, 753–765.
- Beckstead, R., Prokopenko, S.N., Losson, R., Chambon, P., Bellen, H., 2000. *Bonus*, a *Drosophila* RIF1 homolog, plays a role in hormone response. *Drosophila Res. Conf.* 41, 54.
- Bede, J.C., Goodman, W.G., Tobe, S.S., 1999. Developmental distribution of insect juvenile hormone III in the sedge, *Cyperus iria* L. *Phytochemistry* 52, 1269–1274.
- Bede, J.C., Teal, P.E.A., Goodman, W.G., Tobe, S.S., 2001. Biosynthetic pathway of insect juvenile hormone III in cell suspension cultures of the sedge *Cyperus iria*. *Plant Physiol.* 127, 584–593.
- Beintema, J.J., Stam, W.T., Hazes, B., Smidt, M.P., 1994. Evolution of arthropod hemocyanins and insect storage proteins (hexamerins). *Mol. Biol. Evol.* 11, 493–503.
- Bellés, X., Graham, L.A., Bendena, W.G., Ding, Q., Edwards, J.P., *et al.*, 1999. The molecular evolution of the allatostatin precursor in cockroaches. *Peptides* 20, 11–22.
- Bendena, W.G., Garside, C.S., Yu, C.G., Tobe, S.S., 1997. Allatostatins: diversity in structure and function of an insect neuropeptide family. *Ann. New York Acad. Sci.* 814, 53–66.
- Bergamasco, R., Horn, D.H.S., 1983. Distribution and role of insect hormones in plants. In: Downer, R.G.H., Laufer, H. (Eds.), *Invertebrate Endocrinology*. Alan R. Liss, New York, pp. 627–656.
- Bergot, B.J., Baker, F.C., Cerf, D.C., Jamieson, G., Schooley, D.A., 1981a. Qualitative and quantitative aspects of juvenile hormone titers in developing embryos of several insect species: discovery of a new JH-like substance extracted from eggs of *Manduca sexta*. In: Pratt, G.E., Brooks, G.T. (Eds.), *Juvenile Hormone Biochemistry*. Elsevier/North-Holland, Amsterdam, pp. 33–45.
- Bergot, B.J., Ratcliff, M., Schooley, D.A., 1981b. A method for quantitative determination of juvenile hormones by mass spectroscopy. *J. Chromatogr.* 204, 231–244.
- Berridge, M.J., 1993. Inositol trisphosphate and calcium signaling. *Nature* 361, 315–325.
- Bhaskaran, G., Dahm, K.H., Barrera, P., Pacheco, J.L., Peck, K.E., *et al.*, 1990. Allatohibin, a neurohormonal inhibitor of juvenile hormone biosynthesis in *Manduca sexta*. *Gen. Comp. Endocrinol.* 78, 123–136.
- Bhatt, T.R., Horodyski, F., 1999. Expression of the *Manduca sexta* allatotropin gene in cells of the central and enteric nervous system. *J. Comp. Neurol.* 403, 407–420.
- Bidmon, H.J., Stumpf, W.E., Granger, N.A., 1992. Ecdysteroid receptors in the neuroendocrine–endocrine axis of a moth. *Experientia* 48, 42–47.
- Billas, I.M.L., Moulinier, L., Rochel, N., Moras, D., 2001. Crystal structure of the ligand-binding domain of the ultraspiracle protein, usp, the ortholog of retinoid x receptors in insects. *J. Biol. Chem.* 276, 7465–7474.
- Birgul, N., Weise, C., Kreienkamp, H.J., Richter, D., 1999. Reverse physiology in *Drosophila*: identification of a novel allatostatin-like neuropeptide and its cognate receptor structurally related to the mammalian somatostatin/galanin/opioid receptor family. *EMBO J.* 18, 5892–5900.
- Blackmore, P.F., Beebe, S.J., Danforth, D.R., Alexander, N., 1990. Progesterone and 17 alpha-hydroxyprogesterone: novel stimulators of calcium influx in human sperm. *J. Biol. Chem.* 265, 1376–1380.
- Blenau, W., Baumann, A., 2001. Molecular and pharmacological properties of insect biogenic amine receptors: lessons from *Drosophila melanogaster* and *Apis mellifera*. *Arch. Insect Biochem. Physiol.* 48, 13–38.
- Bogus, M., Scheller, K., 1996. Allatotropin released by the brain controls larval molting *Galleria mellonella* by affecting juvenile hormone synthesis. *Int. J. Devel. Biol.* 40, 205–210.
- Bollenbacher, W.E., 1988. The interendocrine regulation of larval–pupal development in the tobacco hornworm, *Manduca sexta*: a model. *J. Insect Physiol.* 34, 941–948.
- Bollenbacher, W.E., Granger, N.A., Katahira, E.J., O'Brien, M.A., 1987. Developmental endocrinology of larval moulting in the tobacco hornworm, *Manduca sexta*. *J. Exp. Biol.* 128, 175–192.
- Bollenbacher, W.E., Smith, S.L., Goodman, W.G., Gilbert, L.I., 1981. Ecdysteroid titer during larval–pupal–adult development of the tobacco hornworm, *Manduca sexta*. *Gen. Comp. Endocrinol.* 44, 302–306.
- Bonning, B.C., Hammock, B.D., 1996. Development of recombinant baculoviruses for insect control. *Annu. Rev. Entomol.* 41, 191–210.

- Bonning, B.C., Ward, V.K., Vanmeer, M.M.M., Booth, T.F., Hammock, B.D., 1997. Disruption of lysosomal targeting is associated with insecticidal potency of juvenile hormone esterase. *Proc. Natl Acad. Sci. USA* 94, 6007–6012.
- Boore, J.L., Collins, T.M., Stanton, D., Daehler, L.L., Brown, W.M., 1995. Deducing the pattern of arthropod phylogeny from mitochondrial DNA rearrangements. *Nature* 376, 163–165.
- Borovsky, D., Carlson, D.A., Hancock, R.G., Rembold, H., Vanhandel, E., 1994a. *De novo* biosynthesis of juvenile hormone III and I by the accessory glands of the male mosquito. *Insect Biochem. Mol. Biol.* 24, 437–444.
- Borovsky, D., Carlson, D.A., Ujvary, I., Prestwich, G.D., 1994b. Biosynthesis of (10R)-juvenile hormone III from farnesoic acid by *Aedes aegypti* ovary. *Arch. Insect Biochem. Physiol.* 27, 11–25.
- Borst, D.W., Laufer, H., Landau, M., Chang, E.S., Hertz, W.A., et al., 1987. Methyl farnesoate and its role in crustacean reproduction and development. *Insect Biochem.* 17, 1123–1127.
- Boulay, R., Hooper-Bui, L.M., Woodring, J., 2001. Oviposition and oogenesis in virgin fire ant females, *Solenopsis invicta*, are associated with a high level of dopamine in the brain. *Physiol. Entomol.* 26, 294–299.
- Bowers, W.S., Thompson, M.J., Vebel, E., 1965. Juvenile and gonadotropic hormone activity of 10,11-epoxyfarnesoic acid methyl ester. *Life Sci.* 4, 2323–2331.
- Bownes, M., Rembold, H., 1987. The titer of juvenile hormone during the pupal and adult stages of the life cycle of *Drosophila melanogaster*. *Eur. J. Biochem.* 164, 709–712.
- Bowser, P.R.F., Tobe, S.S., 2000. Partial characterization of a putative allatostatin receptor in the midgut of the cockroach *Diploptera punctata*. *Gen. Comp. Endocrinol.* 119, 1–10.
- Branden, C., Tooze, J., 1999. Introduction to Protein Structure, 2nd edn. Garland Press, New York.
- Braun, R.P., Wyatt, G.R., 1996. Sequence of the hexameric juvenile hormone-binding protein from the hemolymph of *Locusta migratoria*. *J. Biol. Chem.* 271, 31756–31762.
- Brindle, P.A., Baker, F.C., Tsai, L.W., Reuter, C.C., Schooley, D.A., 1987. Sources of propionate for the biogenesis of ethyl-branched insect juvenile hormone: role of isoleucine and valine. *Proc. Natl Acad. Sci. USA* 84, 7906–7910.
- Brindle, P.A., Baker, F.C., Tsai, L.W., Schooley, D.A., 1992. Comparative metabolism of isoleucine by corpora allata of nonlepidopteran insects versus lepidopteran insects in relation to juvenile hormone biosynthesis. *Arch. Insect Biochem. Physiol.* 19, 1–15.
- Brindle, P.A., Schooley, D.A., Tsai, L.W., Baker, F.C., 1988. Comparative metabolism of branched-chain amino acids to precursors of juvenile hormone biogenesis in corpora allata of lepidopteran versus nonlepidopteran insects. *J. Biol. Chem.* 263, 10653–10657.
- Broadie, K.S., Bate, M., Tublitz, N.J., 1991. Quantitative staging of embryonic development of the tobacco hawkmoth, *Manduca sexta*. *Roux's Arch. Dev. Biol.* 199, 327–334.
- Brockhouse, A.C., Horner, H.T., Booth, T.F., Bonning, B.C., 1999. Pericardial cell ultrastructure in the tobacco hornworm, *Manduca sexta*. *Int. J. Insect Morphol. Embryol.* 28, 261–271.
- Bronsert, M., Bingol, H., Atkins, G., Stout, J., 2003. Prolonged response to calling songs by the L3 auditory interneuron in female crickets (*Acheta domesticus*): possible roles in regulating phontactic threshold and selectiveness for call carrier frequency. *J. Exp. Zool.* 296, 72–85.
- Browder, M.H., D'Amico, L.J., Nijhout, H.F., 2001. The role of low levels of juvenile hormone esterase in the metamorphosis of *Manduca sexta*. *J. Insect Sci.* 1, 1–4.
- Brüning, E., Lanzrein, B., 1987. Function of juvenile hormone III in embryonic development of the cockroach, *Nauphoeta cinera*. *Int. J. Invertebr. Reprod. Devel.* 12, 29–44.
- Brüning, E., Saxer, A., Lanzrein, B., 1985. Methyl farnesoate and juvenile hormone III in normal and precocene treated embryos of the ovoviparous cockroach *Nauphoeta cinerea*. *Int. J. Invertebr. Reprod. Devel.* 8, 269–278.
- Bürgin, C., Lanzrein, B., 1988. Stage dependent biosynthesis of methyl farnesoate and juvenile hormone III and metabolism of juvenile hormone III in embryos of the cockroach, *Nauphoeta cinera*. *Insect Biochem.* 18, 3–9.
- Burmester, T., 2002. Origin and evolution of arthropod hemocyanins and related proteins. *J. Comp. Physiol. B* 172, 95–107.
- Burmester, T., Massey, H.C., Zakharkin, S.O., Benes, H., 1998. The evolution of hexamerins and the phylogeny of insects. *J. Mol. Evol.* 47, 93–108.
- Burns, S.N., Teal, P.E.A., Meer, R.K.V., Nation, J.L., Vogt, J.T., 2002. Identification and action of juvenile hormone III from sexually mature alate females of the red imported fire ant, *Solenopsis invicta*. *J. Insect Physiol.* 48, 357–365.
- Buszczak, M., Segraves, W.A., 2000. Insect metamorphosis: out with the old, in with the new. *Curr. Biol.* 10, R830–R833.
- Campbell, P.M., Harcourt, R.L., Crone, E.J., Claudianos, C., Hammock, B.D., et al., 2001. Identification of a juvenile hormone esterase gene by matching its peptide mass fingerprint with a sequence from the *Drosophila* genome project. *Insect Biochem. Mol. Biol.* 31, 513–520.
- Campbell, P.M., Healy, M.J., Oakeshott, J.G., 1992. Characterisation of juvenile hormone esterase in *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* 22, 665–677.

- Capella, I.C.S., Hartfelder, K., 2002. Juvenile-hormone-dependent interaction of actin and spectrin is crucial for polymorphic differentiation of the larval honey bee ovary. *Cell Tissue Res.* 307, 265–272.
- Carey, M., Smale, S.T., 2000. Transcriptional Regulation in Eukaryotes: Concepts, Strategies, Techniques. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Casas, J., Harshman, L.G., Hammock, B.D., 1991. Epoxide hydrolase activity on juvenile hormone in *Manduca sexta*. *Insect Biochem.* 21, 17–26.
- Cascone, P.J., Schwartz, L.M., 2001. Post-transcriptional regulation of gene expression during the programmed death of insect skeletal muscle. *Devel. Genes Evol.* 211, 397–405.
- Castillo-Gracia, M., Couillaud, F., 1999. Molecular cloning and tissue expression of an insect farnesyl fdiophosphate synthase. *Eur. J. Biochem.* 262, 365–370.
- Caveney, S., 1970. Juvenile hormone and wound modeling of *Tenebrio* cuticle architecture. *J. Insect Physiol.* 16, 1087–1107.
- Champlin, D.T., Reiss, S.E., Truman, J.W., 1999. Hormonal control of ventral diaphragm myogenesis during metamorphosis of the moth, *Manduca sexta*. *Devel. Genes Evol.* 209, 265–274.
- Chau, P.L., Vanaalten, D.M.F., Bywater, R.P., Findlay, J.B.C., 1999. Functional concerted motions in the bovine serum retinol-binding protein. *J. Comp. Aided Mol. Des.* 13, 11–20.
- Chawla, A., Repa, J.J., Evans, R.M., Mangelsdorf, D.J., 2001. Nuclear receptors and lipid physiology: opening the X-files. *Science* 294, 1866–1870.
- Chefurka, W., 1978. Sesquiterpene juvenile hormones: novel uncouplers of oxidative phosphorylation. *Biochem. Biophys. Res. Commun.* 83, 571–578.
- Chen, L., O'Keefe, S.L., Hodgetts, R.B., 2002a. Control of dopa decarboxylase gene expression by the Broad-Complex during metamorphosis in *Drosophila*. *Mech. Devel.* 119, 145–156.
- Chen, L., Reece, C., O'Keefe, S.L., Hawryluk, G.W.L., Engstrom, M.M., et al., 2002b. Induction of the early-late *Ddc* gene during *Drosophila* metamorphosis by the ecdysone receptor. *Mech. Devel.* 114, 95–107.
- Chen, P.S., Stumm-Zollinger, E., Aigaki, T., Balmer, J., Bienz, M., et al., 1988. A male accessory gland peptide that regulates reproductive behavior of female *D. melanogaster*. *Cell* 54, 291–298.
- Cheon, H.M., Hwang, S.J., Kim, H.J., Jin, B.R., Chae, K.S., et al., 2002. Two juvenile hormone suppressible storage proteins may play different roles in *Hyphantria cunea*. *Arch. Insect Biochem. Physiol.* 50, 157–172.
- Chiang, A.S., Lin, W.Y., Liu, H.P., Pszczolkowski, M.A., Fu, T.F., et al., 2002a. Insect NMDA receptors mediate juvenile hormone biosynthesis. *Proc. Natl Acad. Sci. USA* 99, 37–42.
- Chiang, A.S., Gadot, M., Burns, E.L., Schal, C., 1991. Developmental regulation of juvenile hormone synthesis: ovarian synchronization of volumetric changes of corpus allatum cells in cockroaches. *Mol. Cell. Endocrinol.* 75, 141–147.
- Chiang, A.S., Pszczolkowski, M.A., Liu, H.P., Lin, S.C., 2002b. Ionotropic glutamate receptors mediate juvenile hormone synthesis in the cockroach, *Diploptera punctata*. *Insect Biochem. Mol. Biol.* 32, 669–678.
- Chiang, A.S., Gadot, M., Schal, C., 1989. Morphometric analysis of corpus allatum cells in adult females of three cockroach species. *Mol. Cell. Endocrinol.* 67, 179–184.
- Clarke, K.U., Baldwin, R.W., 1960. The effect of insect hormones and of 2:2-dinitrophenol on the mitochondrion of *Locusta migratoria*. *J. Insect Physiol.* 5, 37–46.
- Clayton, G.M., Peak-Chew, S.Y., Evans, R.M., Schwabe, J.W.R., 2001. The structure of the ultraspiracle ligand-binding domain reveals a nuclear receptor locked in an inactive conformation. *Proc. Natl Acad. Sci. USA* 98, 1549–1554.
- Cnaani, J., Robinson, G.E., Hefetz, A., 2000. The critical period for caste determination in *Bombus terrestris* and its juvenile hormone correlates. *J. Comp. Physiol. A* 186, 1089–1094.
- Cole, T.J., Beckage, N.E., Tan, F.F., Srinivasan, A., Ramaswamy, S.B., 2002. Parasitoid–host endocrine relations: self-reliance or co-option? *Insect Biochem. Mol. Biol.* 32, 1673–1679.
- Cotton, G., Anstee, J.H., 1991. A biochemical and structural study on the effects of methoprene on fat body development in *Locusta migratoria* L. *J. Insect Physiol.* 37, 525–539.
- Coudron, T.A., 1981. Preparation of homogeneous juvenile hormone specific esterase from the haemolymph of the tobacco hornworm, *Manduca sexta*. *Insect Biochem.* 11, 453–461.
- Couillaud, F., 1995. Juvenile hormone biosynthesis by insect corpora allata: variations on a central theme. *Netherlands J. Zool.* 45, 79–82.
- Couillaud, F., Girardie, J., Tobe, S.S., Girardie, A., 1984. Activity of disconnected corpora allata in *Locusta migratoria*: juvenile hormone biosynthesis *in vitro* and physiological effects *in vitro*. *J. Insect Physiol.* 30, 551–556.
- Crossley, A.C., 1985. Nephrocytes and pericardial cells. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 3. Pergamon, Oxford, pp. 487–515.
- Cusson, M., Lepage, A., McNeil, J.N., Tobe, S.S., 1996. Rate of isoleucine metabolism in lepidopteran corpora allata: Regulation of the proportion of juvenile hormone homologues released. *Insect Biochem. Mol. Biol.* 26, 195–201.
- Cusson, M., Miller, D., Goodman, W.G., 1997. Characterization of antibody 444 using chromatographically purified enantiomers of juvenile hormones I, II, and III: Implications for radioimmunoassays. *Analyt. Biochem.* 249, 83–87.
- Cusson, M., Prestwich, G.D., Stay, B., Tobe, S.S., 1991b. Photoaffinity labeling of allatostatin receptor proteins in the corpora allata of the cockroach, *Diploptera*

- punctata*. *Biochem. Biophys. Res. Commun.* 181, 736–742.
- Cusson, M., Tobe, S.S., McNeil, J.N., 1994. Juvenile hormones: their role in the regulation of the pheromonal communication system of the armyworm moth, *Pseudaletia unipuncta*. *Arch. Insect Biochem. Physiol.* 25, 329–345.
- Cusson, M., Yagi, K.J., Guan, X.C., Tobe, S.S., 1992. Assessment of the role of cyclic nucleotides in allatostatatin-induced inhibition of juvenile hormone biosynthesis in *Diploptera punctata*. *Mol. Cell. Endocrinol.* 89, 121–125.
- Cusson, M., Yagi, K.J., Ding, Q., Duve, H., Thorpe, A., et al., 1991a. Biosynthesis and release of juvenile hormone and its precursors in insects and crustaceans: The search for a unifying arthropod endocrinology. *Insect Biochem. Mol. Biol.* 21, 1–6.
- Cymborowski, B., Bogus, M., Beckage, N.E., Williams, C.M., Riddiford, L.M., 1982. Juvenile hormone titers and metabolism during starvation-induced supernumerary larval moulting of the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol.* 28, 129–135.
- Czeisler, C.A., Klerman, E.B., 1999. Circadian and sleep-dependent regulation of hormone research in humans. *Rec. Progr. Hormone Res.* 57, 97–130.
- Dahm, K.H., Bhaskaran, G., Peter, M.G., Shirk, P.D., Seshan, K.R., et al., 1976. On the identity of the juvenile hormones in insects. In: Gilbert, L.I. (Ed.), *The Juvenile Hormones*. Plenum, New York, pp. 19–47.
- Dahm, K.H., Röller, H., Trost, B.M., 1968. The JH: IV. Stereochemistry of JH and biological activity of some of its isomers and related compounds. *Life Sci.* 7, 129–137.
- Dai, J.D., Gilbert, L.I., 1998. Juvenile hormone prevents the onset of programmed cell death in the prothoracic glands of *Manduca sexta*. *Gen. Comp. Endocrinol.* 109, 155–165.
- Dai, J.D., Gilbert, L.I., 1999. An *in vitro* analysis of ecdysteroid-elicited cell death in the prothoracic gland of *Manduca sexta*. *Cell Tissue Res.* 297, 319–327.
- Dale, J.F., Tobe, S.S., 1988a. Differences in the stimulation by calcium ionophore of juvenile hormone-III release from corpora allata of solitary and gregarious *Locusta migratoria*. *Experientia* 44, 240–241.
- Dale, J.F., Tobe, S.S., 1988b. The effect of a calcium ionophore, a calcium channel blocker and calcium-free medium on juvenile hormone release *in vitro* from corpora allata of *Locusta migratoria*. *J. Insect Physiol.* 34, 451–456.
- Darrouzet, E., Rossignol, F., Couillaud, F., 1998. The release of isoprenoids by locust corpora allata *in vitro*. *J. Insect Physiol.* 44, 103–111.
- Darrouzet, E., Mauchamp, B., Prestwich, G.D., Kerhoas, L., Ujvary, I., et al., 1997. Hydroxy juvenile hormones: new putative juvenile hormones biosynthesized by locust corpora allata *in vitro*. *Biochem. Biophys. Res. Commun.* 240, 752–758.
- Davey, K.G., 2000a. The modes of action of juvenile hormones: some questions we ought to ask. *Insect Biochem. Mol. Biol.* 30, 663–669.
- Davey, K.G., 2000b. Do thyroid hormones function in insects? *Insect Biochem. Mol. Biol.* 30, 877–884.
- Davey, K.G., Gordon, D.R.B., 1996. Fenoxycarb and thyroid hormones have JH-like effects on the follicle cells of *Locusta migratoria in vitro*. *Arch. Insect Biochem. Physiol.* 32, 613–622.
- Davey, K.G., Sevala, V.L., Gordon, D.R.B., 1993. The action of juvenile hormone and antigonadotropin on the follicle cells of *Locusta migratoria*. *Invertebr. Reprod. Devel.* 24, 39–46.
- Davis, N.T., Veenstra, J.A., Feyereisen, R., Hildebrand, J.G., 1997. Allatostatatin-like-immunoreactive neurons of the tobacco hornworm, *Manduca sexta*, and isolation and identification of a new neuropeptide related to cockroach allatostatins. *J. Comp. Neurol.* 385, 265–284.
- Dean, R.L., Locke, M., Collins, J.V., 1985. Structure of the fat body. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 3. Pergamon, Oxford, pp. 155–210.
- Debernard, S., Morisseau, C., Severson, T.F., Feng, L., Wojtasek, H., et al., 1998. Expression and characterization of the recombinant juvenile hormone epoxide hydrolase (JHEH) from *Manduca sexta*. *Insect Biochem. Mol. Biol.* 28, 409–419.
- Debernard, S., Rossignol, F., Malosse, C., Mauchamp, B., Couillaud, F., 1995. Transesterification of juvenile hormone occurs *in vivo* in locust when injected in alcoholic solvents. *Experientia* 51, 1220–1224.
- Dedos, S.G., Fugo, H., 1996. Effects of fenoxycarb on the secretory activity of the prothoracic glands in the fifth instar of the silkworm *Bombyx mori*. *Gen. Comp. Endocrinol.* 104, 213–225.
- deKort, C.A.D., 1990. Thirty-five years of diapause research with the Colorado potato beetle. *Entomol. Exp. Applic.* 56, 1–13.
- deKort, C.A.D., Bergot, B.J., Schooley, D.A., 1982. The nature and titre of juvenile hormone in the Colorado potato beetle *Leptinotarsa decemlineata*. *J. Insect Physiol.* 28, 471–474.
- deKort, C.A.D., Granger, N.A., 1996. Regulation of JH titers: the relevance of degradative enzymes and binding proteins. *Arch. Insect Biochem. Physiol.* 33, 1–26.
- Della-Cioppa, G., Engelmann, F., 1984. Juvenile hormone regulation of phospholipid synthesis in the endoplasmic reticulum of vitellogenic fat body cells from *Leucophaea maderae*. *Insect Biochem.* 14, 27–36.
- deWilde, J., Staal, G.B., deKort, C.A.D., deLoof, A., Baard, G., 1968. JH titer in the hemolymph as a function of photoperiodic treatment in the adult Colorado beetle (*Leptinotarsa decemlineata* Say). *Proc. Kon. Ned. Wetensch. Ser. C Biol. Med. Sci.* 71C, 321–326.
- Ding, Q., Donly, B.C., Tobe, S.S., Bendena, W.G., 1995. Comparison of the allatostatatin neuropeptide precursors

- in the distantly related cockroaches *Periplaneta americana* and *Diploptera punctata*. *Eur. J. Biochem.* 234, 737–746.
- Dingle, H., Winchell, R., 1997. Juvenile hormone as a mediator of plasticity in insect life histories. *Arch. Insect Biochem. Physiol.* 35, 359–373.
- Dirksen, H., Skiebe, P., Abel, B., Agricola, H., Buchner, K., et al., 1999. Structure, distribution, and biological activity of novel members of the allatostatin family in the crayfish *Orconectes limosus*. *Peptides* 20, 695–712.
- Donly, B.C., Ding, Q., Tobe, S.S., Bendena, W.G., 1993a. Molecular cloning of the gene for the allatostatin family of neuropeptides from the cockroach *Diploptera punctata*. *Proc. Natl Acad. Sci. USA* 90, 8807–8811.
- Donly, B.C., Ding, Q., Tobe, S.S., Bendena, W.G., 1993b. Molecular cloning of the gene for the allatostatin family of neuropeptides from the cockroach *Diploptera punctata*. *Proc. Natl Acad. Sci. USA* 90, 12055 (correction of 1993a).
- Dorn, A., 1975. Struktur und funktion des embryonalen corpus allatum von *Oncopeltus fasciatus*. *Verhandl. Deutsche Zool. Gesell.* 67, 85–89.
- Dorn, A., 1982. Precocene-induced effects and possible role of juvenile hormone during embryogenesis of the milkweed bug *Oncopeltus fasciatus*. *Gen. Comp. Endocrinol.* 42, 42–52.
- Dorn, A., Bishoff, S.T., Gilbert, L.I., 1987. An incremental analysis of the embryonic development of the tobacco hornworm, *Manduca sexta*. *Int. J. Invertebr. Reprod. Devel.* 11, 137–158.
- Duk, M., Krotkiewski, H., Forest, E., Parkitna, J.M.R., Kochman, M., et al., 1996. Evidence for glycosylation of the juvenile-hormone-binding protein from *Galleria mellonella* hemolymph. *Eur. J. Biochem.* 242, 741–746.
- Duportets, L., Dufour, M.C., Couillaud, F., Gadenne, C., 1998. Biosynthetic activity of corpora allata, growth of sex accessory glands and mating in the male moth *Agrotis ipsilon* (Hufnagel). *J. Exp. Biol.* 201, 2425–2432.
- Duve, H., Johnsen, A.H., Scott, A.G., Thorpe, A., 2002. Allatostatins of the tiger prawn, *Penaeus monodon*. *Peptides* 23, 1039–1051.
- Duve, H., Audsley, N., Weaver, R.J., Thorpe, A., 2000. Triple co-localisation of two types of allatostatin and an allatotropin in the frontal ganglion of the lepidopteran *Lacanobia oleracea*: innervation and action on the foregut. *Cell Tissue Res.* 300, 153–163.
- Duve, H., East, P.D., Thorpe, A., 1999. Regulation of lepidopteran foregut movement by allatostatins and allatotropin from the frontal ganglion. *J. Comp. Neurol.* 413, 405–416.
- Dyby, S., Silhacek, D.L., 1997. Juvenile hormone agonists cause abnormal midgut closure and other defects in the moth *Plodia interpunctella*. *Invertebr. Reprod. Devel.* 32, 231–244.
- East, P.D., Sutherland, T.D., Trowell, S.C., Herlt, A.J., Rickards, R.W., 1997. Juvenile hormone synthesis by ring glands of the blowfly *Lucilia cuprina*. *Arch. Insect Biochem. Physiol.* 34, 239–253.
- Edwards, J.P., Weaver, R.J., Marris, G.C., 2001. Endocrine changes in lepidopteran larvae: potential challenges to parasitoid development and survival. In: Edwards, J.P., Weaver, R.J. (Eds.), *Endocrine Interactions of Insect Parasites and Pathogens*. Bios Scientific Publishers, Oxford, pp. 1–32.
- Elekovich, M.M., Robinson, G.E., 2000. Organizational and activational effects of hormones on insect behavior. *J. Insect Physiol.* 46, 1509–1515.
- Elekovich, M.M., Schulz, D.J., Bloch, G., Robinson, G.E., 2001. Juvenile hormone levels in honey bee (*Apis mellifera* L.) foragers: foraging experience and diurnal variation. *J. Insect Physiol.* 47, 1119–1125.
- Engelmann, F., Mala, J., 2000. The interactions between juvenile hormone (JH), lipophorin, vitellogenin, and JH esterases in two cockroach species. *Insect Biochem. Mol. Biol.* 30, 793–803.
- Enslee, E.C., Riddiford, L.M., 1977. Morphological effects of JH mimics on embryonic development in the bug *Pyrrochoris apterus*. *Roux's Arch. Devel. Biol.* 181, 163–181.
- Evans, P.D., 1985. Octopamine. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology Biochemistry and Pharmacology*, vol. 11. Pergamon, Oxford, pp. 500–530.
- Everson, R.D., Feir, D., 1976. Juvenile hormone regulation of cyclic AMP and cAMP phosphodiesterase activity in *Oncopeltus fasciatus*. *J. Insect Physiol.* 22, 781–784.
- Fain, M., Riddiford, L.M., 1975. Juvenile hormone titers in the hemolymph during late larval development of the tobacco hornworm, *Manduca sexta*. *Biol. Bull.* 149, 506–521.
- Fan, Y.L., Rafaeli, A., Moshitzky, P., Kubli, E., Choffat, Y., et al., 2000. Common functional elements of *Drosophila melanogaster* seminal peptides involved in reproduction of *Drosophila melanogaster* and *Helicoverpa armigera* females. *Insect Biochem. Mol. Biol.* 30, 805–812.
- Fan, Y.L., Rafaeli, A., Gileadi, C., Kubli, E., Applebaum, S.W., 1999. *Drosophila melanogaster* sex peptide stimulates juvenile hormone synthesis and depresses sex pheromone production in *Helicoverpa armigera*. *J. Insect Physiol.* 45, 127–133.
- Farkas, R., Sutakova, G., 2001. Swelling of mitochondria induced by juvenile hormone in larval salivary glands of *Drosophila melanogaster*. *Biochem. Cell Biol.* 79, 755–764.
- Farkas, R., Knopp, J., 1998. Genetic and hormonal control of cytosolic malate dehydrogenase activity in *Drosophila melanogaster*. *Gen. Physiol. and Biophys.* 17, 37–50.
- Farkas, R., Knopp, J., 1997. Ecdysone-modulated response of *Drosophila* cytosolic malate dehydrogenase to juvenile hormone. *Arch. Insect Biochem. Physiol.* 35, 71–83.
- Farkas, R., Danis, P., Medvedova, L., Mechler, B.M., Knopp, J., 2002. Regulation of cytosolic malate dehydrogenase by juvenile hormone in *Drosophila melanogaster*. *Cell Biochem. Biophys.* 37, 37–52.

- Faulkner, D.J., Petersen, H.R., 1971. Synthesis of C18 cecropia juvenile hormone to obtain optically active forms of known absolute configuration. *J. Am. Chem. Soc.* 93, 3766–3767.
- Feng, Q.L., Ladd, T.R., Tomkins, B.L., Sundaram, M., Sohi, S.S., et al., 1999. Spruce budworm (*Choristoneura fumiferana*) juvenile hormone esterase: hormonal regulation, developmental expression and cDNA cloning. *Mol. Cell. Endocrinol.* 148, 95–108.
- Ferenz, H.J., Aden, E., 1993. Ovarian control of juvenile hormone biosynthesis in *Locusta migratoria*. *Entomol. General.* 18, 9–17.
- Fersht, A., 1985. Enzyme Structure and Mechanism. W.H. Freeman, San Francisco, CA.
- Feyereisen, R., 1985. Regulation of juvenile hormone titer: Synthesis. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology Biochemistry and Pharmacology*, vol. 7. Pergamon, Oxford, pp. 391–430.
- Feyereisen, R., Farnsworth, D.E., 1988. Forced synthesis of trace amounts of juvenile hormone II from propionate by corpora allata of a juvenile hormone III producing insect. *Experientia* 44, 47–49.
- Feyereisen, R., Farnsworth, D.E., 1987. Inhibition of insect juvenile hormone synthesis by phorbol 12-myristate 13-acetate. *FEBS Lett.* 222, 345–348.
- Feyereisen, R., Tobe, S.S., 1981. A rapid partition assay for routine analysis of juvenile hormone release by insect corpora allata. *Analyt. Biochem.* 111, 372–375.
- Flower, D.R., 1996. The lipocalin protein family: structure and function. *Biochem. J.* 318, 1–14.
- Flower, D.R., 2000. Experimentally determined lipocalin structures. *Biochim. Biophys. Acta* 1482, 46–56.
- Forman, B.M., Goode, E., Chen, J., Oro, A.E., Bradley, D.J., et al., 1995. Identification of a nuclear receptor that is activated by farnesol metabolites. *Cell* 81, 687–693.
- Friedrich, M., Tautz, D., 1995. Ribosomal DNA phylogeny of the major extant arthropod classes and the evolution of myriapods. *Nature* 376, 165–167.
- Gade, G., 2002. Allatostatin peptides: molecules with multiple functions. *Invertebr. Reprod. Devel.* 41, 127–135.
- Gade, G., Hoffmann, K.H., Spring, J.H., 1997. Hormonal regulation in insects: facts, gaps, and future directions. *Physiol. Rev.* 77, 963–1032.
- Gadot, M., Rafaeli, A., Applebaum, S.W., 1987. Partial purification and characterization of locust allatotropin I. *Arch. Insect Biochem. Physiol.* 4, 213–224.
- Garside, C.S., Koladich, P.M., Bendena, W.G., Tobe, S.S., 2002. Expression of allatostatin in the oviducts of the cockroach *Diploptera punctata*. *Insect Biochem. Mol. Biol.* 32, 1089–1099.
- Garside, C.S., Hayes, T.K., Tobe, S.S., 1997a. Degradation of dip-allatostatins by hemolymph from the cockroach, *Diploptera punctata*. *Peptides* 18, 17–25.
- Garside, C.S., Hayes, T.K., Tobe, S.S., 1997b. Inactivation of Dip-allatostatin 5 by membrane preparations from the cockroach *Diploptera punctata*. *Gen. Comp. Endocrinol.* 108, 258–270.
- Garside, C.S., Nachman, R.J., Tobe, S.S., 2000. Injection of dip-allatostatin or dip-allatostatin pseudopeptides into mated female *Diploptera punctata* inhibits endogenous rates of JH biosynthesis and basal oocyte growth. *Insect Biochem. Mol. Biol.* 30, 703–710.
- Gharib, B., deReggi, M., Connat, J.L., Chaix, J.C., 1983. Ecdysteroid and juvenile hormone changes in *Bombyx mori* eggs, related to the initiation of diapause. *FEBS Lett.* 160, 119–123.
- Gilbert, L.I., 1962. Maintenance of the prothoracic gland by the juvenile hormone in insects. *Nature* 193, 1205–1207.
- Gilbert, L.I., Granger, N.A., Roe, R.M., 2000. The juvenile hormones: historical facts and speculations on future research directions. *Insect Biochem. Mol. Biol.* 30, 617–644.
- Gilbert, L.I., Schneiderman, H.A., 1961. The content of juvenile hormone and lipid in Lepidoptera: sexual differences and developmental changes. *Gen. Comp. Endocrinol.* 1, 453–472.
- Gingrich, J.A., Caron, M.G., 1993. Recent advances in the molecular biology of dopamine receptors. *Annu. Rev. Neurosci.* 16, 299–321.
- Girardie, J., Girardie, A., 1996. Lom OMP, a putative ecdysiotropic factor for the ovary in *Locusta migratoria*. *J. Insect Physiol.* 42, 215–222.
- Girardie, J., Huet, J.C., Atay-Kadiri, Z., Ettaouil, S., Delbecq, J.P., et al., 1998. Isolation, sequence determination, physical and physiological characterization of the neuroparsins and ovary maturing parsins of *Schistocerca gregaria*. *Insect Biochem. Mol. Biol.* 28, 641–650.
- Gold, S.M.W., Davey, K.G., 1989. The effect of juvenile hormone on protein synthesis in the transparent accessory gland of male *Rhodnius prolixus*. *Insect Biochem.* 19, 139–143.
- Goodman, W.G., 1983. Hemolymph transport of ecdysteroids and juvenile hormones. In: Downer, R.G.H., Laufer, H. (Eds.), *Endocrinology of Insects*. Alan R. Liss, New York, pp. 147–160.
- Goodman, W.G., 1990. Biosynthesis, titer regulation and transport of juvenile hormones. In: Gupta, A.P. (Ed.), *Morphogenetic Hormones of Arthropods*. Rutgers University Press, New Brunswick, NJ, pp. 83–124.
- Goodman, W.G., Adams, B., Trost, J.T., 1985. Purification and characterization of a biliverdin-associated protein from the hemolymph of *Manduca sexta*. *Biochem.* 24, 1168–1175.
- Goodman, W.G., Bollenbacher, W.E., Zvenko, H.L., Gilbert, L.I., 1976. A competitive binding protein assay for juvenile hormone. In: Gilbert, L.I. (Ed.), *The Juvenile Hormones*. Plenum, New York, pp. 75–95.
- Goodman, W.G., Huang, Z.H., Robinson, G.E., Strambi, C., Strambi, A., 1993. Comparison of two juvenile hormone radioimmunoassays. *Arch. Insect Biochem. Physiol.* 23, 147–152.

- Goodman, W.G., Maxfield, B., Park, Y.C., 1991. Immunochemical similarities among the hemolymph juvenile hormone binding proteins of moths. *Experientia* 47, 945–948.
- Goodman, W.G., O'Hern, P.A., Zaug, R.H., Gilbert, L.I., 1978a. Purification and characterization of a juvenile hormone binding protein from the hemolymph of the fourth instar tobacco hornworm, *Manduca sexta*. *Mol. Cell. Endocrinol.* 11, 225–242.
- Goodman, W.G., Orth, A.P., Toong, Y.C., Ebersohl, R., Hiruma, K., et al., 1995. Recent advances in radioimmunoassay technology for the juvenile hormones. *Arch. Insect Biochem. Physiol.* 30, 295–305.
- Goodman, W.G., Schooley, D.A., Gilbert, L.I., 1978b. Specificity of the juvenile hormone binding protein: the geometrical isomers of juvenile hormone I. *Proc. Natl Acad. Sci. USA* 75, 185–189.
- Goodman, W.G., Tatham, G., Nesbit, D.J., Bultmann, H., Sutton, R.D., 1987. The role of juvenile hormone in endocrine control of pigmentation in *Manduca sexta*. *Insect Biochem.* 17, 1065–1070.
- Granger, N.A., 2003. Chemistry of juvenile hormones. In: Henry, H.L., Norman, A.W. (Eds.), *Encyclopedia of Hormones*. Elsevier Science, New York, pp. 547–554.
- Granger, N.A., Allen, L.G., Sturgis, S.L., Combest, W., Ebersohl, R., 1995b. Corpora allata of the larval tobacco hornworm contain a calcium/calmodulin-sensitive adenylyl cyclase. *Arch. Insect Biochem. Physiol.* 30, 149–164.
- Granger, N.A., Bollenbacher, W.E., Vince, R., Gilbert, L.I., Baehr, J.-C., et al., 1979. *In vitro* biosynthesis of juvenile hormone by the larval corpora allata of *Manduca sexta*. *Mol. Cell. Endocrinol.* 16, 1–17.
- Granger, N.A., Ebersohl, R., Sparks, T.C., 2000. Pharmacological characterization of dopamine receptors in the corpus allatum of *Manduca sexta* larvae. *Insect Biochem. Mol. Biol.* 30, 755–766.
- Granger, N.A., Goodman, W.G., 1983. Juvenile hormone radioimmunoassays: theory and practice. *Insect Biochem.* 13, 333–340.
- Granger, N.A., Goodman, W.G., 1988. Radioimmunoassays: Juvenile Hormones. In: Gilbert, L.I., Miller, T.A. (Eds.), *Immunological Techniques in Insect Biology*. Springer, New York, pp. 215–252.
- Granger, N.A., Janzen, W.P., 1987. Inhibition of *Manduca sexta* corpora allata *in vitro* by a cerebral allatostatic neuropeptide. *Mol. Cell. Endocrinol.* 49, 237–248.
- Granger, N.A., Janzen, W.P., Ebersohl, R., 1995a. Biosynthetic products of the corpus allatum of the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol.* 25, 427–439.
- Granger, N.A., Macdonald, J.D., Menold, M., Ebersohl, R., Hiruma, K., et al., 1994. Evidence of a stimulatory effect of cyclic AMP on corpus allatum activity in *Manduca sexta*. *Mol. Cell. Endocrinol.* 103, 73–80.
- Granger, N.A., Mitchell, L.J., Niemiec, S.M., 1986. Biosynthesis of juvenile hormones I and III by the corpora allata of *Manduca sexta*: effects of *in vitro* conditions on gland activity. In: Kurstak, E. (Ed.), *In Vitro Invertebrate Hormones and Genes*. Elsevier, London, pp. 1–15.
- Granger, N.A., Niemiec, S.M., Gilbert, L.I., Bollenbacher, W.E., 1982. Juvenile hormone III biosynthesis *in vitro* by larval and pupal corpora allata of *Manduca sexta*. *Mol. Cell. Endocrinol.* 28, 587–604.
- Granger, N.A., Sehnal, F., 1974. Regulation of larval corpora allata in *Galleria mellonella*. *Nature* 21, 415–417.
- Granger, N.A., Sturgis, S.L., Ebersohl, R., Geng, C.X., Sparks, T.C., 1996. Dopaminergic control of corpora allata activity in the larval tobacco hornworm, *Manduca sexta*. *Arch. Insect Biochem. Physiol.* 32, 449–466.
- Granger, N.A., Whisenton, L.R., Janzen, W.P., Bollenbacher, W.E., 1987. Interendocrine control by 20-hydroxyecdysone of the corpora allata of *Manduca sexta*. *Insect Biochem.* 17, 949–954.
- Grasberger, H., Golcher, H.M.B., Fingerhut, A., Janssen, O.E., 2002. Loop variants of the serpin thyroxine-binding globulin: implications for hormone release upon limited proteolysis. *Biochem. J.* 365, 311–316.
- Grieneisen, M.L., Mok, A., Kieckbusch, T.D., Schooley, D.A., 1997. The specificity of juvenile hormone esterase revisited. *Insect Biochem. Mol. Biol.* 27, 365–376.
- Grieneisen, M.L., Kieckbusch, T.D., Mok, A., Dorman, G., Latli, B., et al., 1995. Characterization of the juvenile hormone epoxide hydrolase (JHEH) and juvenile hormone diol phosphotransferase (JHDPT) from *Manduca sexta* Malpighian tubules. *Arch. Insect Biochem. Physiol.* 30, 255–270.
- Grishkovskaya, I., Avvakumov, G.V., Hammond, G.L., Catalano, M.G., Muller, Y.A., 2002. Steroid ligands bind human sex hormone-binding globulin in specific orientations and produce distinct changes in protein conformation. *J. Biol. Chem.* 277, 32086–32093.
- Grossniklaus-Bürgin, C., Lanzrein, B., 1990. Qualitative and quantitative analyses of juvenile hormone and ecdysteroids from the egg to the pupal molt in *Trichoplusia ni*. *Arch. Insect Biochem. Physiol.* 14, 14–30.
- Gruetzmacher, M.C., Gilbert, L.I., Granger, N.A., Goodman, W., Bollenbacher, W.E., 1984. The effect of juvenile hormone on prothoracic gland function during the larval–pupal development of *Manduca sexta*: an *in situ* and *in vitro* analysis. *J. Insect Physiol.* 30, 331–340.
- Gruntenko, N.E., Wilson, T.G., Monastirioti, M., Rauschenbach, I.Y., 2000. Stress-reactivity and juvenile hormone degradation in *Drosophila melanogaster* strains having stress-related mutations. *Insect Biochem. Mol. Biol.* 30, 775–783.
- Gu, S.H., Chow, Y.S., Yin, C.-M., 1997. Involvement of juvenile hormone in regulation of prothoracicotrophic hormone transduction during the early last larval instar of *Bombyx mori*. *Mol. Cell. Endocrinol.* 127, 109–116.

- Hagedorn, H.H., 1983. The role of ecdysteroids in adult insects. In: Downer, R.G.H., Laufer, H. (Eds.), *Endocrinology of Insects*. Alan R. Liss, New York, pp. 271–304.
- Halarnkar, P.P., Jackson, G.P., Straub, K.M., Schooley, D.A., 1993. Juvenile hormone catabolism in *Manduca sexta*: homologue selectivity of catabolism and identification of a diol-phosphate conjugate as a major end product. *Experientia* 49, 988–994.
- Halarnkar, P.P., Schooley, D.A., 1990. Reversed-phase liquid chromatographic separation of juvenile hormone and its metabolites and its application for an *in vivo* juvenile hormone catabolism study in *Manduca sexta*. *Analyt. Biochem.* 188, 394–397.
- Hammock, B.D., 1985. Regulation of juvenile hormone titer: Degradation. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 7. Pergamon, Oxford, pp. 431–472.
- Hammock, B.D., Nowock, J., Goodman, W.G., Stamoudis, V., Gilbert, L.I., 1975. The influence of hemolymph-binding protein on juvenile hormone stability and distribution in *Manduca sexta* fat body and imaginal discs *in vitro*. *Mol. Cell. Endocrinol.* 3, 167–184.
- Hammock, B.D., Wing, K.D., McLaughlin, J., Lovell, V.M., Sparks, T.C., 1982. Trifluoromethylketones as possible transition state analog inhibitors of juvenile hormone esterase. *Pestic. Biochem. Physiol.* 17, 76–88.
- Hanzlik, T.N., Abdel-aal, Y.A.I., Harshman, L.G., Hammock, B.D., 1989. Isolation and sequencing of cDNA clones coding for juvenile hormone esterase from *Heliothis virescens*: evidence for a catalytic mechanism for the serine carboxylesterases different from that of the serine proteases. *J. Biol. Chem.* 264, 12419–12425.
- Hanzlik, T.N., Hammock, B.D., 1987. Characterization of affinity purified JHE from *Trichoplusia ni*. *J. Biol. Chem.* 262, 13584–13589.
- Hanzlik, T.N., Hammock, B.D., 1988. Characterization of juvenile hormone hydrolysis in early larval development of *Trichoplusia ni*. *Arch. Insect Biochem. Physiol.* 9, 135–156.
- Hardie, J., 1987. Juvenile hormone stimulation of oocyte development and embryogenesis in the parthenogenetic ovaries of an aphid, *Aphis fabae*. *Int. J. Invertebr. Reprod. Devel.* 11, 189–202.
- Hardie, J., Mallory, A.C.L., Quashie-Williams, C.A., 1990. Juvenile hormone and host-plant colonization by the black bean aphid, *Aphis fabae*. *Physiol. Entomol.* 15, 331–336.
- Harmon, M.A., Boehm, M.F., Heyman, R.A., Mangelsdorf, D.J., 1995. Activation of mammalian retinoid X receptors by the insect growth regulator methoprene. *Proc. Natl Acad. Sci. USA* 92, 6157–6160.
- Harris, S.V., Thompson, D.M., Linderman, R.J., Tomalski M.D., Roe, R.M., 1999. Cloning and expression of a novel juvenile hormone-metabolizing epoxide hydrolase during larval-pupal metamorphosis of the cabbage looper, *Trichoplusia ni*. *Insect Mol. Biol.* 8, 85–96.
- Harshman, L.G., Ward, V.K., Beetham, J.K., Grant, D.F., Grahah, L.J., et al., 1994. Cloning, characterization, and genetics of the juvenile hormone esterase gene from *Heliothis virescens*. *Insect Biochem. Mol. Biol.* 24, 671–676.
- Harshman, L.G., Casas, J., Dietze, E.C., Hammock, B.D., 1991. Epoxide hydrolase activities in *Drosophila melanogaster*. *Insect Biochem.* 21, 887–894.
- Hartmann, R., Jendrszczok, C., Peter, M.G., 1987. The occurrence of a juvenile hormone binding protein and *in vitro* synthesis of juvenile hormone by the serosa of *Locusta migratoria* embryos. *Roux's Arch. Devel. Biol.* 196, 347–355.
- Hauerland, N.H., Shirk, P.D., 1995. Regional and functional differentiation in the insect fat body. *Annu. Rev. Entomol.* 40, 121–145.
- Hayakawa, Y., 1990. Juvenile hormone esterase activity repressive factor in the plasma of parasitized insect larvae. *J. Biol. Chem.* 265, 10813–10816.
- Heftmann, E., Mosettig, E., 1960. *Biochemistry of Steroids*. Reinhold Publishing, New York, NY.
- Hegstrom, C.D., Riddiford, L.M., Truman, J.W., 1998. Steroid and neuronal regulation of ecdysone receptor expression during metamorphosis of muscle in the moth, *Manduca sexta*. *J. Neurosci.* 18, 1786–1794.
- Heming, B.S., 2003. *Insect Development and Evolution*. Cornell University Press, Ithaca, NY.
- Hewes, R.S., Truman, J.W., 1994. Steroid regulation of excitability in identified insect neurosecretory cells. *J. Neurosci.* 14, 1812–1819.
- Hidayat, P., Goodman, W.G., 1994. Juvenile hormone and hemolymph juvenile hormone binding protein titers and their interaction in the hemolymph of fourth stadium *Manduca sexta*. *Insect Biochem. Mol. Biol.* 24, 709–715.
- Hinton, A.C., Hammock, B.D., 2001. Purification of juvenile hormone esterase and molecular cloning of the cDNA from *Manduca sexta*. *Insect Biochem. Mol. Biol.* 32, 57–66.
- Hinton, A.C., Hammock, B.D., 2003. Juvenile hormone esterase (JHE) from *Tenebrio molitor*: full-length cDNA sequence, *in vitro* expression, and characterization of the recombinant protein. *Insect Biochem. Mol. Biol.* 33, 477–487.
- Hirai, M., Kamimura, M., Kikuchi, K., Yasukochi, Y., Kiuchi, M., et al., 2002. cDNA cloning and characterization of *Bombyx mori* juvenile hormone esterase: an inducible gene by the imidazole insect growth regulator KK-42. *Insect Biochem. Mol. Biol.* 32, 627–635.
- Hiruma, K., Carter, M.S., Riddiford, L.M., 1995. Characterization of the dopa decarboxylase gene of *Manduca sexta* and its suppression by 20-hydroxyecdysone. *Devel. Biol.* 169, 195–209.
- Hiruma, K., Riddiford, L.M., 1984. Regulation of melanization of tobacco hornworm larval cuticle *in vitro*. *J. Exp. Zool.* 230, 393–403.

- Hiruma, K., Riddiford, L.M., 1988. Granular phenoloxidase involved in cuticular melanization in the tobacco hornworm: regulation of its synthesis in the epidermis by juvenile hormone. *Devel. Biol.* 130, 87–97.
- Hiruma, K., Shimada, H., Yagi, S., 1978. Activation of the prothoracic gland by juvenile hormone and prothoracicotrophic hormone in *Mamestra brassicae*. *J. Insect Physiol.* 24, 215–220.
- Ho, H.Y., Tu, M.P., Chang, C.Y., Yin, C.-M., Kou, R., 1995a. Identification of *in vitro* release products of corpora allata in female and male loreyi leafworms, *Leucania loreyi*. *Experientia* 51, 601–605.
- Ho, H.Y., Tu, M.P., Chang, C.Y., Yin, C.-M., Kou, R., 1995b. Identification of *in vitro* release products of corpora allata in female and male loreyi leafworms, *Leucania loreyi*. *Experientia* 51, 1225 (correction of 1995a).
- Hodkova, M., Okuda, T., Wagner, R., 1996. Stimulation of corpora allata by extract from neuroendocrine complex; Comparison of reproducing and diapausing *Pyrrhocoris apterus*. *Eur. J. Entomol.* 93, 535–543.
- Hoffmann, K.H., Lorenz, M.W., Klein, P.M., 1996. Ovarian control of juvenile hormone-III biosynthesis in the cricket species *Gryllus bimaculatus*. *Entomol. General.* 20, 157–167.
- Holbrook, G.L., Chiang, A.S., Schal, C., 1997. Improved conditions for culture of biosynthetically active cockroach corpora allata. *In Vitro Cell Devel. Biol. – Animal* 33, 452–458.
- Homola, E., Chang, E.S., 1997. Crustacean juvenile hormone in search of functions. *Comp. Biochem. Physiol. B* 117, 347–356.
- Hopkins, T.E., Kramer, K.J., 1992. Insect cuticle sclerotization. *Annu. Rev. Entomol.* 37, 273–302.
- Hopkins, T.L., Starkey, S.R., Beckage, N.E., 1998. Tyrosine and catecholamine levels in the hemolymph of tobacco hornworm larvae, *Manduca sexta*, parasitized by the braconid wasp, *Cotesia congregata*. *Arch. Insect Biochem. Physiol.* 38, 193–201.
- Horodyski, F.M., Bhatt, S.R., Lee, K.Y., 2001. Alternative splicing of transcripts expressed by the *Manduca sexta* allatotropin (Mas-AT) gene is regulated in a tissue-specific manner. *Peptides* 22, 263–269.
- Horodyski, F.M., Riddiford, L.M., 1989. Expression and hormonal control of a new larval cuticular multigene family at the onset of metamorphosis of the tobacco hornworm. *Devel. Biol.* 132, 292–303.
- Horseman, G., Hartmann, R., Virantdoberlet, M., Loher, W., Huber, F., 1994. Nervous control of juvenile hormone biosynthesis in *Locusta migratoria*. *Proc. Natl Acad. Sci. USA* 91, 2960–2964.
- Huang, X.M., Lawler, C.P., Lewis, M.M., Nichols, D.E., Mailman, R.B., 2001. D-1 dopamine receptors. *Int. Rev. Neurobiol.* 48, 65–139.
- Huang, Z.Y., Robinson, G.E., 1995. Seasonal changes in juvenile hormone titers and rates of biosynthesis in honey bees. *Comp. Biochem. Physiol. B* 165, 18–28.
- Huang, Z.Y., Robinson, G.E., Tobe, S.S., Yagi, K.J., Strambi, C., *et al.*, 1991. Hormonal regulation of behavioural development in the honeybee is based on changes in the rate of juvenile hormone biosynthesis. *J. Insect Physiol.* 37, 733–741.
- Hwang, S.J., Cheon, H.M., Kim, H.J., Chae, K.S., Chung, D.H., *et al.*, 2001. cDNA sequence and gene expression of storage protein 2. A juvenile hormone-suppressible hexamerin from the fall webworm, *Hyphantria cunea*. *Comp. Biochem. Physiol. B* 129, 97–107.
- Ichinose, R., Nakamura, A., Yamoto, T., Booth, T.F., Maeda, S., *et al.*, 1992. Uptake of juvenile hormone esterase by pericardial cells of *Manduca sexta*. *Insect Biochem. Mol. Biol.* 22, 893–904.
- Injeyan, H.S., Tobe, S.S., Rapport, E., 1979. The effects of exogenous juvenile hormone treatment on embryogenesis in *Schistocerca gregaria*. *Can. J. Zool.* 57, 838–845.
- Ismail, S.M., Gillott, C., 1995. Identification, characterization, and developmental profile of a high molecular weight, juvenile hormone binding protein in the hemolymph of the migratory grasshopper, *Melanoplus sanguinipes*. *Arch. Insect Biochem. Physiol.* 29, 415–430.
- Ismail, S.M., Goin, C., Muthumani, K., Kim, M., Dahm, K.H., *et al.*, 2000. Juvenile hormone acid and ecdysteroid together induce competence for metamorphosis of the Verson's gland in *Manduca sexta*. *J. Insect Physiol.* 46, 59–68.
- Ismail, S.M., Satyanarayana, K., Bradfield, J.Y., Dahm, K.H., Bhaskaran, G., 1998. Juvenile hormone acid: evidence for a hormonal function in induction of vitellogenin in larvae of *Manduca sexta*. *Arch. Insect Biochem. Physiol.* 37, 305–310.
- Jansons, I.S., Cusson, M., McNeil, J.N., Tobe, S.S., Bendena, W.G., 1996. Molecular characterization of a cDNA from *Pseudaletia unipuncta* encoding the *Manduca sexta* allatostatin peptide (Mas-AST). *Insect Biochem. Mol. Biol.* 26, 767–773.
- Janzen, W.P., Menold, M., Granger, N.A., 1991. Effects of endogenous esterases and an allatostatin on the products of *Manduca sexta* corpora allata *in vitro*. *Physiol. Entomol.* 16, 283–293.
- Jensen, A.L., Brasch, K., 1985. Nuclear development in locust fat body: the influence of juvenile hormone on inclusion bodies and the nuclear matrix. *Tissue Cell* 17, 117–130.
- Jesudason, P., Anspaugh, D.D., Roe, R.M., 1992. Juvenile hormone metabolism in the plasma, integument, midgut, fat body, and brain during the last instar of the tobacco hornworm, *Manduca sexta*. *Arch. Insect Biochem. Physiol.* 20, 87–105.
- Jiang, H., Wang, Y., Yu, X.-Q., Zhu, Y., 2003. Prophenoloxidase-activating proteinase-3 from *Manduca sexta* hemolymph clip-domain serine proteinase regulated by serpin-1J and serine proteinase homologs. *Insect Biochem. Mol. Biol.* 33, 1049–1060.
- Johannsen, O.A., Butt, F.M., 1941. *Embryology of Insects and Myriapods*. McGraw-Hill, New York.

- Jones, G., 1995. Molecular mechanisms of action of juvenile hormone. *Annu. Rev. Entomol.* 40, 147–169.
- Jones, G., Brown, N., Manczak, M., Hiremath, S., Kafatos, F.C., 1990a. Molecular cloning, regulation, and complete sequence of a hemocyanin-related, juvenile hormone-suppressible protein from insect hemolymph. *J. Biol. Chem.* 265, 8596–8602.
- Jones, G., Hanzlik, T., Hammock, B.D., Schooley, D.A., Miller, C.A., et al., 1990b. The juvenile hormone titre during the penultimate and ultimate larval stadia of *Trichoplusia ni*. *J. Insect Physiol.* 36, 77–83.
- Jones, G., Hiremath, S.T., Hellmann, G.M., Rhoads, R.E., 1988. Juvenile hormone regulation of mRNA levels for a highly abundant hemolymph protein in larval *Trichoplusia ni*. *J. Biol. Chem.* 263, 1089–1092.
- Jones, G., Manczak, M., Schelling, D., Turner, H., Jones, D., 1998. Transcription of the juvenile hormone esterase gene is under the control of both an initiator and AT-rich motif. *Biochem. J.* 335, 79–84.
- Jones, G., Sharp, P.A., 1997. Ultraspiracle: an invertebrate nuclear receptor for juvenile hormones. *Proc. Natl Acad. Sci. USA* 94, 13499–13503.
- Jones, G., Wozniak, M., Chu, Y.X., Dhar, S., Jones, D., 2001. Juvenile hormone III-dependent conformational changes of the nuclear receptor ultraspiracle. *Insect Biochem. Mol. Biol.* 32, 33–49.
- Judy, K.J., Schooley, D.A., Dunham, L.L., Hall, M.S., Bergot, J., et al., 1973. Isolation, structure, and absolute configuration of a new natural insect juvenile hormone from *Manduca sexta*. *Proc. Natl Acad. Sci. USA* 70, 1509–1513.
- Jurka, J., Klonowski, P., 1996. Integration of retroposable elements in mammals: selection of target sites. *J. Mol. Evol.* 43, 685–689.
- Kaatz, H., Eichmuller, S., Kreissl, S., 1994. Stimulatory effect of octopamine on juvenile hormone biosynthesis in honey bees (*Apis mellifera*): physiological and immunocytochemical evidence. *J. Insect Physiol.* 40, 865–872.
- Kahn, S.M., Hryb, D.J., Nakhla, A.M., Romas, N.A., Rosner, W., 2002. Sex hormone-binding globulin is synthesized in target cells. *J. Endocrinol.* 175, 113–120.
- Kallapur, V.L., Majumder, C., Roe, R.M., 1996. *In vivo* and *in vitro* tissue specific metabolism of juvenile hormone during the last stadium of the cabbage looper, *Trichoplusia ni*. *J. Insect Physiol.* 42, 181–190.
- Kamimura, M., Kiuchi, M., 2002. Applying fenoxycarb at the penultimate instar triggers an additional ecdysteroid surge and induces perfect extra larval molting in the silkworm. *Gen. Comp. Endocrinol.* 128, 231–237.
- Kamita, S.G., Hinton, A.C., Wheelock, C.E., Wogulis, M.D., Wilson, D.K., et al., 2003. Juvenile hormone esterase: Why are you so JH specific? *Insect Biochem. Mol. Biol.* 33, 1261–1273.
- Kataoka, H., Toschi, A., Li, J.P., Carney, R.L., Schooley, D.A., et al., 1989. Identification of an allatotropin from adult *Manduca sexta*. *Science* 243, 1481–1483.
- Keiser, K.C.L., Brandt, K.S., Silver, G.M., Wisniewski, N., 2002. Cloning, partial purification and *in vivo* developmental profile of expression of the juvenile hormone epoxide hydrolase of *Ctenocephalides felis*. *Arch. Insect Biochem. Physiol.* 50, 191–206.
- Kelly, G.M., Huebner, E., 1987. Juvenoid effects on *Rhodnius prolixus* embryogenesis. *Insect Biochem.* 17, 1079–1083.
- Kensler, T.W., Verma, A.K., Boutwell, R.K., Mueller, G.C., 1978. Effects of retinoic acid and juvenile hormone on the induction of ornithine decarboxylase activity by 12-O-tetradecanoylphorbol-13-acetate. *Cancer Res.* 38, 2896–2899.
- Kikuchi, K., Hirai, M., Shiotsuki, T., 2001. Molecular cloning and tissue distribution of farnesyl pyrophosphate synthase from the silkworm, *Bombyx mori*. *J. Insect Biotechnol. Sericol.* 70, 167–172.
- Kikukawa, S., Tobe, S.S., Solowiej, S., Rankin, S.M., Stay, B., 1987. Calcium as a regulator of juvenile hormone biosynthesis and release in the cockroach *Diploptera punctata*. *Insect Biochem.* 17, 179–187.
- Kim, Y., Davari, E.D., Sevala, V., Davey, K.G., 1999. Functional binding of a vertebrate hormone, L-3,5,3'-triiodothyronine (T-3), on insect follicle cell membranes. *Insect Biochem. Mol. Biol.* 29, 943–950.
- King, L.E., Ding, Q., Prestwich, G.D., Tobe, S.S., 1995. The characterization of a haemolymph methyl farnesoate binding protein and the assessment of methyl farnesoate metabolism by the haemolymph and other tissues from *Procambrus clarkii*. *Insect Biochem. Mol. Biol.* 25, 495–501.
- King, L.E., Tobe, S.S., 1988. The identification of an enantioselective JH-III binding protein from the haemolymph of the cockroach, *Diploptera punctata*. *Insect Biochem.* 18, 793–805.
- King, L.E., Tobe, S.S., 1993. Changes in the titre of a juvenile hormone III binding lipophorin in the hemolymph of *Diploptera punctata* during development and reproduction. *J. Insect Physiol.* 39, 241–252.
- Kiss, I., Beaton, A.H., Tardiff, J., Fristrom, D., Fristrom, J.W., 1988. Interactions and developmental effects of mutations in the Broad-Complex of *Drosophila melanogaster*. *Genetics* 118, 247–259.
- Klein, P.M., Lorenz, M.W., Huang, K.H., Hoffmann, K.H., 1993. Age dependency and regulatory properties of juvenile hormone III biosynthesis in adult male crickets, *Gryllus bimaculatus*. *J. Insect Physiol.* 39, 315–324.
- Kobayashi, M., Ishikawa, H., 1994. Involvement of juvenile hormone and ubiquitin-dependent proteolysis in flight muscle breakdown of an alate aphid (*Acyrtosiphon pisum*). *J. Insect Physiol.* 40, 107–111.
- Koladich, P.M., Cusson, M., Bendena, W.G., Tobe, S.S., McNeil, J.N., 2002. Cardioacceleratory effects of *Manduca sexta* allatotropin in the true armyworm moth, *Pseudaletia unipuncta*. *Peptides* 23, 645–651.
- Kolodziejczyk, R., Dobryszycycki, P., Ozyhar, A., Kochman, M., 2001. Two disulphide bridges are present in juvenile hormone binding protein from *Galleria mellonella*. *Acta Biochim. Polon.* 48, 917–920.
- Kolodziejczyk, R., Kochman, M., Bujacz, G., Dobryszycycki, P., Ozyhar, A., et al., 2003. Crystallization

- and preliminary crystallographic studies of juvenile hormone-juvenile hormone binding protein from *Galleria mellonella*. *Acta Crystallogr. D* 59, 519–521.
- Koopmanschap, A.B., deKort, C.A.D., 1988. Isolation and characterization of a high molecular weight JH-III transport protein in the hemolymph of *Locusta migratoria*. *Arch. Insect Biochem. Physiol.* 7, 105–118.
- Kotaki, T., 1993. Biosynthetic products by heteropteran corpora allata *in vitro*. *Appl. Entomol. Zool.* 28, 242–245.
- Kotaki, T., 1996. Evidence for a new juvenile hormone in a stink bug, *Plautia stali*. *J. Insect Physiol.* 42, 279–286.
- Kotaki, T., 1997. A putative juvenile hormone in a stink bug, *Plautia stali*: the corpus allatum releases a JH-active product different from any known JHs *in vitro*. *J. Invertebr. Reprod. Devel.* 31, 225–230.
- Kou, R., Chen, S.J., 2000. Allatotropic activity in the suboesophageal ganglia and corpora cardiaca of the adult male loreyi leafworm, *Mythimna loreyi*. *Arch. Insect Biochem. Physiol.* 43, 78–86.
- Krakauer, D.C., Plotkin, J.B., 2002. Redundancy, antiredundancy, and the robustness of genomes. *Proc. Natl Acad. Sci. USA* 99, 1405–1409.
- Kramer, K.J., Dunn, P.E., Peterson, R., Law, J.H., 1976. Interaction of juvenile hormone with binding proteins in insect hemolymph. In: Gilbert, L.I. (Ed.), *The Juvenile Hormones*. Plenum, New York, pp. 327–341.
- Kramer, S.J., Toschi, A., Miller, C.A., Kataoka, H., Quistad, G.B., *et al.*, 1991. Identification of an allatotatin from the tobacco hornworm *Manduca sexta*. *Proc. Natl Acad. Sci. USA* 88, 9458–9462.
- Kroeger, H., 1968. Gene activities during insect metamorphosis and their control by hormones. In: Etkin, W., Gilbert, L.I. (Eds.), *Metamorphosis: A Problem in Developmental Biology*. Appleton-Century-Crofts, New York, NY, pp. 185–220.
- Krzyzanowska, D., Lisowski, M., Kochman, M., 1998. UV-difference and CD spectroscopy studies on juvenile hormone binding to its carrier protein. *J. Peptide Res.* 51, 96–102.
- Kumaran, A.K., 1990. Action of JH at cellular and molecular levels. In: Gupta, A.P. (Ed.), *Morphogenetic Hormones of Arthropods*. Rutgers University Press, New Brunswick, NJ, pp. 183–227.
- Lacourciere, G.M., Armstrong, R.N., 1993. The catalytic mechanism of microsomal epoxide hydrolase involves an ester intermediate. *J. Am. Chem. Soc.* 115, 10466–10467.
- Lafon-Cazal, M., Baehr, J.-C., 1988. Octopaminergic control of corpora allata activity in an insect. *Experientia* 44, 895–896.
- Lafont, R., 2000. Understanding insect endocrine systems: molecular approaches. *Entomol. Exp. Applic.* 97, 123–136.
- Lamer, A., Dorn, A., 2001. The serosa of *Manduca sexta*: ontogeny, secretory activity, structural changes, and functional consideration. *Tissue Cell* 33, 580–595.
- Lanzrein, B., Imboden, H., Bürgin, C., Brüning, E., Gfeller, G., 1984. On titers, origin, and functions of juvenile hormone III, methyl farnesoate and ecdysteroids in embryonic development of the ovoviparous cockroach, *Nauphoeta cinerea*. In: Hoffmann, J., Porchet, M. (Eds.), *Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones*. Springer, Berlin, pp. 454–465.
- Larsen, M.J., Burton, K.J., Zantello, M.R., Smith, V.G., Lowery, D.L., *et al.*, 2001. Type A allatostatins from *Drosophila melanogaster* and *Diptera punctata* activate two *Drosophila* allatostatin receptors, DAR-1 and DAR-2, expressed in CHO cells. *Biochem. Biophys. Res. Commun.* 286, 895–901.
- Lassiter, M.T., Apperson, C.S., Crawford, C.L., Roe, R.M., 1994. Juvenile hormone metabolism during adult development of *Culex quinquefasciatus*. *J. Med. Entomol.* 32, 586–93.
- Lassiter, M.T., Apperson, C.S., Roe, R.M., 1995. Juvenile hormone metabolism during the fourth stadium and pupal stage of the southern house mosquito, *Culex quinquefasciatus*. *J. Insect Physiol.* 41, 869–876.
- Laufer, H., Ahl, J.S.B., Sagi, A., 1993. The role of juvenile hormones in crustacean reproduction. *Am. Zool.* 33, 365–374.
- Lauga-Reyrel, F., 1985. Experimental establishment and rupture of the ecomorphosis of *Hypogastrura tullbergi* by exogenous contribution of precocene or juvenile hormone. *Bull. Soc. Hist. Nat. Toulouse* 120, 61–74.
- Law, J.H., 1980. Lipid-protein interactions in insects. In: Locke, M., Smith, D.S. (Eds.), *Insect Biology in the Future: VBW80*. Academic Press, New York, pp. 295–310.
- Lee, C.Y., Simon, C.R., Woodard, C.T., Baehrecke, E.H., 2002. Genetic mechanism for the stage- and tissue-specific regulation of steroid triggered programmed cell death in *Drosophila*. *Devel. Biol.* 252, 138–148.
- Lee, K.Y., Chamberlin, M.E., Horodyski, F.M., 2002. Biological activity of *Manduca sexta* allatotropin-like peptides, predicted products of tissue-specific and developmentally regulated alternatively spliced mRNAs. *Peptides* 23, 1933–1941.
- Lee, K.Y., Horodyski, F.M., 2002. Restriction of nutrient intake results in the increase of a specific *Manduca sexta* allatotropin (Manse-AT) mRNA in the larval nerve cord. *Peptides* 23, 653–661.
- Lee, K.Y., Horodyski, F.M., Chamberlin, M.E., 1998. Inhibition of midgut ion transport by allatotropin (Mas-AT) and *Manduca* FLRFamides in the tobacco hornworm *Manduca sexta*. *J. Exp. Biol.* 201, 3067–3074.
- Lee, Y.J., Chiang, A.S., 1997. Ultrastructural changes in corpora allata during a cycle of juvenile hormone biosynthesis in embryos of the viviparous cockroach *Diptera punctata*. *Tissue Cell* 29, 715–726.
- Lefevre, K.S., Lacey, M.J., Smith, P.H., Roberts, B., 1993. Identification and quantification of juvenile hormone biosynthesized by larval and adult Australian sheep blowfly *Lucilia cuprina*. *Insect Biochem. Mol. Biol.* 23, 713–720.

- Lehmberg, E., Ferenz, H.J., Applebaum, S.W., 1992. Maturation and responsiveness to extracts of corpora allata from male *Locusta migratoria* containing allatotrophic factors. *Z. Naturforsch.* 47C, 449–452.
- Lenz, C., Williamson, M., Hansen, G.N., Grimmelikhuijzen, C.J.P., 2001. Identification of four *Drosophila* allatostatins as the cognate ligands for the *Drosophila* orphan receptor DAR-2. *Biochem. Biophys. Res. Commun.* 286, 1117–1122.
- Lenz, C., Williamson, M., Grimmelikhuijzen, C.J.P., 2000. Molecular cloning and genomic organization of an allatostatin preprohormone from *Drosophila melanogaster*. *Biochem. Biophys. Res. Commun.* 273, 1126–1131.
- Levenbook, L., 1985. Insect storage proteins. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology Biochemistry and Pharmacology*, vol. 10. Pergamon, Oxford, pp. 307–346.
- Lezzi, M., Bergman, T., Henrich, V.C., Vogtli, M., Fromel, C., et al., 2002. Ligand-induced heterodimerization between the ligand binding domains of the *Drosophila* ecdysteroid receptor and ultraspiracle. *Eur. J. Biochem.* 269, 3237–3245.
- Li, H., Borst, D.W., 1991. Characterization of a methyl farnesoate binding protein in hemolymph from *Libinia emarginata*. *Gen. Comp. Endocrinol.* 81, 335–342.
- Li, W.C., 1996. Differential expression of the two duplicated insecticyanin genes, ins-a and ins-b, in the black mutant of *Manduca sexta*. *Arch. Biochem. Biophys.* 330, 65–70.
- Li, W.C., Riddiford, L.M., 1992. Two distinct genes encode two major isoelectric forms of insecticyanin in the tobacco hornworm, *Manduca sexta*. *Eur. J. Biochem.* 205, 491–499.
- Li, W.C., Riddiford, L.M., 1994. The two duplicated insecticyanin genes, ins-a and ins-b are differentially expressed in the tobacco hornworm, *Manduca sexta*. *Nucl. Acids Res.* 22, 2945–2950.
- Li, Y., Unnithan, G.C., Veenstra, J.A., Feyereisen, R., Noriega, F.G., 2003. Stimulation of JH biosynthesis by the corpora allata of adult female *Aedes aegypti* in vitro: effect of farnesoic acid and *Aedes* allatotropin. *J. Exp. Biol.* 206, 1825–1832.
- Linderman, R.J., Roe, R.M., Harris, S. V., Thompson, D.M., 2000. Inhibition of insect juvenile hormone epoxide hydrolase: asymmetric synthesis and assay of glycidol-ester and epoxy-ester inhibitors of *Trichoplusia ni* epoxide hydrolase. *Insect Biochem. Mol. Biol.* 30, 767–774.
- Locke, M., 1984. The structure and development of the vacuolar system in the fat body of insects. In: King, R.C., Akai, H. (Eds.), *Insect Ultrastructure*. Plenum, New York, pp. 151–197.
- Loher, W., Ruzo, F., Baker, F.C., Miller, C.A., Schooley, D.A., 1983. Identification of the juvenile hormone from the cricket, *Teleogryllus commodus*, and juvenile hormone titre changes. *J. Insect Physiol.* 29, 585–589.
- Lonard, D.M., Bhaskaran, G., Dahm, K. H., 1996. Control of prothoracic gland activity by juvenile hormone in fourth instar *Manduca sexta* larvae. *J. Insect Physiol.* 42, 205–214.
- Lorenz, M.W., Hoffmann, K.H., 1995. Allatotrophic activity in the subesophageal ganglia of crickets, *Gryllus bimaculatus* and *Acheta domesticus*. *J. Insect Physiol.* 41, 191–196.
- Lorenz, M.W., Hoffmann, K.H., Gade, G., 1999. Juvenile hormone biosynthesis in larval and adult stick insects, *Carausius morosus*. *J. Insect Physiol.* 45, 443–452.
- Lorenz, M.W., Kellner, R., Hoffmann, K.H., Gade, G., 2000. Identification of multiple peptides homologous to cockroach and cricket allatostatins in the stick insect *Carausius morosus*. *Insect Biochem. Mol. Biol.* 30, 711–718.
- Mauchamp, B., Darrouzet, E., Malosse, C., Couillaud, F., 1999. 4'-OH-JH-III: an additional hydroxylated juvenile hormone produced by locust corpora allata in vitro. *Insect Biochem. Mol. Biol.* 29, 475–480.
- Maxwell, R.A., Welch, W.H., Horodyski, F.M., Schegg, K.M., Schooley, D.A., 2002b. Juvenile hormone diol kinase. II. Sequencing, cloning, and molecular modeling of juvenile hormone-selective diol kinase from *Manduca sexta*. *J. Biol. Chem.* 277, 21882–21890.
- Maxwell, R.A., Welch, W.H., Schooley, D.A., 2002a. Juvenile hormone diol kinase. I. Purification, characterization, and substrate specificity of juvenile hormone-selective diol kinase from *Manduca sexta*. *J. Biol. Chem.* 277, 21874–21881.
- Meller, V., Aucoin, R.R., Tobe, S. S., Feyereisen, R., 1985. Evidence for an inhibitory role of cyclic AMP in the control of juvenile hormone biosynthesis by cockroach corpora allata. *Mol. Cell. Endocrinol.* 43, 155–163.
- Mendel, C.M., 1989. The free hormone hypothesis: a physiologically based mathematical model. *Endocrine Rev.* 10, 232–74.
- Meyer, A.S., Hanzmann, E., Murphy, R. C., 1971. Absolute configuration of cecropia juvenile hormone. *Proc. Natl Acad. Sci. USA* 68, 2312–2315.
- Meyer, A.S., Schneiderman, H.A., Hanzmann, E., Ko, J., 1968. The two juvenile hormones from the cecropia silk moth. *Proc. Natl Acad. Sci. USA* 60, 853–860.
- Meyer, W.R., Lanzrein, B., 1989a. Degradation of juvenile hormone and methylation of juvenile hormone acid by corpora cardiaca–corpora allata of the cockroach, *Nauphoeta cinerea*. 1. Biochemical aspects. *Arch. Insect Biochem. Physiol.* 10, 303–316.
- Meyer, W.R., Lanzrein, B., 1989b. Degradation of juvenile hormone and methylation of juvenile hormone acid by corpora cardiaca–corpora allata of the cockroach, *Nauphoeta cinerea*. 2. Physiological aspects. *Arch. Insect Biochem. Physiol.* 10, 317–331.
- Meyering-Vos, M., Wu, X., Huang, J., Jindra, M., Hoffmann, K.H., et al., 2001. The allatostatin gene of the cricket *Gryllus bimaculatus*. *Mol. Cell. Endocrinol.* 184, 103–114.
- Miura, K., Shinoda, T., Yura, M., Nomura, S., Kamiya, K., et al., 1998. Two hexameric cyanoprotein subunits from an insect, *Riptortus clavatus*, sequence, phylogeny

- and developmental and juvenile hormone regulation. *Eur. J. Biochem.* 258, 929–940.
- Mizoguchi, A., 2001. Effects of juvenile hormone on the secretion of prothoracicotropic hormone in the last- and penultimate-instar larvae of the silkworm *Bombyx mori*. *J. Insect Physiol.* 47, 767–775.
- Moshitzky, P., Applebaum, S.W., 1995. Pathway and regulation of JH III-bisepoxide biosynthesis in adult *Drosophila melanogaster* corpus allatum. *Arch. Insect Biochem. Physiol.* 30, 225–238.
- Moshitzky, P., Gilbert, L.I., Applebaum, S.W., 2003. Biosynthetic maturation of the corpus allatum of the female adult medfly, *Ceratitis capitata*, and its putative control. *J. Insect Physiol.* 49, 603–609.
- Moshitzky, P., Fleischmann, I., Chaimov, N., Saudan, P., Klausner, S., et al., 1996. Sex-peptide activates juvenile hormone biosynthesis in the *Drosophila melanogaster* corpus allatum. *Arch. Insect Biochem. Physiol.* 32, 363–374.
- Mugat, B., Brodu, V., Kejzlarova-Lepesant, J., Antoniewski, C., Bayer, C.A., et al., 2000. Dynamic expression of Broad-complex isoforms mediates temporal control of an ecdysteroid target gene at the onset of *Drosophila* metamorphosis. *Devel. Biol.* 227, 104–117.
- Nachman, R.J., Garside, C.S., Tobe, S.S., 1999. Hemolymph and tissue-bound peptidase-resistant analogs of the insect allatostatins. *Peptides* 20, 23–29.
- Nakanishi, K., Schooley, D.A., Koreeda, M., Dillon, J., 1971. Absolute configuration of the C18-juvenile hormone: application of a new circular dichroism method using tris(dipivaloylmethanato) praseodymium. *J. Chem. Soc., Chem. Commun.* 1235–1236.
- Nardi, F., Spinsanti, G., Boore, J.L., Carapelli, A., Dallai, R., et al., 2003. Hexapod origins: monophyletic or paraphyletic. *Science* 299, 1887–1889.
- Neese, P.A., Sonenshine, D.E., Kallapur, V.L., Apperson, C.S., Roe, R.M., 2000. Absence of insect juvenile hormones in the American dog tick, *Dermacentor variabilis* (Say) (Acari: Ixodidae), and in *Ornithodoros parkeri* Cooley (Acari: Argasidae). *J. Insect Physiol.* 46, 477–490.
- Nelson, J.A., 1915. The Embryology of the Honeybee. Princeton University Press, Princeton, NJ.
- Newman, J.W., Morisseau, C., Harris, T.R., Hammock, B.D., 2003. The soluble epoxide hydrolase encoded by EPXH2 is a bifunctional enzyme with novel lipid phosphate phosphatase activity. *Proc. Natl Acad. Sci. USA* 100, 1558–1563.
- Niimi, S., Sakurai, S., 1997. Developmental changes in juvenile hormone and juvenile hormone acid titers in the hemolymph and *in vitro* juvenile hormone synthesis by corpora allata of the silkworm, *Bombyx mori*. *J. Insect Physiol.* 43, 875–884.
- Nijhout, H.F., 1994. Insect Hormones. Princeton, University Press, Princeton, NJ.
- Nijhout, H.F., Wheeler, D.E., 1982. Juvenile hormone and the physiological basis of insect polymorphisms. *Q. Rev. Biol.* 57, 109–133.
- Nijhout, H.F., Williams, C.M., 1974. Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta*: cessation of juvenile hormone secretion as a trigger for pupation. *J. Exp. Biol.* 61, 493–501.
- Noguchi, H., Hayakawa, Y., 1997. Role of dopamine at the onset of pupal diapause in the cabbage armyworm *Mamestra brassicae*. *FEBS Lett.* 413, 157–161.
- Noguchi, H., Hayakawa, Y., 1996. Mechanism of parasitism-induced elevation of dopamine levels in host insect larvae. *Insect Biochem. Mol. Biol.* 26, 659–665.
- Noguchi, H., Hayakawa, Y., Downer, R.G.H., 1995. Elevation of dopamine levels in parasitized insects. *Insect Biochem. Mol. Biol.* 25, 197–201.
- Noriega, F.G., Edgar, K.A., Goodman, W.G., Shah, D.K., Wells, M.A., 2001. Neuroendocrine factors affecting the steady-state levels of early trypsin mRNA in *Aedes aegypti*. *J. Insect Physiol.* 47, 515–522.
- Nowock, J., Gilbert, L.I., 1976. *In vitro* analysis of factors regulating the juvenile hormone titer of insects. In: Kurstak, E., Maramorosch, K. (Eds.), Invertebrate Tissue Culture: Applications in Medicine, Biology, and Agriculture. Academic Press, New York, pp. 203–212.
- Nowock, J., Goodman, W.G., Bollenbacher, W.E., Gilbert, L.I., 1975. Synthesis of juvenile hormone binding proteins by the fat body of *Manduca sexta*. *Gen. Comp. Endocrinol.* 27, 230–239.
- Numata, H., Numata, A., Takahashi, C., Nakagawa, Y., Iwatani, K., et al., 1992. Juvenile hormone I is the principal juvenile hormone in a hemipteran insect, *Riptortus clavatus*. *Experientia* 48, 606–610.
- Oeh, U., Dyker, H., Losel, P., Hoffmann, K.H., 2001. *In vivo* effects of *Manduca sexta* allatotropin and allatostatin on development and reproduction in the fall armyworm, *Spodoptera frugiperda* (Lepidoptera, Noctuidae). *Invertebr. Reprod. Devel.* 39, 239–247.
- Oeh, U., Lorenz, M.W., Dyker, H., Losel, P., Hoffmann, K.H., 2000. Interaction between *Manduca sexta* allatotropin and *Manduca sexta* allatostatin in the fall armyworm *Spodoptera frugiperda*. *Insect Biochem. Mol. Biol.* 30, 719–727.
- Oeh, U., Lorenz, M.W., Dyker, H., Loesel, P.H.K.H., 1999. Interaction between *Manduca* allatotropin and *Manduca* allatostatins in the fall armyworm *Spodoptera frugiperda*. *Insect Biochem. Mol. Biol.* 30, 719–727.
- Oro, A.E., McKeown, M., Evans, R.M., 1990. Relationship between the product of the *Drosophila ultraspiracle* locus and the vertebrate retinoid X receptor. *Nature* 347, 298–301.
- Orth, A.P., Doll, S.C., Goodman, W.G., 2003a. Sequence, structure and expression of the hemolymph juvenile hormone binding protein gene in the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol.* 33, 93–102.
- Orth, A.P., Tauchman, S.J., Doll, S.C., Goodman, W.G., 2003b. Embryonic expression of juvenile hormone binding protein and its relationship to the toxic effects of juvenile hormone in *Manduca sexta*. *Insect Biochem. Mol. Biol.* 33, 1275–1284.

- Orth, A.P., Lan, Q., Goodman, W.G., 1999. Ligand regulation of juvenile hormone binding protein mRNA in mutant *Manduca sexta*. *Mol. Cell. Endocrinol.* 149, 61–69.
- Owen, M.D., Foster, J., 1988. The association of changes in cerebral ganglion dopamine metabolism with ootheca development in *Periplaneta americana*. *Comp. Biochem. Physiol. C* 91, 403–408.
- Panchapakesan, K., Lampert, E.P., Granger, N.A., Goodman, W.G., Roe, R.M., 1994. Biology and physiology of the white mutant of the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol.* 40, 423–429.
- Park, C., Hwang, J.S., Kang, S.W., Lee, B.H., 2002. Molecular characterization of a cDNA from the silk moth *Bombyx mori* encoding *Manduca sexta* allatotropin peptide. *Zool. Sci.* 19, 287–292.
- Park, Y.C., Goodman, W.G., 1993. Analysis and modification of thiols in the hemolymph juvenile hormone binding protein of *Manduca sexta*. *Arch. Biochem. Biophys.* 302, 12–18.
- Park, Y.C., Tesch, M.J., Toong, Y.C., Goodman, W.G., 1993. Affinity purification and binding analysis of the hemolymph juvenile hormone binding protein from *Manduca sexta*. *Biochemistry* 32, 7909–7915.
- Pastor, D., Artigas, F., Martinez, E., Bellés, X., 1991a. Brain levels of 5-hydroxytryptamine and dopamine in *Blattella germanica* adult females during the first gonadotropic cycle. *Biogen. Amines* 8, 101–114.
- Pastor, D., Piulachs, M.-D., Cassier, P., Andres, M., Bellés, X., 1991b. Etude *in vivo* et *in vitro* de l'action de la dopamine sur la croissance des oocytes et la production d'hormone juvenile chez *Blattella germanica*. *C. R. Acad. Sci. Paris* 313, 207–212.
- Pener, M.P., Dessberg, D., Lazarovici, P., Reuter, C.C., Tsai, L.W., et al., 1986. The effect of a synthetic precocene on juvenile hormone III titer in late *Locusta* eggs. *J. Insect Physiol.* 32, 853–857.
- Peralta, E., Vilaplana, L., Pascual, N., Carreno, C., Piulachs, M.D., et al., 2000. A microdialysis study of allatostatin degradation in *Blattella germanica*. *Physiol. Entomol.* 25, 254–259.
- Peter, M.G., 1990. Chiral recognition in insect juvenile hormone metabolism. In: Testa, B. (Ed.), *Chirality and Biological Activity*. Alan, R. Liss, New York, pp. 111–117.
- Peter, M.G., Shirk, P.D., Dahm, K.H., Röller, H., 1981. On the specificity of juvenile hormone biosynthesis in the male cecropia. *Z. Naturforsch.* 36C, 579–585.
- Peter, M.G., Sunawan, S., Gellissen, G., Emmerich, H., 1979. Difference in hydrolysis and binding of homologous juvenile hormones in *Locusta migratoria* hemolymph. *Z. Naturforsch.* 34C, 588–598.
- Peterson, R.C., Dunn, P.E., Seballos, H.L., Barbeau, B.K., Keim, P.S., et al., 1982. Juvenile hormone carrier protein of *Manduca sexta* haemolymph. Improved purification procedure: protein modification studies and sequence of the amino terminus of the protein. *Insect Biochem.* 12, 643–650.
- Peterson, R.C., Reich, M.F., Dunn, P.E., Law, J.H., Katzenellenbogen, J.A., 1977. Binding specificity of the juvenile hormone carrier protein from the hemolymph of the tobacco hornworm *Manduca sexta*. *Biochemistry* 16, 2305–2311.
- Petri, B., Homberg, U., Loesel, R., Stengl, M., 2002. Evidence for a role of GABA and Mas-allatotropin in photic entrainment of the circadian clock of the cockroach *Leucophaea maderae*. *J. Exp. Biol.* 205, 1459–1469.
- Pratt, G.E., Tobe, S.S., 1974. Juvenile hormone radiobiosynthesized by corpora allata of adult female locusts *in vitro*. *Life Sci.* 14, 575–586.
- Pratt, G.E., Unnithan, G.C., Fok, K.F., Siegel, N.R., Feyerisen, R., 1997. Structure–activity studies reveal two allatostatin receptor types in corpora allata of *Diploptera punctata*. *J. Insect Physiol.* 43, 627–634.
- Pratt, G.E., Farnsworth, D.E., Fok, K.F., Siegel, N.R., McCormack, A.L., et al., 1991. Identity of a second type of allatostatin from cockroach brains: An octadecapeptide amide with a tyrosine-rich address sequence. *Proc. Natl Acad. Sci. USA* 88, 2412–2416.
- Pratt, G.E., Farnsworth, D.E., Feyerisen, R., 1990. Changes in the sensitivity of adult cockroach corpora allata to a brain allatostatin. *Mol. Cell. Endocrinol.* 70, 185–195.
- Pratt, G.E., Farnsworth, D.E., Siegel, N.R., Fok, K.F., Feyerisen, R., 1989. Identification of an allatostatin from adult *Diploptera punctata*. *Biochem. Biophys. Res. Commun.* 163, 1243–1247.
- Prestwich, G.D., 1987. Chemistry of pheromone and hormone metabolism in insects. *Science* 237, 999–1006.
- Prestwich, G.D., Touhara, K., Riddiford, L.M., Hammock, B.D., 1994. Larva lights: a decade of photo-affinity labeling with juvenile hormone analogues. *Insect Biochem. Mol. Biol.* 24, 747–761.
- Prestwich, G.D., Wawrzęńczyk, C., 1985. High specific activity enantiomerically enriched juvenile hormones: synthesis and binding assay. *Proc. Natl Acad. Sci. USA* 82, 5290–5294.
- Pszczolkowski, M.A., Lee, W.S., Liu, H.P., Chiang, A.S., 1999. Glutamate-induced rise in cytosolic calcium concentration stimulates *in vitro* rates of juvenile hormone biosynthesis in corpus allatum of *Diploptera punctata*. *Mol. Cell. Endocrinol.* 158, 163–171.
- Pursley, S., Ashok, M., Wilson, T.G., 2000. Intracellular localization and tissue specificity of the methoprene-tolerant (*met*) gene product in *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* 30, 839–845.
- Rachinsky, A., 1994. Octopamine and serotonin influence on corpora allata activity in honey bee (*Apis mellifera*) larvae. *J. Insect Physiol.* 40, 549–554.
- Rachinsky, A., Feldlaufer, M.F., 2000. Responsiveness of honey bee (*Apis mellifera*) corpora allata to allatostatin regulatory peptides from four insect species. *J. Insect Physiol.* 46, 41–46.
- Rachinsky, A., Hartfelder, K., 1991. Differential production of juvenile hormone and its deoxy precursor by corpora allata of honeybees during a critical

- period of caste development. *Naturwissenschaften* 78, 270–272.
- Rachinsky, A., Tobe, S.S., 1996. Role of second messengers in the regulation of juvenile hormone production in insects, with particular emphasis on calcium and phosphoinositide signaling. *Arch. Insect Biochem. Physiol.* 33, 259–282.
- Rachinsky, A., Tobe, S.S., Feldlaufer, M.F., 2000. Terminal steps in JH biosynthesis in the honey bee (*Apis mellifera*): developmental changes in sensitivity to JH precursors and allatotropin. *Insect Biochem. Mol. Biol.* 30, 729–737.
- Rachinsky, A., Zhang, J., Tobe, S.S., 1994. Signal transduction in the inhibition of juvenile hormone biosynthesis by allatostatins: roles of diacylglycerol and calcium. *Mol. Cell. Endocrinol.* 105, 89–96.
- Rankin, S.M., Stay, B., 1985. Ovarian inhibition of juvenile hormone synthesis in the viviparous cockroach *Diploptera punctata*. *Gen. Comp. Endocrinol.* 59, 230–238.
- Rankin, S.M., Stay, B., Aucoin, R.R., Tobe, S.S., 1986. *In vitro* inhibition of juvenile hormone synthesis by corpora allata of the viviparous cockroach *Diploptera punctata*. *J. Insect Physiol.* 32, 151–156.
- Rao, K.D.P., Krishnakumaran, A., 1974. Effect of juvenile hormone on DNA synthesis during embryogenesis in *Acheta domesticus*. *Roux's Arch. Devel. Biol.* 174, 276–284.
- Reagan, J.D., Miller, W.H., Kramer, S.J., 1992. Allatotropin-induced formation of inositol phosphates in the corpora allata of the moth, *Manduca sexta*. *Arch. Insect Biochem. Physiol.* 20, 145–155.
- Rebers, J.E., Niu, J., Riddiford, L.M., 1997. Structure and spatial expression of the *Manduca sexta* MSCP14.6 cuticle gene. *Insect Biochem. Mol. Biol.* 27, 229–240.
- Rebers, J.E., Riddiford, L.M., 1988. Structure and expression of a *Manduca sexta* larval cuticle gene homologous to *Drosophila* cuticle genes. *J. Mol. Biol.* 203, 411–423.
- Rees, H.H., 1985. Biosynthesis of ecdysones. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology Biochemistry and Pharmacology*, vol. 7. Pergamon, Oxford, pp. 249–293.
- Rembold, H., Czoppelt, C., Grune, M., Lackner, B., Pfeffer, J., et al., 1992. Juvenile hormone titers during honeybee embryogenesis and metamorphosis. In: Mauchamp, B., Couillaud, F., Baehr, J.-C. (Eds.), *Insect Juvenile Hormone Research: Chemistry, Biochemistry and Mode of Action*. INRA, Paris, pp. 37–43.
- Rembold, H., Hagenguth, H., Rascher, J., 1980. A sensitive method for detection and estimation of juvenile hormones from biological samples by glass capillary combined gas chromatography-selected ion monitoring mass spectrometry. *Analyt. Biochem.* 101, 356–363.
- Rensing, L., 1964. Daily rhythmicity of corpus allatum and neurosecretory cells in *Drosophila melanogaster*. *Science* 144, 1586–1587.
- Restifo, L.L., Wilson, T.G., 1998. A juvenile hormone agonist reveals distinct developmental pathways mediated by ecdysone-inducible Broad complex transcription factors. *Devel. Genet.* 22, 141–159.
- Richard, D.S., Gilbert, L.I., 1991. Reversible juvenile hormone inhibition of ecdysteroid and juvenile hormone synthesis by the ring gland of *Drosophila melanogaster*. *Experientia* 47, 1063–1066.
- Richard, D.S., Applebaum, S.W., Gilbert, L.I., 1990. Allatostatic regulation of juvenile hormone production *in vitro* by the ring gland of *Drosophila melanogaster*. *Mol. Cell. Endocrinol.* 68, 153–161.
- Richard, D.S., Applebaum, S.W., Sliter, T.J., Baker, F.C., Schooley, D.A., et al., 1989. Juvenile hormone bisepoxide biosynthesis *in vitro* by the ring gland of *Drosophila melanogaster*: a putative juvenile hormone in the higher Diptera. *Proc. Natl Acad. Sci. USA* 86, 1421–1425.
- Richard, D.S., Watkins, N.L., Serafin, R.B., Gilbert, L.I., 1998. Ecdysteroids regulate yolk protein uptake by *Drosophila melanogaster* oocytes. *J. Insect Physiol.* 44, 637–644.
- Richter, K., Gronert, M., 1999. Neurotropic effect of juvenile hormone III in larvae of the cockroach, *Periplaneta americana*. *J. Insect Physiol.* 45, 1065–1071.
- Riddiford, L.M., 1970. Effects of juvenile hormone on the programming of postembryonic development in eggs of the silkworm, *Hyalophora cecropia*. *Devel. Biol.* 22, 249–263.
- Riddiford, L.M., 1986. Hormonal regulation of sequential larval cuticular gene expression. *Arch. Insect Biochem. Physiol.* 3(Suppl. 1), 75–86.
- Riddiford, L.M., 1994. Cellular and molecular actions of the juvenile hormones. 1. General considerations and premetamorphic actions. *Adv. Insect Physiol.* 24, 213–274.
- Riddiford, L.M., 1996. Molecular aspects of juvenile hormone action in insect metamorphosis. In: Gilbert, L.I., Tata, J.R., Atkinson, B.G. (Eds.), *Metamorphosis*. Academic Press, New York, pp. 223–251.
- Riddiford, L.M., Ashburner, M., 1991. Effects of juvenile hormone mimics on larval development and metamorphosis of *Drosophila melanogaster*. *Gen. Comp. Endocrinol.* 82, 172–183.
- Riddiford, L.M., Cherbas, P., Truman, J.W., 2001. Ecdysone receptors and their biological actions. *Vitamins and Hormones – Adv. Res. Applic.* 60, 1–73.
- Riddiford, L.M., Hiruma, K., Zhou, X., 2003. Insights into the molecular basis of the hormonal control of molting and metamorphosis from *Manduca sexta* and *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* 33, 1327–1338.
- Riddiford, L.M., Palli, S.R., Hiruma, K., Li, W., Green, J., et al., 1990. Developmental expression, synthesis, and secretion of insecticyanin by the epidermis of the tobacco hornworm, *Manduca sexta*. *Arch. Insect Biochem. Physiol.* 14, 171–190.
- Riley, C.T., Barbeau, B.K., Keim, P.S., Kezdy, F.J., Heinrikson, R.L., et al., 1984. The covalent protein

- structure of insecticyanin, a blue biliprotein from the hemolymph of the tobacco hornworm, *Manduca sexta*. *J. Biol. Chem.* 259, 13159–13165.
- Robertson, H.M., Lampe, D.J., 1995. Distribution of transposable elements in arthropods. *Annu. Rev. Entomol.* 40, 333–357.
- Robinson, G.E., Vargo, E.L., 1997. Juvenile hormone in adult eusocial Hymenoptera: gonadotropin and behavioral pacemaker. *Arch. Insect Biochem. Physiol.* 35, 559–583.
- Rodriguez Parkitna, J.M., Ozyhar, A., Wisniewski, J.R., Kochman, M., 2002. Cloning and sequence analysis of *Galleria mellonella* juvenile hormone binding protein: a search for ancestors and relatives. *Biol. Chem.* 383, 1343–1355.
- Roe, R.M., Venkatesh, K., 1990. Metabolism of juvenile hormones: degradation and titer regulation. In: Gupta, A.P. (Ed.), *Morphogenetic Hormones of Arthropods*. Rutgers University Press, New Brunswick, NJ, pp. 126–179.
- Roe, R.M., Anspaugh, D.D., Venkatesh, K., Linderman, R.J., Graves, D.M., 1997. A novel geminal diol as a highly specific and stable *in vivo* inhibitor of insect juvenile hormone esterase. *Arch. Insect Biochem. Physiol.* 36, 165–179.
- Roe, R.M., Crawford, C.L., Clifford, C.W., Woodring, J.P., Sparks, T.C., *et al.*, 1987a. Characterization of the juvenile hormone esterases during embryogenesis of the house cricket, *Acheta domesticus*. *Int. J. Invertebr. Reprod. Dev.* 12, 57–72.
- Roe, R.M., Crawford, C.L., Clifford, C.W., Woodring, J.P., Sparks, T.C., *et al.*, 1987b. Role of juvenile hormone metabolism during embryogenesis of the house cricket, *Acheta domesticus*. *Insect Biochem.* 17, 1023–1026.
- Roe, R.M., Jesudason, P., Venkatesh, K., Kallapur, V.L., Anspaugh, D.D., *et al.*, 1993. Developmental role of juvenile hormone metabolism in Lepidoptera. *Am. Zool.* 33, 375–383.
- Roe, R.M., Kallapur, V., Linderman, R.J., Viviani, F., Harris, S.V., *et al.*, 1996. Mechanism of action and cloning of epoxide hydrolase from the cabbage looper, *Trichoplusia ni*. *Arch. Insect Biochem. Physiol.* 32, 527–535.
- Rohdendorf, E.B., Sehnal, F., 1973. Inhibition of reproduction and embryogenesis in the firebrat, *Thermobia domestica*, by juvenile hormone analogues. *J. Insect Physiol.* 19, 37–56.
- Röller, H., Dahm, K.H., Sweely, C.C., Trost, B.M., 1967. The structure of the juvenile hormone (giant silkworm moth *Hyalophora cecropia*). *Angew. Chem. Int. Edn* 6, 179–180.
- Rose, U., Ferber, M., Hustert, R., 2001. Maturation of muscle properties and its hormonal control in an adult insect. *J. Exp. Biol.* 204, 3531–3545.
- Rountree, D., Bollenbacher, W.E., 1986. The release of the PTH in the tobacco hornworm, *Manduca sexta*, is controlled intrinsically by juvenile hormone. *J. Exp. Biol.* 120, 41–59.
- Roy, S., VijayRaghavan, K., 1999. Muscle pattern diversification in *Drosophila*: the story of imaginal myogenesis. *BioEssays* 21, 486–498.
- Rudnicka, M., Kochman, M., 1984. Purification of the juvenile hormone esterase from the haemolymph of the wax moth *Galleria mellonella*. *Insect Biochem.* 14, 189–198.
- Rudolph, P.H., Stay, B., 1997. Cockroach allatostatin-like immunoreactivity in the central nervous system of the freshwater snails *Bulinus globosus* (Planorbidae) and *Stagnicola elodes* (Lymnaeidae). *Gen. Comp. Endocrinol.* 106, 241–250.
- Rudwall, A.J., Sliwowska, J., Nassel, D.R., 2000. Allatotropin-like neuropeptide in the cockroach abdominal nervous system: myotropic actions, sexually dimorphic distribution and co-localization with serotonin. *J. Comp. Neurol.* 428, 159–173.
- Safranek, L., Cymborowski, B., Williams, C.M., 1980. Effects of juvenile hormone on ecdysone-dependent development in the tobacco hornworm, *Manduca sexta*. *Biol. Bull.* 158, 248–256.
- Safranek, L., Riddiford, L.M., 1975. The biology of the black larval mutant of the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol.* 21, 1931–1938.
- Sakurai, S., Williams, C.M., 1989. Short-loop negative and positive feedback on ecdysone secretion by prothoracic gland in the tobacco hornworm, *Manduca sexta*. *Gen. Comp. Endocrinol.* 75, 204–216.
- Sakurai, S., Okuda, M., Ohtaki, T., 1989. Juvenile hormone inhibits ecdysone secretion and responsiveness to prothoracicotropic hormone in prothoracic glands of *Bombyx mori*. *Gen. Comp. Endocrinol.* 75, 222–230.
- Sanburg, L.L., Kramer, K.J., Kezdy, F.J., Law, J.H., Oberlander, H., 1975. Role of juvenile hormone esterases and carrier proteins in insect development. *Nature* 253, 266–267.
- Sasorith, S., Billas, I.M.L., Iwema, T., Moras, D., Wurtz, J.M., 2002. Structure-based analysis of the Ultraspiracle protein and docking studies of putative ligands. *J. Insect Sci.* 2, 25–00.
- Sbrenna-Micciarelli, A., 1977. Effects of farnesyl methyl ether on embryos of *Schistocerca gregaria*. *Acta Embryol. Exp.* 3, 295–303.
- Schmidt, T., Choffat, Y., Klausner, S., Kubli, E., 1993. The *Drosophila melanogaster* sex peptide: a molecular analysis of structure–function relationships. *J. Insect Physiol.* 39, 361–368.
- Schneider, M., Wiesel, G., Dorn, A., 1995. Effects of JH III and JH analogues on phase-related growth, egg maturation and lipid metabolism in *Schistocerca gregaria* females. *J. Insect Physiol.* 41, 23–31.
- Schooley, D.A., Baker, F.C., 1985. Juvenile hormone biosynthesis. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology Biochemistry and Pharmacology*, vol. 7. Pergamon, Oxford, pp. 363–390.
- Schooley, D.A., Baker, F.C., Tsai, L.W., Miller, C.A., Jamieson, G.C., 1984. Juvenile hormones 0, I, II exist only in Lepidoptera. In: Hoffmann, J., Porchet, M.

- (Eds.), *Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones*. Springer, New York, pp. 373–383.
- Schooley, D.A., Judy, K.J., Baker, F.C., Lee, E., Bergot, B.J., *et al.*, 1978a. Biosynthesis of the juvenile hormones: the role of the homoisoprenoid intermediates. In: Gaillard, P.J., Boer, H.H. (Eds.), *Comparative Endocrinology*. Elsevier, London, pp. 499–502.
- Schooley, D.A., Bergot, B.J., Goodman, W.G., Gilbert, L.I., 1978b. Synthesis of both optical isomers of insect juvenile hormone JHIII and their affinity for the JH-specific binding protein of *Manduca sexta*. *Biochem. Biophys. Res. Commun.* 81, 743–749.
- Schooley, D.A., Judy, K., Bergot, B.J., Hall, M.S., Jennings, R.C., 1976. Determination of the physiological levels of juvenile hormone in several insects and biosynthesis of the carbon skeleton of the juvenile hormones. In: Gilbert, L.I. (Ed.), *The Juvenile Hormones*. Plenum, New York, pp. 101–117.
- Schulz, D.J., Robinson, G.E., 2001. Octopamine influences division of labor in honey bee colonies. *J. Comp. Physiol. A* 187, 53–61.
- Schulz, D.J., Sullivan, J.P., Robinson, G.E., 2002. Juvenile hormone and octopamine in the regulation of division of labor in honeybee colonies. *Hormones Behav.* 42, 222–231.
- Schwartz, L.M., 1992. Insect muscle as a model for programmed cell death. *J. Neurobiol.* 23, 1312–1326.
- Secher, T., Lenz, C., Cazzamali, G., Sorensen, G., Williamson, M., *et al.*, 2001. Molecular cloning of a functional allatostatin gut/brain receptor and an allatostatin prohormone from the silkworm *Bombyx mori*. *J. Biol. Chem.* 276, 47052–47060.
- Sedlak, B.J., 1985. Structure of endocrine glands. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology Biochemistry and Pharmacology*, vol. 7. Pergamon, Oxford, pp. 25–60.
- Sedlak, B.J., Marchione, L., Devorkin, B., Davino, R., 1983. Correlations between endocrine gland ultrastructure and hormone titers in the fifth larval instar of *Manduca sexta*. *Gen. Comp. Endocrinol.* 52, 291–310.
- Sehnal, F., 1984. The juvenile hormones of insects. *Nov. Acta Leopold.* 56, 251–266.
- Sehnal, F., Granger, N.A., 1975. Control of corpora allata functions in the last instar larvae of *Galleria mellonella*. *Biol. Bull.* 148, 106–116.
- Sehnal, F., Svacha, P., Zrzavy, J., 1996. Evolution of insect metamorphosis. In: Gilbert, L.I., Tata, J.R., Atkinson, B.G. (Eds.), *Metamorphosis*. Academic Press, London, pp. 3–58.
- Sen, S.E., Sperry, A.E., 2002. Partial purification of a farnesyl diphosphate synthase from whole-body *Manduca sexta*. *Insect Biochem. Mol. Biol.* 32, 889–899.
- Sen, S.E., Ewing, G.J., 1997. Natural and unnatural terpenoid precursors of insect juvenile hormone. *J. Organ. Chem.* 62, 3529–3536.
- Sen, S.E., Garvin, G.M., 1995. Substrate requirements for lepidopteran farnesol dehydrogenase. *J. Agric. Food Chem.* 43, 820–826.
- Severson, T.F., Goodrow, M.H., Morisseau, C., Dowdy, D.L., Hammock, B.D., 2002. Urea and amide-based inhibitors of the juvenile hormone epoxide hydrolase of the tobacco hornworm *Manduca sexta*. *Insect Biochem. Mol. Biol.* 32, 1741–1756.
- Shanmugavelu, M., Baytan, A.R., Chesnut, J.D., Bonning, B.C., 2000. A novel protein that binds juvenile hormone esterase in fat body tissue and pericardial cells of the tobacco hornworm *Manduca sexta*. *J. Biol. Chem.* 275, 1802–1806.
- Share, M.R., Venkatesh, K., Jesudason, P., Roe, R.M., 1988. Juvenile hormone metabolism during embryogenesis in the tobacco hornworm, *Manduca sexta*. *Arch. Insect Biochem. Physiol.* 8, 173–186.
- Shemshedini, L., Lanoue, M., Wilson, T.G., 1990. Evidence for a juvenile hormone receptor involved in protein synthesis in *Drosophila melanogaster*. *J. Biol. Chem.* 265, 1913–1918.
- Shiga, S., Yasuyama, K., Okamura, N., Yamaguchi, T., 2002. Neural- and endocrine control of flight muscle degeneration in the adult cricket, *Gryllus bimaculatus*. *J. Insect Physiol.* 48, 15–24.
- Shirk, P., Bhaskaran, G., Röller, H., 1983. Developmental physiology of corpora allata and accessory sex glands in the cecropia silkworm. *J. Exp. Zool.* 227, 69–79.
- Short, J.E., Edwards, J.P., 1992. Levels of juvenile hormone III during embryonic development in the oriental cockroach, *Blattella orientalis* (L.). In: Mauchamp, B., Couillaud, F., Baehr, J.-C. (Eds.), *Insect Juvenile Hormone Research: Chemistry, Biochemistry and Mode of Action*. INRA, Paris, pp. 19–25.
- Siew, Y.C., Gilbert, L.I., 1971. Effects of molting hormone and juvenile hormone on insect endocrine gland activity. *J. Insect Physiol.* 17, 2095–2104.
- Skiebe, P., 1999. Allatostatin-like immunoreactivity in the stomatogastric nervous system and the pericardial organs of the crab *Cancer pagurus*, the lobster *Homarus americanus*, and the crayfish *Cherax destructor* and *Procambarus clarkii*. *J. Comp. Neurol.* 403, 85–105.
- Skinner, J.R., Fairbairn, S.E., Woodhead, A.P., Bendena, W.G., Stay, B., 1997. Allatostatin in hemocytes of the cockroach *Diploptera punctata*. *Cell Tissue Res.* 290, 119–128.
- Sláma, K., Romaňuk, M., Šorm, F., 1974. *Insect Hormones and Bioanalogues*. Springer, Berlin.
- Sliter, T.J., Sedlak, B.J., Baker, F.C., Schooley, D.A., 1987. Juvenile hormone in *Drosophila melanogaster*: identification and titer determination during development. *Insect Biochem.* 17, 161–166.
- Smith, P.A., Clare, A.S., Rees, H.H., Prescott, M.C., Wainwright, G., *et al.*, 2000. Identification of methyl farnesoate in the cypris larva of the barnacle, *Balanus amphitrite*, and its role as a juvenile hormone. *Insect Biochem. Mol. Biol.* 30, 885–890.
- Smith, R.F., Arking, R., 1975. The effects of juvenile hormone analogues on embryogenesis in *Drosophila melanogaster*. *J. Insect Physiol.* 21, 723–732.

- Sondergaard, L., 1993. Homology between the mammalian liver and the *Drosophila* fat body. *Trends Genet.* 9, 193–200.
- Song, Q.S., Gilbert, L.I., 1998. Alterations in Ultraspiracle (USP) content and phosphorylation state accompany feedback regulation of ecdysone synthesis in the insect prothoracic gland. *Insect Biochem. Mol. Biol.* 28, 849–860.
- Soulages, J.L., Wells, M.A., 1994. Lipophorin: the structure of an insect lipoprotein and its role in lipid transport in insects. *Adv. Protein Chem.* 45, 371–415.
- Sparagana, S.P., Bhaskaran, G., Barrera, P., 1985. Juvenile hormone acid methyltransferase activity in imaginal discs of *Manduca sexta* prepupae. *Arch. Insect Biochem. Physiol.* 2, 191–202.
- Sparagana, S.P., Bhaskaran, G., Dahm, K.H., Riddle, V., 1984. Juvenile hormone production, juvenile hormone esterase, and juvenile hormone acid methyltransferase in corpora allata of *Manduca sexta*. *J. Exp. Zool.* 230, 309–314.
- Sparks, T.C., Rose, R.L., 1983. Inhibition and substrate specificity of the haemolymph juvenile hormone esterase of the cabbage looper, *Trichoplusia ni*. *Insect Biochem.* 13, 633–640.
- Sparks, T.C., Allen, L.G., Schneider, F., Granger, N.A., 1989. Juvenile hormone esterase activity from *Manduca sexta* corpora allata *in vitro*. *Arch. Insect Biochem. Physiol.* 11, 93–108.
- Sparks, T.C., Hammock, B.D., Riddiford, L.M., 1983. The hemolymph juvenile hormone esterase of *Manduca sexta* inhibition and regulation. *Insect Biochem.* 13, 529–541.
- Sparks, T.C., Willis, W.S., Shorey, H.H., Hammock, B.D., 1979. Haemolymph juvenile hormone esterase activity in synchronous last instar larvae of the cabbage looper, *Trichoplusia ni*. *J. Insect Physiol.* 25, 125–132.
- Sperry, A.E., Sen, S.E., 2001. Farnesol oxidation in insects: evidence that the biosynthesis of insect juvenile hormone is mediated by a specific alcohol oxidase. *Insect Biochem. Mol. Biol.* 31, 171–178.
- Spitz, F., Gonzalez, F.D., Duboule, D., 2003. A global control region defines a chromosomal regulatory landscape containing the HoxD cluster. *Cell* 113, 405–417.
- Stahl, G.B., 1975. Insect growth regulators with juvenile hormone activity. *Annu. Rev. Entomol.* 20, 417–460.
- Stay, B., 2000. A review of the role of neurosecretion in the control of juvenile hormone synthesis: a tribute to Berta Scharrer. *Insect Biochem. Mol. Biol.* 30, 653–662.
- Stay, B., Zhang, J.R., Tobe, S.S., 2002. Methyl farnesoate and juvenile hormone production in embryos of *Diploptera punctata* in relation to innervation of corpora allata and their sensitivity to allatostatin. *Peptides* 23, 1981–1990.
- Stay, B., Chan, K.K., Woodhead, A.P., 1992. Allatostatatin-immunoreactive neurons projecting to the corpora allata of adult *Diploptera punctata*. *Cell Tissue Res.* 270, 15–23.
- Stay, B., Woodhead, A.P., Joshi, S., Tobe, S.S., 1991a. Allatostatins, neuropeptide inhibitors of juvenile hormone biosynthesis in brain and corpora allata of cockroaches, *Diploptera punctata*. In: Menn, J.J., Kelly, T.J., Masler, E.P. (Eds.), *Insect Neuropeptides*. American Chemical Society, Washington, DC, pp. 164–176.
- Stay, B., Joshi, S., Woodhead, A.P., 1991b. Sensitivity to allatostatins of corpora allata from larval and adult female *Diploptera punctata*. *J. Insect Physiol.* 37, 63–70.
- Stay, B., Friedel, T., Tobe, S.S., Mundall, E.C., 1980. Feedback control of juvenile hormone synthesis in cockroaches: possible role for ecdysterone. *Science* 207, 898–900.
- Stael, C.G.H., Ampleford, E.J., 1984. Circadian control of haemolymph ecdysteroid titers and ecdysis rhythms in *Rhodnius prolixus*. In: Porter, R., Collins, G.M. (Eds.), *Photoperiodic Regulation of Insect and Molluscan Hormones*. Pitman, London, pp. 150–169.
- Steiner, B., Pfisterwilhelm, R., Grossniklaus-Bürgin, C., Rembold, H., Treiblmayr, K., et al., 1999. Titres of juvenile hormone I, II and III in *Spodoptera littoralis* (Noctuidae) from the egg to the pupal moult and their modification by the egg-larval parasitoid *Chelonus inanitus* (Braconidae). *J. Insect Physiol.* 45, 401–413.
- Stoclet, J.C., Gerard, D., Kilhoffer, M.C., Lugnier, C., Miller, R., et al., 1987. Calmodulin and its role in intracellular calcium regulation. *Progr. Neurobiol.* 29, 321–364.
- Stout, J., Hao, J., Kim, P., Mbungu, D., Bronsert, M., et al., 1998. Regulation of the phonotactic threshold of the female cricket, *Acheta domesticus*: juvenile hormone III, allatectomy, auditory neuron thresholds and environmental factors. *J. Comp. Physiol. A* 182, 635–645.
- Stout, J., Hayes, V., Zacharias, D., Henley, J., Stumper, A., et al., 1992. Juvenile hormone controls phonotactic responsiveness of female crickets by genetic regulation of the response properties of identified auditory interneurons. In: Mauchamp, B., Couillaud, F., Baehr, J.-C. (Eds.), *Insect Juvenile Hormone Research: Fundamental and Applied Approaches*. INRA, Paris, pp. 265–283.
- Strambi, C., 1981. Some data obtained by radioimmunoassay of juvenile hormones. In: Pratt, G.E., Brooks, G.E. (Eds.), *Juvenile Hormone Biochemistry*. Elsevier/North Holland, Amsterdam, pp. 59–66.
- Strambi, C., Strambi, A., De Reggi, M.L., Hirn, M.H., DeLagge, M.A., 1981. Radioimmunoassay of insect juvenile hormones and of their diol derivatives. *Eur. J. Biochem.* 118, 401–406.
- Sundaram, M., van Aalten, D.M.F., Findlay, J.B.C., Sivaprasadarao, A., 2002. The transfer of transthyretin and receptor-binding properties from the plasma retinol-binding protein to the epididymal retinoic acid-binding protein. *Biochem. J.* 362, 265–271.
- Sundaram, M., Sivaprasadarao, A., Desousa, M.M., Findlay, J.B.C., 1998. The transfer of retinol from serum retinol-binding protein to cellular retinol-binding protein is mediated by a membrane receptor. *J. Biol. Chem.* 273, 3336–3342.

- Sutherland, T.D., Feyereisen, R., 1996. Target of cockroach allatostatin in the pathway of juvenile hormone biosynthesis. *Mol. Cell. Endocrinol.* 120, 115–123.
- Sutherland, T.D., Unnithan, G.C., Andersen, J.F., Evans, P.H., Murataliev, M.B., *et al.*, 1998. A cytochrome P450 terpenoid hydroxylase linked to the suppression of insect juvenile hormone synthesis. *Proc. Natl Acad. Sci. USA* 95, 12884–12889.
- Tanaka, S., 1994. Endocrine control of ovarian development and flight muscle histolysis in a wing dimorphic cricket, *Modicogryllus confirmatus*. *J. Insect Physiol.* 40, 483–490.
- Taylor, P.A., Bhatt, T.R., Horodyski, F.M., 1996. Molecular characterization and expression analysis of *Manduca sexta* allatotropin. *Eur. J. Biochem.* 239, 588–596.
- Teal, P.E.A., 2002. Effects of allatotropin and allatostatin on *in vitro* production of juvenile hormones by the corpora allata of virgin females of the moths of *Heliothis virescens* and *Manduca sexta*. *Peptides* 23, 663–669.
- Teal, P.E.A., Proveaux, A.T., Heath, R.R., 2000. Analysis and quantitation of insect juvenile hormones using chemical ionization ion-trap mass spectrometry. *Analyt. Biochem.* 277, 206–213.
- Temin, G., Zander, M., Roussel, J.P., 1986. Physico-chemical (GC-MS) measurements of juvenile hormone III titers during embryogenesis of *Locusta migratoria*. *Int. J. Invertebr. Reprod. Devel.* 9, 105–112.
- Thomas, B.A., Church, W.B., Lane, T.R., Hammock, B.D., 1999. Homology model of juvenile hormone esterase from the crop pest, *Heliothis virescens*. *Proteins – Struct. Funct. Genet.* 34, 184–196.
- Thomas, H.E., Stunnenberg, H.G., Stewart, A.F., 1993. Heterodimerization of the *Drosophila* ecdysone receptor with retinoid-x receptor and Ultraspiracle. *Nature* 362, 471–475.
- Thompson, C.S., Tobe, S.S., 1986. Electrical properties of membranes and cells of the corpora allata of the cockroach *Diploptera punctata*: evidence for the presence of voltage-sensitive calcium channels. In: Borkovec, A.B., Gelman, D.B. (Eds.), *Insect Neurochemistry and Neurophysiology*. Humana Press, Totowa, NJ, pp. 375–378.
- Thompson, C.S., Yagi, K.J., Chen, Z.F., Tobe, S.S., 1990. The effects of octopamine on juvenile hormone biosynthesis, electrophysiology, and cAMP content of the corpora allata of the cockroach *Diploptera punctata*. *J. Comp. Physiol. B* 160, 241–249.
- Tobe, S.S., 1990. Role of intracellular messengers in the regulation of juvenile hormone biosynthesis in the cockroach, *Diploptera punctata*. In: Eppler, A., Scanes, C.G., Stetson, M.H. (Eds.), *Progress in Comparative Endocrinology*. Wiley-Liss, New York, pp. 174–179.
- Tobe, S.S., Stay, B., 2004. Neuropeptides: roles in the regulation of juvenile hormone production. In: Henry, H.L., Norman, A.W. (Eds.), *Encyclopedia of Hormones*. Elsevier Science, Oxford. (in press).
- Tobe, S.S., Bendena, W.G., 1999. The regulation of juvenile hormone production in arthropods. *Ann. New York Acad. Sci.* 897, 300–310.
- Tobe, S.S., Stay, B., 1985. Structure and regulation of the corpus allatum. *Adv. Insect Physiol.* 18, 305–432.
- Tobe, S.S., Feyereisen, R., 1983. Juvenile hormone biosynthesis: Regulation and assay. In: Downer, R.G.H., Laufer, H. (Eds.), *Endocrinology of Insects*. Alan R. Liss, New York, pp. 161–178.
- Tobe, S.S., Pratt, G.E., 1974. The influence of substrate concentrations on the rate of insect juvenile hormone biosynthesis by corpora allata of the desert locust *in vitro*. *Biochem. J.* 144, 107–113.
- Toong, Y.C., Schooley, D.A., Baker, F.C., 1988. Isolation of insect juvenile hormone III from a plant. *Nature* 333, 170–171.
- Touhara, K., Lerro, K.A., Bonning, B.C., Hammock, B.D., Prestwich, G.D., 1993. Ligand binding by a recombinant insect juvenile hormone binding protein. *Biochemistry* 32, 2068–2075.
- Touhara, K., Prestwich, G.D., 1992. Binding site mapping of a photoaffinity-labeled juvenile hormone binding protein. *Biochem. Biophys. Res. Commun.* 182, 466–473.
- Touhara, K., Prestwich, G.D., 1993. Juvenile hormone epoxide hydrolase: photoaffinity labeling, purification, and characterization from tobacco hornworm eggs. *J. Biol. Chem.* 268, 19604–19609.
- Touhara, K., Prestwich, G.D., 1994. Role of juvenile hormone binding protein in modulating function of JH epoxide hydrolase in eggs of *Manduca sexta*. *Insect Biochem. Mol. Biol.* 24, 641–646.
- Touhara, K., Soroker, V., Prestwich, G.D., 1994. Photoaffinity labeling of juvenile hormone epoxide hydrolase and JH-binding proteins during ovarian and egg development in *Manduca sexta*. *Insect Biochem. Mol. Biol.* 24, 633–640.
- Touhara, K., Wojtasek, H., Prestwich, G.D., 1996. *In vitro* modeling of the ternary interaction in juvenile hormone metabolism. *Arch. Insect Biochem. Physiol.* 32, 399–406.
- Trautmann, K.H., 1972. *In vitro* Studium der Tragerproteine von ³H-markierten Juvenilhormonwirksamen Verbindungen in der Hämolymphe von *Tenebrio molitor* L. *Larvae. Z. Naturforsch.* 27c, 263–273.
- Trautmann, K.H., Masner, P., Schuler, A., Suchy, M., Wipf, H.K., 1974. Evidence of the juvenile hormone III in insects of four orders. *Z. Naturforsch.* 29c, 757–759.
- Trowell, S.C., 1992. High affinity juvenile hormone carrier proteins in the haemolymph of insects. *Comp. Biochem. Physiol. B* 103, 795–808.
- Trowell, S.C., Hines, E.R., Herlt, A.J., Rickards, R.W., 1994. Characterization of a juvenile hormone binding lipophorin from the blowfly, *Lucilia cuprina*. *Comp. Biochem. Physiol. B* 109, 339–359.
- Truesdell, P.F., Koladich, P.M., Kataoka, H., Kojima, K., Suzuki, A., *et al.*, 2000. Molecular characterization of a cDNA from the true armyworm *Pseudaletia unipuncta*

- encoding *Manduca sexta* allatotropin peptide. *Insect Biochem. Mol. Biol.* 30, 691–702.
- Truman, J.W., 1996. Metamorphosis of the insect nervous system. In: Gilbert, L.I., Tata, J.R., Atkinson, B.G. (Eds.), *Metamorphosis*. Academic Press, New York, pp. 283–320.
- Truman, J.W., Reiss, S.E., 1988. Hormonal regulation of the shape of identified motorneurons in the moth *Manduca sexta*. *J. Neurosci.* 8, 765–775.
- Truman, J.W., Riddiford, L.M., 1999. The origins of insect metamorphosis. *Nature* 401, 447–452.
- Truman, J.W., Riddiford, L.M., 2002. Endocrine insights into the evolution of metamorphosis in insects. *Annu. Rev. Entomol.* 47, 467–500.
- Tu, M.P., Kou, R., O'Remus, G., Yin, C.-M., Stoffolano, J.G., 2002. Allatotrophic activity in the brain of female *Phormia regina*. *J. Insect Physiol.* 48, 733–741.
- Tu, M.P., Kou, R., Wang, Z.S., Stoffolano, J.G., Yin, C.-M., 2001. Immunolocalization and possible effect of a moth allatotropin-like substance in a fly, *Phormia regina*. *J. Insect Physiol.* 47, 233–244.
- Ulrich, G.M., 1985. Elimination of the allatotrophic activity in locusts by microsurgical and immunological methods: evidence for humoral control of the corpora allata, hemolymph proteins and ovary development. *Gen. Comp. Endocrinol.* 59, 120–130.
- Unni, B., Barrera, P., Muszynskapitel, M., Bhaskaran, G., Dahm, K.H., 1993. Partial characterization of allatostatin, a neurohormone of *Manduca sexta*. *Arch. Insect Biochem. Physiol.* 24, 173–185.
- Unnithan, G.C., Feyereisen, R., 1995. Experimental acquisition and loss of allatostatin sensitivity by corpora allata of *Diploptera punctata*. *J. Insect Physiol.* 41, 975–980.
- Unnithan, G.C., Sutherland, T.D., Cromey, D.W., Feyereisen, R., 1998. A factor causing stable stimulation of juvenile hormone synthesis by *Diploptera punctata* corpora allata *in vitro*. *J. Insect Physiol.* 44, 1027–1037.
- Vafopoulou, X., Steel, C.G.H., 2001. Induction of rhythmicity in prothoracicotropic hormone and ecdysteroids in *Rhodnius prolixus*: roles of photic and neuroendocrine zeitgebers. *J. Insect Physiol.* 47, 935–941.
- Valaitis, A.P., 1991. Characterization of hemolymph juvenile hormone esterase from *Lymantria dispar*. *Insect Biochem.* 21, 583–595.
- Varjas, L., Kulcsar, P., Fekete, J., Bihatsi-Karsai, E., Lelik, L., 1992. JH titres measured by GC-MS in the hemolymph of *Mamestra oleracea* larvae reared under different photoperiodic conditions. In: Mauchamp, B., Couillaud, F., Baehr, J.-C. (Eds.), *Insect Juvenile Hormone Research: Fundamental and Applied Approaches*. INRA, Paris, pp. 45–50.
- Veenstra, J.A., Costes, L., 1999. Isolation and identification of a peptide and its cDNA from the mosquito *Aedes aegypti* related to *Manduca sexta* allatotropin. *Peptides* 20, 1145–1151.
- Veenstra, J.A., Lehman, H.K., Davis, N.T., 1994. Allatotropin is a cardioacceleratory peptide in *Manduca sexta*. *J. Exp. Biol.* 188, 347–354.
- Veenstra, J.A., Noriega, F.G., Graf, R., Feyereisen, R., 1997. Identification of three allatostatins and their cDNA from the mosquito *Aedes aegypti*. *Peptides* 18, 937–942.
- Venkataraman, V., Omahony, P.J., Manzcak, M., Jones, G., 1994. Regulation of juvenile hormone esterase gene transcription by juvenile hormone. *Devel. Genet.* 15, 391–400.
- Venkatesh, K., Roe, R.M., 1988. The role of juvenile hormone and brain factor(s) in the regulation of plasma juvenile hormone esterase activity during the last larval stadium of the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol.* 34, 415–426.
- Vermunt, A.M.W., Vermeesch, A.M.G., deKort, C.A.D., 1997a. Purification and characterization of juvenile hormone esterase from hemolymph of the Colorado potato beetle. *Arch. Insect Biochem. Physiol.* 35, 261–277.
- Vermunt, A.M.W., Koopmanschap, A.B., Vlask, J.M., deKort, C.A.D., 1997b. Cloning and sequence analysis of cDNA encoding a putative juvenile hormone esterase from the Colorado potato beetle. *Insect Biochem. Mol. Biol.* 27, 919–928.
- Vernier, P., Cardinaud, B., Valdenaire, O., Philippe, H., Vincent, J.D., 1995. An evolutionary view of drug-receptor interaction: the bioamine receptor family. *Trends Pharmacol. Sci.* 16, 375–381.
- Vince, R., Gilbert, L.I., 1977. Juvenile hormone esterase activity in precisely timed instar larvae and pharate pupae of *Manduca sexta*. *Insect Biochem.* 7, 115–120.
- von Nickisch-Rosengck, E., Krieger, J., Kubick, S., Laage, R., Strobel, J., et al., 1996. Cloning of biogenic amine receptors from moths (*Bombyx mori* and *Heliothis virescens*). *Insect Biochem. Mol. Biol.* 26, 817–827.
- Walter, W.R., Sing, G.B., Krawetz, S.A., 1998. MARs mission update. *Biochem. Biophys. Res. Commun.* 242, 419–422.
- Wang, X., Chen, X., Haunerland, N.H., 1993. Flight muscle development in juvenile and adult forms of the desert locust, *Schistocerca gregaria*. *J. Insect Physiol.* 39, 325–333.
- Wang, Z.W., Ding, Q., Yagi, K.J., Tobe, S.S., 1994. Terminal stages in juvenile hormone biosynthesis in corpora allata of *Diploptera punctata*: developmental changes in enzyme activity and regulation by allatostatins. *J. Insect Physiol.* 40, 217–223.
- Ward, V.K., Bonning, B.C., Huang, T., Shiotsuki, T., Griffeth, V.N., et al., 1992. Analysis of the catalytic mechanism of juvenile hormone esterase by site-directed mutagenesis. *Int. J. Biochem.* 24, 1933–1942.
- Watson, R.D., Whinsenton, L.R., Bollenbacher, W.E., Granger, N.A., 1986. Interendocrine regulation of the corpora allata and prothoracic glands of *Manduca sexta*. *Insect Biochem.* 16, 149–155.

- Weeks, J.C., Truman, J.W., 1986a. Hormonally mediated reprogramming of muscles and motoneurons during the larval-pupal transformation of the tobacco hornworm, *Manduca sexta*. *J. Exp. Biol.* 125, 1–14.
- Weeks, J.C., Truman, J.W., 1986b. Steroid control of neuron and muscle development during the metamorphosis of an insect. *J. Neurobiol.* 17, 249–267.
- Weinberger, C., 1996. A model for farnesoid feedback control in the mevalonate pathway. *Trends Endocrinol. Metab.* 7, 1–6.
- Weller, J., Sun, G.C., Zhou, B.H., Lan, Q., Hiruma, K., et al., 2001. Isolation and developmental expression of two nuclear receptors, MHR4 and beta FTZ-F1, in the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol.* 31, 827–837.
- Westphal, U., 1986. Steroid-Protein Interactions II. Springer, Berlin.
- Wheeler, D.E., Nijhout, H.F., 1981. Soldier determination in ants: new role for juvenile hormone. *Science* 213, 361–363.
- Wheeler, D.E., Nijhout, H.F., 2003. A perspective for understanding the modes of juvenile hormone action as a lipid signaling system. *BioEssays* 25, 994–1001.
- Wheelock, C.E., Severson, T.F., Hammock, B.D., 2001. Synthesis of new carboxylesterase inhibitors and evaluation of potency and water solubility. *Chem. Res. Toxicol.* 14, 1536–1572.
- Whisenton, L.R., Bowen, M.F., Granger, N.A., Gilbert, L.I., Bollenbacher, W.E., 1985. Brain-mediated 20-hydroxyecdysone regulation of juvenile hormone synthesis by the corpora allata of the tobacco hornworm, *Manduca sexta*. *Gen. Comp. Endocrinol.* 58, 311–318.
- Whisenton, L.R., Granger, N.A., Bollenbacher, W.E., 1987. A kinetic analysis of brain-mediated 20-hydroxyecdysone stimulation of the corpora allata of *Manduca sexta*. *Mol. Cell. Endocrinol.* 54, 171–178.
- Whitmore, E., Gilbert, L.I., 1972. Haemolymph lipoprotein transport of juvenile hormone. *J. Insect Physiol.* 18, 1153–1167.
- Whitmore, D., Gilbert, L.I., Ittycheriah, P.I., 1974. The origin of hemolymph carboxylesterases “induced” by the insect juvenile hormone. *Mol. Cell. Endocrinol.* 1, 37–54.
- Wieczorek, E., Kochman, M., 1991. Conformational change of the haemolymph juvenile hormone binding protein from *Galleria mellonella*. *Eur. J. Biochem.* 201, 347–353.
- Wigglesworth, V.B., 1936. The function of the corpus allatum in the growth and reproduction of *Rhodnius prolixus*. *Q. J. Microsc. Sci.* 79, 91–121.
- Williams, C.M., 1952. Physiology of insect diapause IV. The brain and prothoracic glands as an endocrine system in the cecropia silkworm. *Biol. Bull.* 103, 120–138.
- Wigglesworth, V.B., 1959. Metamorphosis, polymorphism, differentiation. *Sci. Am.* 200, 100–106.
- Wigglesworth, V.B., 1970. Insect Hormones. W.H. Freeman, San Francisco.
- Wigglesworth, V.B., 1972. The Principles of Insect Physiol. Chapman and Hall, London.
- Williams, C.M., 1976. Juvenile hormone . . . in retrospect and prospect. In: Gilbert, L.I. (Ed.), The Juvenile Hormones. Plenum, New York, pp. 1–14.
- Williamson, M., Lenz, C., Winther, A.M.E., Nassel, D.R., Gimmelikhuijzen, C.J.P., 2001a. Molecular cloning, genomic organization and expression of a B-Type (Cricket-Type) allatostatin preprohormone from *Drosophila melanogaster*. *Biochem. Biophys. Res. Commun.* 281, 544–550.
- Williamson, M., Lenz, C., Winther, A.M.E., Nassel, D.R., Gimmelikhuijzen, C.J.P., 2001b. Molecular cloning, genomic organization, and expression of a C-type (*Manduca sexta*-type) allatostatin preprohormone from *Drosophila melanogaster*. *Biochem. Biophys. Res. Commun.* 282, 124–130.
- Williamson, M., Lenz, C., Winther, A.M.E., Nassel, D.R., Gimmelikhuijzen, C.J.P., 2001c. Molecular cloning, genomic organization and expression of a B-Type (Cricket-Type) allatostatin preprohormone from *Drosophila melanogaster*. *Biochem. Biophys. Res. Commun.* 282, 368 (correction of 2001b).
- Willis, J.H., 1996. Metamorphosis of the cuticle, its proteins, and their genes. In: Gilbert, L.I., Tata, J.R., Atkinson, B.G. (Eds.), Metamorphosis. Academic Press, New York, pp. 253–282.
- Wilson, T.G., Ashok, M., 1998. Insecticide resistance resulting from an absence of target-site gene product. *Proc. Natl Acad. Sci. USA* 95, 14040–14044.
- Wilson, T.G., Fabian, J., 1986. A *Drosophila melanogaster* mutant resistant to a chemical analog of juvenile hormone. *Devel. Biol.* 118, 190–201.
- Wilson, T.G., DeMoor, S., Lei, J., 2003. Juvenile hormone involvement in *Drosophila melanogaster* male reproduction. *Insect Biochem. Mol. Biol.* 33, 1167–1175.
- Wing, K.D., Sparks, T.C., Lovell, V.M., Levinson, S.O., Hammock, B.D., 1981. The distribution of juvenile hormone esterase and its interrelationship with other proteins influencing juvenile hormone metabolism in the cabbage looper, *Trichoplusia ni*. *Insect Biochem.* 11, 473–485.
- Wisniewski, J.R., Muszynska-Pytel, M., Kochman, M., 1986. Juvenile hormone degradation in brain and corpora cardiaca-corpora allata complex during the last larval instar of *Galleria mellonella*. *Experientia* 42, 167–168.
- Witek, G., Hoffmann, K.H., 2001. Immunological evidence for FGLamide- and W₂W₉-allatostatins in the ovary of *Gryllus bimaculatus*. *Physiol. Entomol.* 26, 49–57.
- Wojtasek, H., Prestwich, G.D., 1995. Key disulfide bonds in an insect hormone binding protein: cDNA cloning of a juvenile hormone binding protein of *Heliothis virescens* and ligand binding by native and mutant forms. *Biochemistry* 34, 5234–5241.
- Wojtasek, H., Prestwich, G.D., 1996. An insect juvenile hormone-specific epoxide hydrolase is related to vertebrate microsomal epoxide hydrolases. *Biochem. Biophys. Res. Commun.* 220, 323–329.

- Woodring, J., Hoffmann, K.H., 1994. The effects of octopamine, dopamine and serotonin on juvenile hormone synthesis, *in vitro*, in the cricket, *Gryllus bimaculatus*. *J. Insect Physiol.* 40, 797–802.
- Wroblewski, V.J., Harshman, L.G., Hanzlik, T.N., Hammock, B.D., 1990. Regulation of juvenile hormone esterase gene expression in the tobacco budworm. *Arch. Biochem. Biophys.* 278, 461–466.
- Wyatt, G.R., Davey, K.G., 1996. Cellular and molecular actions of juvenile hormone. 2. Roles of juvenile hormone in adult insects. *Adv. Insect Physiol.* 26, 1–155.
- Xu, Y., Fang, F., Chu, Y.X., Jones, D., Jones, G., 2002. Activation of transcription through the ligand-binding pocket of the orphan nuclear receptor ultraspiracle. *Eur. J. Biochem.* 269, 6026–6036.
- Yagi, K.J., Tobe, S.S., 2001. The radiochemical assay for juvenile hormone biosynthesis in insects: problems and solutions. *J. Insect Physiol.* 47, 1227–1234.
- Yagi, K.J., Konz, K.G., Stay, B., Tobe, S.S., 1991. Production and utilization of farnesoic acid in the juvenile hormone biosynthetic pathway by corpora allata of larval *Diploptera punctata*. *Gen. Comp. Endocrinol.* 81, 284–294.
- Yagi, S., Kuramochi, K., 1976. The role of juvenile hormone in larval duration and spermiogenesis in relation to phase variation in the tobacco cutworm, *Spodoptera litura*. *Appl. Entomol. Zool.* 11, 133–138.
- Yamamoto, C.K., Chadarevian, A., Pellegrini, M., 1988. Juvenile hormone action mediated in male accessory glands of *Drosophila* by calcium and kinase C. *Science* 239, 916–919.
- Yang, J.H., Park, J.-C., Lee, B.H., 2000. Molecular characterization of allatostatins cDNA in central nervous system and midgut from the German cockroach *Blattella germanica*. *Korean J. Entomol.* 30, 131–137.
- Yao, T.P., Forman, B.M., Jiang, Z.Y., Cherbas, L., Chen, J.D., *et al.*, 1993. Functional ecdysone receptor is the product of EcR and ultraspiracle genes. *Nature* 366, 476–479.
- Yin, C.-M., Zou, B.X., Jiang, M.G., Li, M.F., Qin, W.H., *et al.*, 1995. Identification of juvenile hormone III bisepoxide (JHB₃), JH III and methyl farnesoate secreted by the corpus allatum of *Phormia regina*, *in vitro* and function of JHB₃ either applied alone or as a part of a juvenoid blend. *J. Insect Physiol.* 41, 473–488.
- Yin, C.-M., Takeda, M., Wang, Z.-S., 1987. Juvenile hormone analogue, methoprene as a circadian and development modulator in *Diatraea grandiosella*. *J. Insect Physiol.* 33, 95–102.
- Young, J.K., Orth, A.P., Goodman, W.G., 2003. Allelic variation in the hemolymph juvenile hormone binding protein gene of *Manduca sexta*. *Mol. Cell. Endocrinol.* 208, 41–50.
- Yu, C.G., Hayes, T.K., Strey, A., Bendena, W.G., Tobe, S.S., 1995. Identification and partial characterization of receptors for allatostatins in brain and corpora allata of the cockroach *Diploptera punctata* using a binding assay and photoaffinity labeling. *Regul. Peptides* 57, 347–358.
- Yu, S.J., Terriere, L.C., 1978. Metabolism of juvenile hormone I by microsomal oxidase, esterase and epoxide hydratase of *Musca domestica* and some comparisons with *Phormia regina* and *Sarcophaga bullata*. *Pestic. Biochem. Physiol.* 9, 237–246.
- Zera, A.J., Sanger, T., Hanes, J., Harshman, L., 2002. Purification and characterization of hemolymph juvenile hormone esterase from the cricket, *Gryllus assimilis*. *Arch. Insect Biochem. Physiol.* 49, 41–55.
- Zhou, B.H., Hiruma, K., Shinoda, T., Riddiford, L.M., 1998. Juvenile hormone prevents ecdysteroid-induced expression of broad complex RNAs in the epidermis of the tobacco hornworm, *Manduca sexta*. *Devel. Biol.* 203, 233–244.
- Zhou, C., Liu, B., 2001. Identification and characterization of a silk gland-related matrix association region in *Bombyx mori*. *Gene* 277, 139–144.
- Zhou, X.F., Riddiford, L.M., 2002. Broad specifies pupal development and mediates the “status quo” action of juvenile hormone on the pupal–adult transformation in *Drosophila* and *Manduca*. *Development* 129, 2259–2269.
- Ziese, S., Dorn, A., 2003. Embryonic integument and “molts” in *Manduca sexta*. *J. Morphol.* 255, 146–161.
- Zimowska, G., Rembold, H., Bayer, G., 1989. Juvenile hormone identification, titer, and degradation during the last larval stadium of *Spodoptera littoralis*. *Arch. Insect Biochem. Physiol.* 12, 1–14.
- Žitňan, D., Kingan, T.G., Kramer, S.J., Beckage, N.E., 1995. Accumulation of neuropeptides in the cerebral neurosecretory system of *Manduca sexta* larvae parasitized by the braconid wasp *Cotesia congregata*. *J. Comp. Neurol.* 356, 83–100.

9 Circadian Organization of the Endocrine System

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9.1. Introduction

The subject of this chapter has not been reviewed previously. In the 1985 edition of *Comprehensive Insect Physiology, Biochemistry, and Pharmacology*, circadian biology (Page, 1985) was not included in the two volumes on endocrinology. This fact illustrates the enormous extent of the changes in both fields that have taken place since that time.

Since 1985, endocrinologists have encountered increasingly complex patterns of regulatory interactions among insect hormones, the dissection of which has revealed circadian rhythms in the synthesis and hemolymph titer of numerous hormones. The quest to decipher the functional significance of these phenomena has drawn endocrinologists into the relatively unfamiliar territory of circadian biology (discussed by Steel and Vafopoulou, 2002). From this quest, a new level of understanding of the roles of hormones is emerging. Once regarded as agents that pulled switches that would activate or inhibit certain processes, hormones are increasingly recognized as agents that convey information about time (of day) to diverse cells and tissues that lack other access to such temporal information. Target cells use this information to organize their cellular activities into temporal sequences. Such internal temporal organization is a prerequisite for life; organisms maintained in continuous light (LL)

often fail to develop or reproduce, or else die prematurely (Pittendrigh, 1993). In addition, rhythms in hormones expose target cells throughout the insect to the same temporal information simultaneously, creating synchronization of the activities of cells in otherwise unconnected anatomical locations. These circadian rhythms are propelled by specialized regions of the insect (often, but not always, in the nervous system) known as circadian clocks, which endogenously generate rhythms of roughly 24 h periodicity and transmit this rhythmicity to other cells, primarily via nerves or hormones. Until 10 years ago, these clocks could be localized only by crude methods such as lesioning areas of tissue, which precluded analysis of both the cellular mechanism of timekeeping and its relationships with the endocrine system.

Even more profound revolutions have occurred since 1985 in the field of circadian biology. A comprehensive survey is given by Saunders (2002). A number of genes has been cloned, initially from *Drosophila melanogaster*, which seem to have no role other than to participate in molecular oscillations that generate circadian rhythms. These genes are known as clock genes, of which *period* (*per*) and *timeless* (*tim*) are the best known. These molecular oscillators appear to be functional only in a limited number of groups of cells in the insect. The ease

with which clock gene transcripts and their proteins can be detected and quantified has resulted in the localization of circadian clocks to identified cells and in the discovery of many novel loci of circadian clock gene expression. Thus, endocrinologists (among others) have been given a powerful set of new tools with which to unravel the relations between circadian clocks and the endocrine system.

The confluence of endocrinology and circadian biology is especially clear in the mechanisms that regulate development and reproduction. Both of these processes involve intricate hormonal controls and both are intimately linked with environmental signals that govern their timing. The nature, significance, and implications of clock control over these sets of hormones comprise the bulk of this chapter. This is relatively a new subject and leaves greater room for speculation and suggestions for future research than the reader may encounter in other chapters in this volume. The confluence of the two fields is most visible at the cellular level, where recent evidence reveals the close physical association between clock cells and endocrine cells in several anatomical locations. But hormones are not only the controlled output of clocks; several hormones are now known to act on clocks, where they regulate critical features of rhythmicity such as phase. Such findings are leading to the recognition that hormones also function to couple circadian clocks together, so that they function as a coordinated timing system which orchestrates the myriad events that occur in an insect every day.

9.2. Clocks and Oscillators: An Introduction for Endocrinologists

The later sections of this chapter describe evidence of the presence of circadian clocks and oscillators in numerous organs and tissues in insects. A variety of different methods has been employed to obtain this evidence. Therefore, it is important to keep in mind the virtues and limitations of the various methods. These are discussed briefly here. Definitive proof that particular cells comprise a circadian clock cannot be derived from any one of these alone, a combination of approaches being needed. The most convincingly documented clocks, such as the optic lobe clock that controls the locomotor rhythm (Page, 1985; Helfrich-Förster *et al.*, 1998; Helfrich-Förster, 2003), have been dissected by all these methods. As with all clocks, the locomotor clock may control various overt rhythms, not merely the rhythm that was used to identify it (see Section 9.3.1).

All efforts to localize clocks must be preceded by a careful analysis of the characteristics of the overt rhythm being studied (e.g., locomotion) under a variety of lighting conditions. Such studies are sometimes referred to as revealing the “formal properties” of the rhythm. Essential information includes the free-running characteristics of the rhythm (in both continuous dark (DD) and continuous light (LL) and measurement of the free-running period lengths. This information is necessary in order to detect perturbations of these characteristics in later experiments. A uniform sequence of experimental steps has then been followed for many rhythms. The traditional first steps to clock localization involve ablation and transplantation of candidate structures. Elimination of photoreceptors (e.g., eyes, ocelli, brain) will lead to a free-running rhythm similar to that seen in DD. The removal of certain structures may lead to arrhythmicity (i.e., loss of rhythmicity in the phenomenon, not loss of the phenomenon). Arrhythmicity can be produced by removal of the clock itself, but will also be produced if the clock is disconnected from the outputs that it controls. For example, removal of the subesophageal ganglion (SEG) leads to arrhythmic locomotion not because the clock is located in the SEG, but because the optic lobe clock controls the locomotor centers in the thoracic ganglia by nerve pathways that run through the SEG. Consequently, several structures will be found whose removal causes arrhythmicity in a phenomenon. The clock is usually assigned tentatively to the site that is anatomically closest to the photoreceptors. This tissue is then examined *in vitro* for its ability to generate circadian rhythmicity of nervous activity, hormone release, etc. When an endocrine output is suspected, the structure is implanted into arrhythmic hosts and the restoration of rhythmicity is examined. The fact that such experiments often work, illustrates that circadian clocks and endocrine cells are frequently located close to each other in the same piece of tissue (see Section 9.3.2). Since this sequence of experimental steps relies on perturbations of an overt rhythm, it is clear that the clock identified has an output that communicates temporal information to other parts of the organism. These clocks are therefore true clocks in the sense that they not only keep time but they communicate timing to other cells. A nervous output is traditionally associated with the control of local, behavioral, rhythms and an endocrine output with more widespread effects in diverse cells and tissues. Consequently, clocks that control hormones are able to exert the most profound and widespread

effects, making such clocks the most important (“master clocks”) in the organism. Thus, hormones represent a key controlled output of master clocks (see Section 9.3.2).

The elucidation of clock genes and molecular oscillators in *Drosophila* has both revolutionized and complicated the methods for localization of clocks. Studies of the expression of the clock genes (mainly *per* and *tim*) and of their coded proteins have resulted in the description of numerous cellular loci that are potential cellular clocks. In situations where traditional techniques have shown that a circadian clock is contained in a particular tissue, the presence of cells within it that exhibit cycling of clock gene products provides compelling evidence that these are the cells within the tissue that are responsible for the generation of rhythmicity, i.e., the “clock cells” in the tissue. Indeed, the molecular oscillator was unraveled in *Drosophila* primarily in the context of the cells in the optic lobe that express clock genes. In addition, molecular oscillators have been found in a number of sites, both within the central nervous system (CNS) and in other tissues, the functional significance of which has not been studied. Consequently, it is not yet known whether or not such cells do in fact convey information about time to other cells. Since communication of temporal information to other cells is an essential feature of true circadian clocks, it is often unclear whether these cells are functional clocks. The functions of these novel loci of clock cells and the mechanisms by which they are coordinated with one another represent important new areas of circadian biology.

It is important to note that the models of the molecular oscillator require that the clock genes are transcribed with circadian periodicity, resulting in circadian changes in the levels of clock gene mRNA and the resulting clock proteins (PER, TIM, etc.) in the cell. These proteins act (indirectly) as transcription regulators that move into the nucleus with circadian periodicity. Consequently, cells that express clock genes are candidate clock cells only if the mRNA and/or protein levels show circadian cycling. Specifically, it should be demonstrated that these levels free-run in DD, and that clock protein migration into the nucleus also occurs with a rhythmicity that free-runs in DD. These criteria must all be fulfilled by true clock cells. These clock cells are normally found in groups of ten or more cells, and the cells are coupled together by gap junctions and/or common synaptic or hormonal inputs (examples in sections below). These features suggest that a single clock cell may not have

all the properties of a complete circadian clock. The only known case of a single cell that has all the properties of a clock is the chick pinealocyte, which is photosensitive and generates a rhythmic output of melatonin *in vitro* (Pickard and Tang, 1994).

In some cells that show daily cycling of clock gene expression and clock protein abundance, these rhythms cease in DD. The failure of rhythmicity to free-run in such cases is evidence that it is not generated endogenously within the cells that express the clock genes. Rhythmicity in such cells must be driven, usually by rhythmic inputs from hormones or nerves. Thus, hormones play an important role as inputs that drive rhythmicity in oscillators of this type (see Section 9.3.3). These cellular oscillators are not clock cells, because their rhythmicity is driven by other cells. In other words, true clocks are responsible for generating the driving inputs to these cellular oscillators. This arrangement leads to the notion that oscillators are organized into a hierarchy, with master clocks driving rhythmicity in other oscillators via hormones or nerves. Driven oscillators are found in the CNS as well as in peripheral tissues and true clocks are found in peripheral tissues as well as the CNS. Examples are found throughout the sections below. In the context of hormones, any feedback loop can become an oscillator (i.e., become rhythmic) if it receives rhythmic driving input from a true clock. Removal of the clock input will cause such oscillations to cease. If oscillations continue after removal of the known clock inputs, the presence of unidentified clocks within the feedback loop is implied.

In yet other cells, the clock genes and their products may show no cycling, even in L:D. Such cells presumably have no molecular oscillators and therefore have no role in timekeeping. That clock genes may be expressed in cells with no role in timekeeping has raised the possibility that clock proteins may be multifunctional and have roles in cells other than as components of the molecular oscillator. This notion gains support from the fact that PER and TIM are normally found in cells in great abundance, at levels comparable to those of secretory proteins. Such high levels would be quite unnecessary for their roles as transcriptional regulators. Further, PER and TIM proteins may be found at great distances from the nucleus, such as along the length of axons (Sauman and Reppert, 1996a). Functions of clock genes in noncircadian phenomena include cocaine sensitization (Andretic *et al.*, 1999) and the ultradian male courtship rhythm of *Drosophila* (Hall, 2002, 2003).

9.3. Brain Clocks and the Endocrine System

There are two distinct areas in the insect brain that are well documented as centers of circadian timekeeping. One is located in the optic lobe and has been studied in depth; the second is located in the protocerebrum (Figure 1). Both of these areas have been elucidated using the adult locomotor rhythm as the monitored output rhythm and are frequently referred to as locomotor clocks. This terminology does not imply that no other rhythmic phenomena are controlled by these areas, but rather that other outputs have received little experimental attention. Recent studies of the neuroarchitecture of these clocks, summarized below, reveal a complex

association between the clock cells and cerebral neuropeptides and reveal pathways by which these clock areas, either separately or in cooperation with each other, control the rhythmic release of various hormones.

The structure, physiology, and molecular biology of these clocks has been reviewed extensively (Helfrich-Förster *et al.*, 1998; Hall, 2003) but always in the context of behavioral rhythms. The treatment below differs significantly from previous reviews because it is focused on the relevance of this information to endocrinologists and the control of endocrine rhythms. Two central conclusions emerge. First, these clocks function to control rhythmicity in both behavioral and endocrine rhythms and constitute a key component of the circadian system in insects, with similarities to the suprachiasmatic nucleus in mammals. Second, hormones perform at least two functions in the circadian system; they are key controlled outputs of clocks which convey temporal signals to distant tissues through the hemolymph, but they also participate in the regulation of clocks (e.g., control of phase of the output) and consequently are components of the circadian timekeeping system itself.

9.3.1. Optic Lobe Clock and Hormones

The optic lobe has been the subject of a comprehensive series of experiments employing the classical techniques of ablation, transplantation, and lesions in several species of cockroach (reviews: Page, 1985, 1988). The optic lobe of *Leucophaea maderae* isolated *in vitro* generates a circadian rhythm of compound action potentials that free-runs in DD (Colwell and Page, 1990). When the optic lobe is transplanted into a cockroach previously rendered behaviorally arrhythmic by bilateral lobectomy, locomotion gradually becomes rhythmic as the transplanted optic lobe develops neural connections with the host brain (Page, 1983). The location of this circadian locomotor clock within the optic lobe started to become apparent when an antiserum to crustacean pigment dispersing hormone (PDH) was employed in immunohistochemical investigations of the brain (Homborg *et al.*, 1991a, 1991b; Nässel *et al.*, 1991). The PDH-immunoreactive perikarya were located between the neuropiles of the medulla and the lobula, adjacent to the accessory medulla (a proximal appendix of the medulla). Immunoreactivity was present throughout the axons, enabling the projections to be traced. The cells possess one projection to the protocerebrum, a second that ramifies in the medulla, and commissures connecting to the contralateral cells (Figure 2). On anatomical

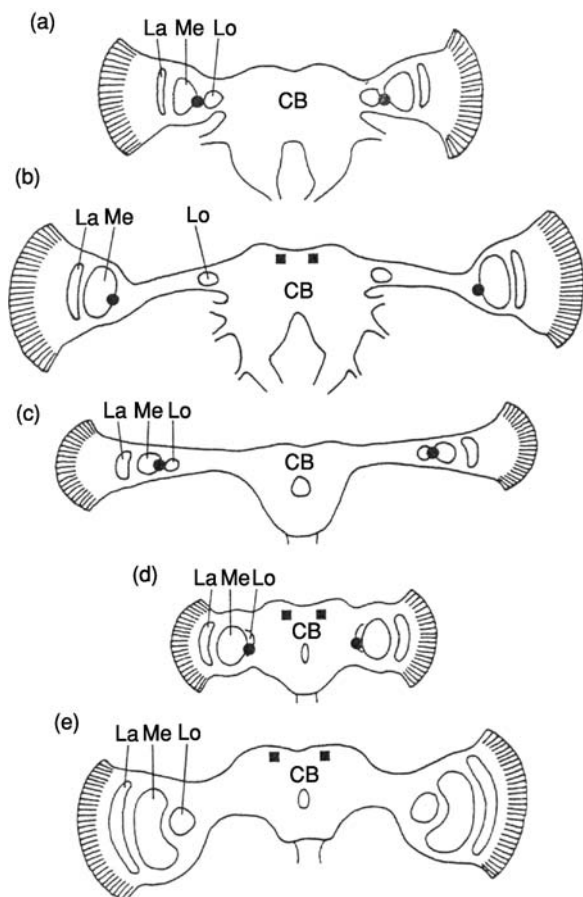


Figure 1 Diagrams of the brains of several insects, indicating the presence and location of the optic lobe clock (filled circles) and protocerebral clock (filled squares). (a) Cockroach; (b) cricket; (c) beetle; (d) fly; and (e) moth. The optic lobe neuropiles are lamina (La), medulla (Me), and lobula (Lo). Optic lobe clock neurons near the accessory medulla project to the protocerebrum in the central brain (CB) and distally to the medulla (see text). (Reproduced from Helfrich-Förster, C., Stengl, M., Homborg, U., 1998. Organization of the circadian system in insects. *Chronobiol. Internat.* 15, 567–597.)

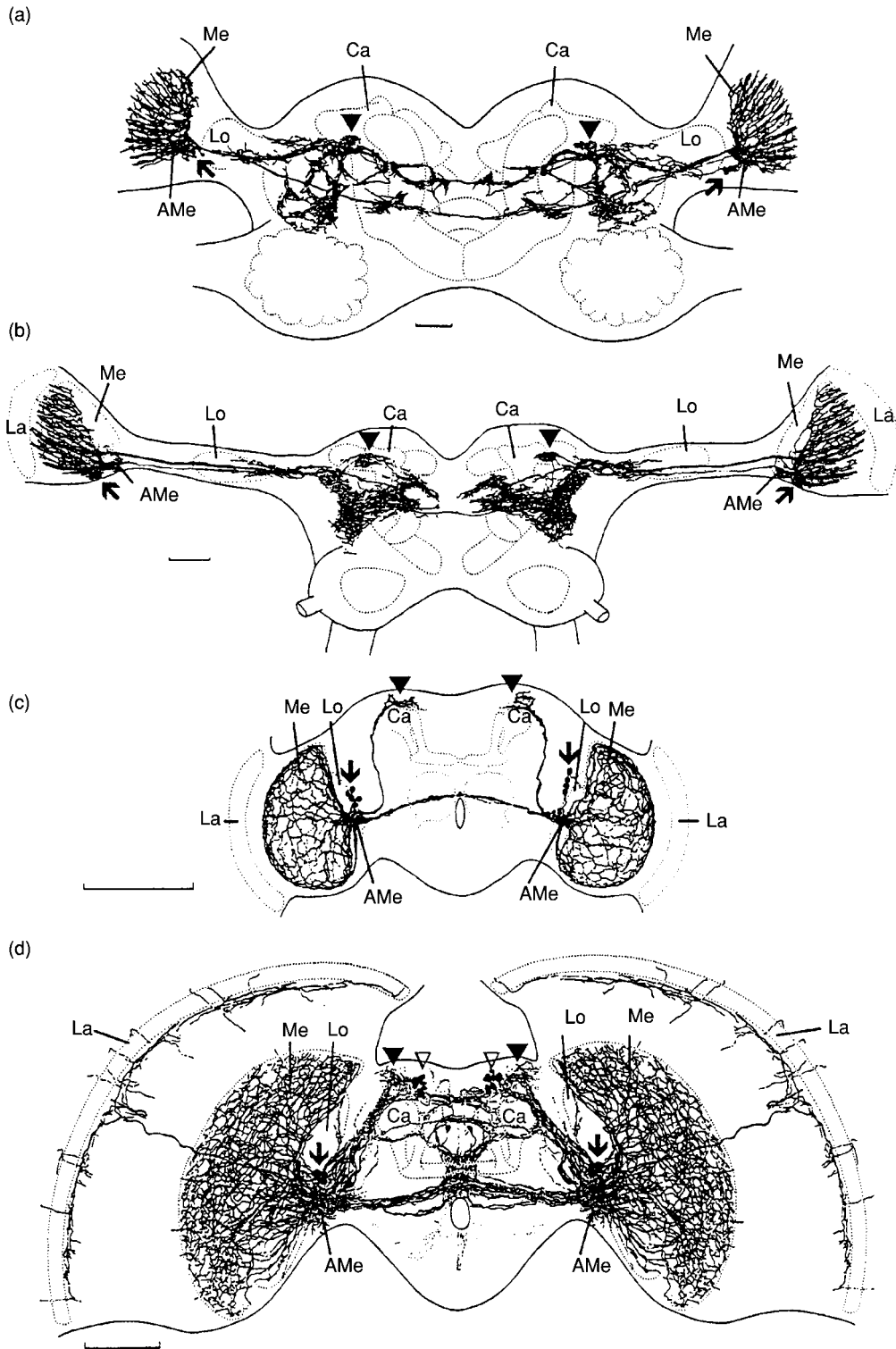


Figure 2 Axonal pathways of the presumed clock neurons of the optic lobe as revealed by pigment dispersing factor (PDF) immunoreactivity. (a) The cockroach *Leucophaea maderae*; (b) the cricket *Teleogryllus commodus*; (c) *Drosophila melanogaster*; (d) the blowfly *Phormia terraenovae*. Arrows point to the perikarya located adjacent to the accessory medulla (AMe). In all species, extensive ramifications of axons occur in the medulla (Me) and central projections occur to an area of the protocerebrum (filled arrowheads) just anterior to the calyces (Ca) of the corpora pedunculata (outlined with dots). Commissures connecting contralateral sets of neurons are also visible. In *P. terraenovae* (d), additional PDF-reactive neurons are found in the protocerebrum (open arrowheads), which project to the corpus cardiacum (not visible in the figure). Scale bar = 200 μ m. (Reproduced from Helfrich-Förster, C., Stengl, M., Homberg, U., 1998. Organization of the circadian system in insects. *Chronobiol. Internat.* 15, 567–597.)

grounds, these neurons are well suited to receive photic input from the eye and to relay it to the central brain (Stengl and Homberg, 1994). The position of these cells corresponded closely to the region of the optic lobe to which the clock had been previously assigned using microlesions (Sokolove, 1975). Following knife cuts between the lobula and protocerebrum, cockroaches regained behavioral rhythmicity in synchrony with regeneration of the PDH-reactive fibers (Stengl and Homberg, 1994). Ablation and transplantation of the accessory medulla confirmed that this small region of the optic lobe contained a clock (Reischig and Stengl, 1997). A similar pattern of branching of PDH-reactive neurons is seen in diverse insect species (Sehadová *et al.*, 2003; Figure 2). Orthologs of PDH are found in insects and are termed pigment dispersing factors (PDFs) (Rao and Riehm, 1993). An intimate association between neuropeptide(s) and circadian clocks was thus established.

Concomitant studies of *per* expression in *Drosophila* had identified two major sites of *per* cycling in the adult brain (Ewer *et al.*, 1992; Frisch *et al.*, 1994). These sites were dubbed the “lateral neurons,” located at the junction of protocerebrum and optic lobes, and the “dorsal neurons,” located above the corpora pedunculata in the protocerebrum. These are the only sites of cycling *per* expression in the brain that are present from the first instar onwards (Kaneko *et al.*, 1997). Critically, the PDF-reactive neurons expressed *per* (Helfrich-Förster, 1995, 1997), showing that these neuropeptide-containing cells were indeed clock cells. But PDF-reactivity was not seen in *all* of the lateral neurons (only in a “subset”) and was absent from all other cells that expressed *per*, including the dorsal neurons. Therefore, no universal role of PDF in clock cells can be expected. Together, these studies show that the optic lobe clock consists of a small group of neurons in which *per* expression cycles. Most, but not all, of these cells coexpress PDF, which enables the axons to be traced both distally towards the eyes and centrally into the protocerebrum. These cells are commonly associated with the accessory medulla.

The accessory medulla contains a variety of neuropeptides other than PDF, including allatostatins, FMRFamide, gastrin/cholecystokinin, leucokinin I, and corazonin (Petri *et al.*, 1995). Local injection close to the accessory medulla of PDF, allatotropin, serotonin and γ -aminobutyric acid (GABA) all induce phase shifts (Petri and Stengl, 1997; Petri *et al.*, 2002; Saifullah and Tomioka, 2002, 2003) (see Figure 20). These recent findings imply that the optic lobe clock is subject to a sophisticated array

of neurochemical manipulations. These seem to act primarily to adjust the phase of the rhythm generated by the clock cells and may either reinforce or contradict the photic information received from the environment. The complexity of organization of the optic lobe clock invites the expectation that it is not solely involved with the locomotor rhythm.

Several points of contact between the PDF-expressing lateral neurons and the neuroendocrine system in the head of adult *Drosophila* were revealed by Siegmund and Korge (2001) using a GAL4 enhancer trap system. The clock cells (sPDFMe cells in Figure 3) form synaptic connections in the protocerebrum with two large neurosecretory cells that terminate on the corpus allatum (CA) cells of the ring gland (Figure 3). At least one of these cells was thought to contain a *Manduca*-allatostatin-like peptide. These connections provide a structural pathway for circadian control of the CA (see Section 9.5.1). The clock cells follow the axons of two other neurosecretory cells for about 70 μ m and seemed to directly contact their collaterals. These cells were very similar in size, position, and morphology to the prothoracicotropic hormone (PTTH) cells of *Bombyx mori* (Mizoguchi *et al.*, 1990) and *Manduca sexta* (Westbrook and Bollenbacher, 1990) and were presumed to contain the PTTH of *Drosophila* (see Chapter 6). Thus, a structural pathway for circadian regulation of PTTH was postulated. A close association between clock cells and PTTH cells has been described in many species (see Section 9.4.1.2) but this association is more commonly with clock cells in the protocerebrum than in the optic lobe. An interesting feature of these cells in *Drosophila* was that the neurosecretory axons formed terminal arborizations within the prothoracic gland (PG) region of the ring gland. This arrangement suggested that *Drosophila* PTTH was delivered directly to the PG cells and might not enter the hemolymph as a classical hormone.

9.3.2. Protocerebral Clock and Hormones

The picture that is emerging from recent studies is that the optic lobe and protocerebral clocks are not the distinct entities assumed in the earlier literature. The presence of a circadian clock in the protocerebrum of various insects has been inferred from traditional lesion experiments and is only now receiving analysis at the level of neuroarchitecture and molecular biology. Some species appear to have both optic lobe and protocerebral clocks, while other species seem to have one but not the other (see Figure 1). But the absence of evidence is not evidence of absence; for example, *per/tim* expressing

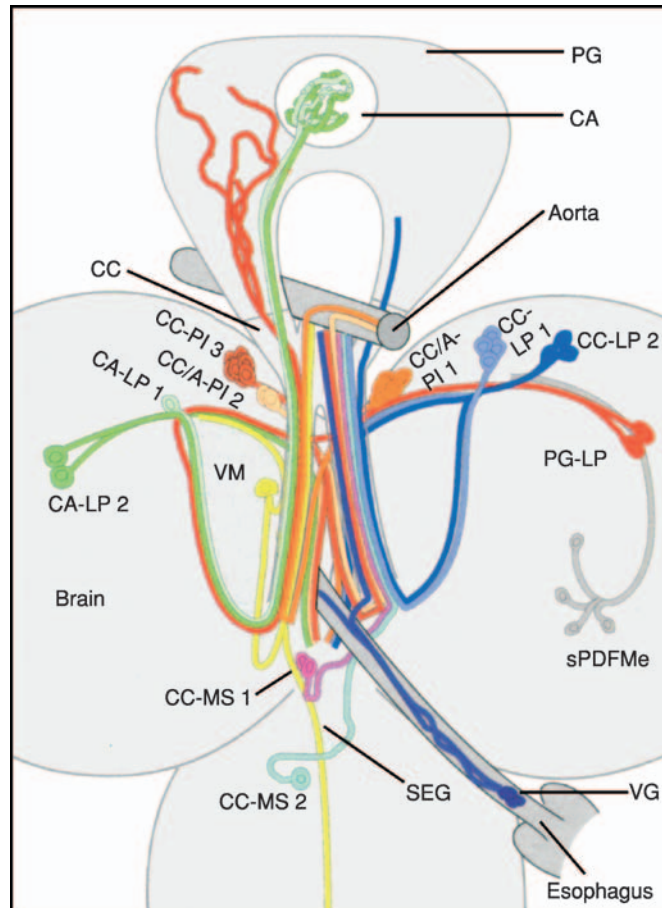


Figure 3 Axon pathways in the brain of *Drosophila melanogaster* that connect the lateral neurons of the optic lobe (sPDFMe) with the cerebral neuroendocrine system. In this diagram, the subesophageal ganglion is depicted in the lower part (SEG) and the ring gland in the upper part. CA is the corpus allatum, CC the corpus cardiacum, and PG the prothoracic gland. Arborizations of the sPDFMe neurons overlap with those of the two PG-LP neurons that directly innervate the PG and probably represent the PTTH neurons (see text). The CA is innervated by three neurons arranged in two groups (CA-LP1 and CA-LP2) and thought to contain allatostatin-like peptide, one of which passes in close proximity to the terminals of the sPDFMe neurons. The CC is innervated from the CC-LP1 and CC/A-PI 1, 2, and 3 neurons, which may receive input from the dorsal group of clock neurons (not shown here; see **Figure 4**). VM neurons are immunoreactive for eclosion hormone. (Siegmund T., Korge G., 2001. Innervation of the ring gland of *Drosophila*. *J. Comp. Neurol.* 431, 481–491; © Wiley. Reprinted by permission of Wiley-Liss Inc., a subsidiary of John Wiley & Sons, Inc.)

neurons close to the accessory medulla of *Manduca* (not shown in **Figure 1e**) have been recently described (Wise *et al.*, 2002). In crickets and flies (**Figure 1b** and **d**) the failure of optic lobe lesions to completely abolish the locomotor rhythm has been ascribed to the presence of a protocerebral clock. In saturniid moths, the optic lobes were entirely unnecessary for rhythmic locomotion (Truman, 1974), which was found to be regulated by a protocerebral clock entrained by extraocular photoreception. Similarly, the eclosion of silkmoths (see Section 9.4.3) was also found to be controlled in this way (Truman and Riddiford, 1970; Truman, 1972b). The release of PTTH that terminates pupal diapause is regulated by a photoperiodic clock in the protocerebrum (Bowen *et al.*, 1984). A close association between

the PTTH cells and “clock cells” in the protocerebrum has been described by Sauman and Reppert (1996b) in *Antheraea pernyi* and by Terry and Steel (2004) in *Rhodnius prolixus* (see Section 9.4.1.2).

Studies of *per* expression in the brain of adult *Drosophila* revealed two groups (of two and 15 cells) of dorsal neurons in the protocerebrum (Ewer *et al.*, 1992). These cells do not express PDF and consequently their connections could not be traced by immunohistochemical methods and the cells were not accessible to targeted molecular lesioning in order to study their functions. Consequently, much less is known of these cells than the lateral neurons. Important information regarding the neuroarchitecture of clock cells in the *Drosophila* brain

was obtained using a GAL4 expression system (Kaneko and Hall, 2000). A third group of dorsal neurons (about 40 cells) was revealed (Figure 4), together with interconnections between the various dorsal and lateral neurons. The lateral neurons of the optic lobe clock (see above) were found to project to terminals in the dorsal protocerebrum, as

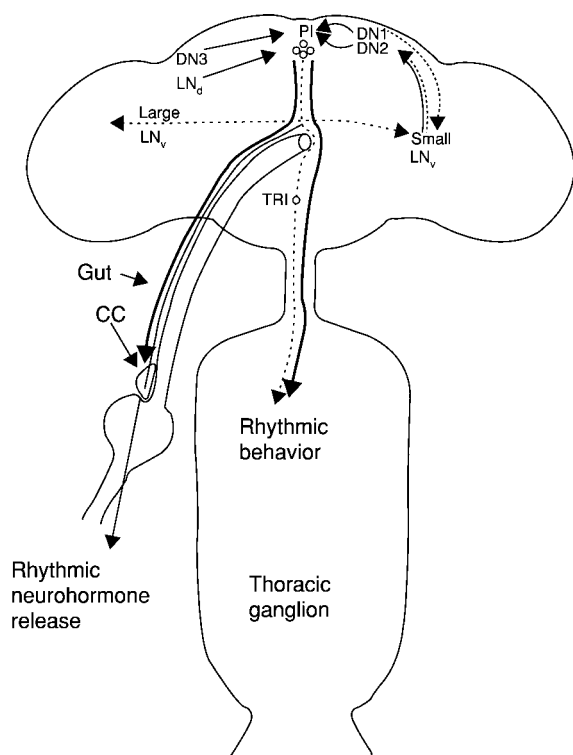


Figure 4 Deduced patterns of interactions between clock neurons and neurosecretory cells in adult *Drosophila melanogaster* and their probable connections with neuroendocrine cells of the pars intercerebralis. These connections may drive the rhythms in neurohormone release reported for other insects (see text). The LN_v (small and large) comprise the putative optic lobe clock of Figure 1. The large cells give rise to the arborizations in the medulla and the commissures between the hemispheres (see Figure 2). The small cells connect with the dorsal neurons (DN1, DN2) and receive reciprocal innervation from the DN1. All three groups of dorsal neurons (DN1, DN2, DN3) innervate the pars intercerebralis where we suggest they drive rhythmic activity in the neurosecretory cells and therefore, rhythmic neurohormone release from the corpus cardiacum (CC). The dorsal lateral neurons (LN_d) may also contribute to this input. Thus, clock cells throughout the brain converge on neurosecretory cells. Rhythmic locomotor behavior is also driven through (unspecified) neurons in the protocerebrum that project to the thoracic ganglion. (Modified with permission from a diagram of the clock neurons that control behavior in *Drosophila* by Kaneko, M., Hall, J.C., 2000. Neuroanatomy of cells expressing clock genes in *Drosophila*: transgenic manipulation of the *period* and *timeless* genes to mark the perikarya of circadian pacemaker neurons and their projections. *J. Comp. Neurol.* 422, 66–94.)

previously found using PDF-immunoreactivity for tracing. These endings appeared to be connections with the dorsal neurons, some of which sent reciprocal processes back to the lateral neurons. Therefore, the protocerebral and optic lobe clocks are not distinct entities, but rather components of an integrated mosaic of clock cells in the brain.

All three groups of dorsal neurons appeared to terminate close to the neurosecretory cells in the pars intercerebralis. Kaneko and Hall (2000) did not consider the relevance of their data to the neuroendocrine system. We interpret their evidence as indicating that the medial neurosecretory cells receive direct circadian input from the “dorsal neurons.” The dorsal neurons are neurally coupled with the lateral neurons of the optic lobe. Therefore, the lateral neurons provide an indirect input to the neurosecretory cells, via the dorsal neurons (Figure 4). In some of the dorsal neurons, *per* expression cycles in antiphase to other dorsal neurons (and to the lateral neurons) (Kaneko *et al.*, 1997), raising the intriguing prospect that the rhythms (in hormones?) driven by this system of clock cells may not all have the same phase. The data of Siegmund and Korge (2001) (see Section 9.3.1) indicate that some of the lateral neurons may bypass the dorsal neurons and make direct synaptic contact with neurosecretory cells. Thus, the neurosecretory cells appear to receive circadian input from all the major groups of “clock cells” in the brain. Therefore, we infer that most, if not all, of the neurosecretory cells would express rhythmicity.

In conclusion, the anatomically separate groups of clock cells in the brain appear to be interconnected. The nature of these interconnections may vary between species and thereby account for apparent species variations in the location and properties of the clock controlling a particular rhythm. The network of clock cells regulates rhythmicity in both behaviors and the endocrine system. As such, it is able to influence, if not drive, rhythmicity throughout the organism. It probably represents a master clock that is analogous to the suprachiasmatic nucleus (SCN) of mammals. The SCN is known to be influenced by an array of inputs, including inputs from other oscillators such as the ocular retinae, pineal gland, and parietal eye. The sections below illustrate that there is accumulating evidence that oscillators outside the brain provide input to the timing system in the brain of insects, which raises the prospect that the insect circadian system will also be comprised of multiple coupled oscillators in several anatomical locations, which communicate with each other primarily by hormones.

9.3.3. Peripheral Oscillators and Hormones

A variety of cells and tissues has been identified that display circadian cycling of a physiological process and/or clock gene expression. Some of these loci are able to generate rhythmicity *in vitro*, free-run in aperiodic conditions, and transmit a rhythmic output to other cells; such cells are therefore true clocks. Three such peripheral clocks have been described. The most fully characterized of these is the PG clock of *Rhodnius* (see Section 9.4.1.3) that generates rhythmic synthesis of ecdysteroids; PGs can be entrained by light *in vitro*, exhibit circadian cycling of PER, and communicate rhythmicity to ecdysteroid-sensitive cells via the rhythm of ecdysteroids in the hemolymph. In *Drosophila*, chemosensory hairs of the antennae exhibit cyclic expression of *per* (Krishnan *et al.*, 1999) (Figure 5) and this cycling is necessary for the circadian rhythm of olfactory responsiveness. In several moths, rhythmic movement of sperm along the duct system is regulated by a circadian clock, but its location is unclear (see Section 9.5.2.2); *per* expression occurs in part of the duct epithelium but this may not be the site of the clock that controls sperm movement.

A number of other loci have been described for which the criteria of a true clock have either not been met or have not been examined. These loci are called peripheral oscillators, not clocks. In *Drosophila*, Plautz *et al.* (1997) used a luciferase reporter driven from the promoter of *per* to identify oscillatory structures. Numerous epidermal structures exhibited a rhythmic glow. Some of these rhythms free-ran in DD and could be reset by light

in pieces of the insect (e.g., a leg). The significance of these oscillators remains obscure since no output from them is known. Hege *et al.* (1997) obtained similar data for Malpighian tubules. Giebultowicz *et al.* (2000) found that tubules that were transplanted into another insect appeared to lose light sensitivity and were unable to respond to humoral signals from the host. The data are consistent with a requirement for entrainment via intact connections between the tubules and the rest of the animal. It is not known whether the tubule rhythm has a functional significance nor whether rhythmicity can be transmitted to other cell types.

In order to determine whether such peripheral oscillators possess clock properties, it will be necessary to employ *in vitro* techniques and eventually to study cell lines for timekeeping properties. These approaches are extensively used in mammalian studies. For example, SCN cell lines produce a wide array of signaling molecules (Hurst *et al.*, 2002) and can communicate rhythmicity to other cells (Allen *et al.*, 2001), confirming the central importance of the SCN cells in circadian timekeeping. Fibroblasts (Yagita *et al.*, 2001) and smooth muscle cells (Nonaka *et al.*, 2001) are able to express the same clock genes as SCN cells and do so with circadian periodicity, but expression of these genes is both induced (Nonaka *et al.*, 2001) and phase-set (McNamara *et al.*, 2001) by hormones. The induced rhythmicity in these cells is not transmitted to other cells. In mammals, peripheral oscillators exhibit circadian expression of clock genes, but this rhythmicity is driven by external factors and is not transmitted to other cells. Such oscillators are not clocks (Allen *et al.*, 2001). The

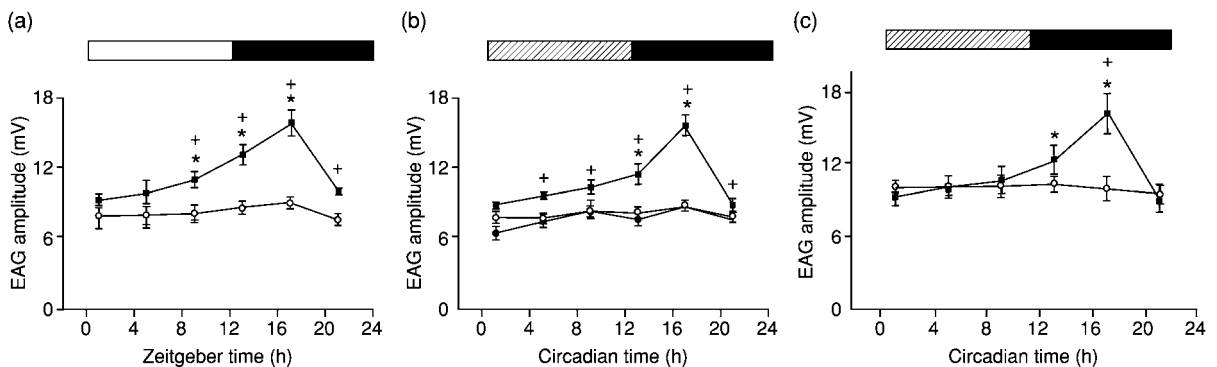


Figure 5 The circadian rhythm of olfactory responsiveness in *Drosophila melanogaster* as seen in electroantennogram records (EAG). Dark portions of horizontal bars indicate scotophase in (a) and subjective scotophase during continuous darkness in (b) and (c); light portions of the bar are photophase in (a), whereas subjective photophase in (b) and (c) is shown as a gray bar. Filled squares in (a), (b), and (c) are wild-type flies that show a rhythm in EAG amplitude that free-runs in DD ((b) and (c)). Flies with null mutations of clock genes (*per*⁰, open circles; *tim*⁰, filled circles) show no rhythmicity. Open squares in panel (c) depict a transgenic *per* strain (7.2.2) in which the peripheral *per* oscillator fails to function. (Reprinted with permission from Nature (Krishnan, B., Dryer, S.E., Hardin, P.E., 1999. Circadian rhythms in olfactory responses of *Drosophila melanogaster*. *Nature* 400, 375–378); © Macmillan Publishers Ltd.)

most common entraining signals to such oscillators are hormones (Herzog and Tosini, 2001; McNamara *et al.*, 2001; Balsalobre, 2002). In insects, the roles of hormones as entraining agents to peripheral oscillators have not yet been studied. This will be a fruitful new area for insect endocrinologists.

In conclusion, insects possess numerous peripheral oscillators, but their properties and significance in all but a few cases have yet to be studied. Related research with mammals creates the expectation that hormones will emerge as the major entraining signals to such oscillators. In insects, a number of peripheral oscillators are photosensitive (Giebultowicz, 2000), but this does not reduce the potential importance of hormones as time signals. A striking example is provided by the PG clock of *Rhodnius*, which is entrainable both by light and by PTTH (see Section 9.4.1.3). Indeed, it has been argued that the known diversity of rhythms in any organism requires complex communication pathways between central timing systems in the brain and tissue-specific peripheral oscillators (Ikonomov *et al.*, 1998; Gillette and Mitchell, 2002; Kalsbeek and Buijs, 2002). Such communication is most readily achieved by hormones.

9.4. Developmental Processes and Hormones

9.4.1. Circadian Regulation of Developmental Hormones

There is evidence that all of the hormones involved in the regulation of development are under circadian control. The literature relating to PTTH, ecdysteroids, the juvenile hormones (JHs), and melatonin is presented below. The circadian regulation of the hormones that control ecdysis behavior is also reviewed as this is a behavior that is under circadian control which is also closely integrated with development. We suspect that it will be found that all of these hormones are integrated together under the control of a circadian system that governs the temporal organization of developmental events. This notion is implied by the pervasive nature of circadian control mechanisms at all levels of development and is supported by various lines of evidence presented below. The literature has yet to integrate these lines of evidence into a comprehensive regulatory scheme.

9.4.1.1. Gated head critical periods One of the oldest observations in the entire field of insect endocrinology was that decapitation resulted in an

arrest of the molting process if performed early in the molt, whereas decapitation later had no effect (Kopeck, 1922). The period of time over which this independence of molting from the head was achieved in a population of insects became known as the head critical period (HCP). The acquisition of head independence was believed to be quite abrupt in individuals, i.e., the HCP did not occur in individual insects, only in populations. To this day, the events that give rise to the HCP are not understood. Initially, it was surmised that release of PTTH gradually activated the PGs and then its release ceased at the HCP once activation was completed (Wigglesworth, 1934). This view prevailed for decades, despite contradictory findings. For example, it was found in *Rhodnius* that brains of insects prior to the HCP (presumably releasing PTTH) were unable to promote molting when implanted into headless hosts (Wigglesworth, 1934). It was later found (Wigglesworth, 1940) that this brain implantation experiment would only promote molting if the brains were taken from animals that had *passed* the HCP. This experiment clearly showed that PTTH release did not cease at the HCP (as later studies confirmed) (see Section 9.4.1.2), but failed to revise the understanding of the significance of the HCP.

Much later, neck ligation experiments showed that the HCP in a population of *Manduca* larvae was gated (Truman, 1972a). This finding was the first experimental evidence that a developmental event in insects was subject to circadian timing. In these experiments, the HCP was assumed to represent the length of time during which release of PTTH occurred (in a population?). In 1972, there was no reliable assay for PTTH with which to test this assumption. It was concluded that the time of PTTH release was controlled by a circadian clock. Several other visible events (prodroma) that occur during the molt to the pupal stage were also found to be gated, including evacuation of the gut when feeding ceases (“gut purge”) and the onset of “wandering” behavior (Truman and Riddiford, 1974). Together, these findings demonstrate that a number of events during molting in *Manduca* are subject to circadian timing and laid a foundation for later studies of the circadian organization of development (see sections below). However, numerous subsequent studies, outlined below, clearly refute the notion that the HCP corresponds to the time of PTTH release.

During the 1980s, doubts were raised about the adequacy of neck ligation experiments for identifying times of release of PTTH (see Chapter 6). Using *Samia cynthia*, Fujishita and Ishizaki (1981) found that the HCP for larval molting could be

phase-shifted and that gating of the HCP persisted in DD and was lost in LL. The HCP for gut purge at the pupal molt was also responsive to phase shifts (Fujishita and Ishizaki, 1982). These findings demonstrated clearly that the gating reported earlier in *Manduca* was indeed subject to formal circadian control. However, Fujishita and Ishizaki (1982) were not convinced that the HCP represented the time of PTTH release and observed that release could begin before the HCP and/or continue after it. Sakurai (1984) made similar observations using *Bombyx* and noted that if release of PTTH took place outside the HCP, traditional neck ligation experiments would be unable to detect it. Overall, these studies showed that the HCP was a manifestation of a circadian control mechanism governing many aspects of development, but its underlying nature remained elusive. Clearly, techniques that quantified the hormones involved were required.

A bioassay for PTTH was employed by Bollenbacher *et al.* (1987), who reported PTTH in the hemolymph of fourth instar *Manduca* during the scotophase of the HCP for gate one larvae. The absence of PTTH from the hemolymph during both the preceding and the subsequent photophases supported the notion of gated PTTH release during the HCP. But no data were obtained for the adjacent scotophases, so it was not known if this release was repeated. The release of PTTH from brains of *Bombyx* was examined every 12 h for all 12 days of the fifth instar by Shirai *et al.* (1993). PTTH release was reported on all but three of the 24 time points studied, clearly showing that PTTH release was not confined to the HCP. Their published data show that PTTH release from the brain was higher during the scotophase than during the following photophase, suggesting that PTTH release occurs with a daily rhythm. However, Shirai *et al.* (1993) preferred to interpret their data as five separate periods of PTTH release over 12 days, not as a rhythm. In *Rhodnius*, PTTH is released throughout larval–adult development with a clear daily rhythm (Vafopoulou and Steel, 1996a). The work with *Rhodnius* is discussed in greater detail in the sections below. In the context of the HCP, it should be noted that the amplitude of the rhythm increased greatly *after* the HCP, showing that most of the PTTH released during development was unrelated to the HCP. This finding incidentally explains the originally paradoxical finding of Wigglesworth (1940) that brains of animals that had passed the HCP were more effective at inducing the molting of decapitated hosts than were brains of animals before the HCP. *Rhodnius* is one of the many insects in which the HCP is not gated (Knobloch and

Steel, 1987). A fluoroimmunoassay (FIA) for PTTH was employed by Dai *et al.* (1995) to examine PTTH release from brains of both fourth and fifth instar larvae of *Bombyx*. PTTH release was found on every day of both instars and again differences are visible in the data between photophase and scotophase. FIA revealed PTTH in the hemolymph on all these days and also throughout the pupal stage (Mizoguchi *et al.*, 2001, 2002). PTTH release for at least three consecutive days after the HCP was found in *Periplaneta americana* (Richter, 2001).

All these reports demonstrate clearly that release of PTTH is not confined to the HCP. Rather, PTTH is released throughout large portions, if not all, of an instar. Therefore, the notion that the HCP represents the time of PTTH release is not correct. This conclusion has been reached by Vafopoulou and Steel (1996a), Sakurai *et al.* (1998), and Mizoguchi *et al.* (2002), with each set of authors employing different pathways of reasoning. Sakurai *et al.* (1998) suggested that the HCP represents a time during which the PGs become more responsive to PTTH and the targets of ecdysteroids become more responsive. Mizoguchi *et al.* (2002) suggested that the HCP might be mediated by the innervation of the PGs found in moths. None of these possibilities requires any change in PTTH release at the HCP. It should be recalled that the HCP represents the time when PGs become able to maintain steroidogenesis without input from the head. As such, the HCP signals a change in the behavior of the PGs, rather than of the head. Despite the continuing enigmatic nature of the HCP, it must be emphasized that its definition and its gated nature (in certain species) have not been questioned. The HCP remains the original well-documented event in insect development that is under circadian control.

9.4.1.2. Prothoracicotropic hormone The timing and pattern of release of PTTH during development has been studied extensively in the blood-feeding bug *Rhodnius*. *Rhodnius* represents a particularly favorable experimental animal for the study of circadian phenomena in the endocrine system because of the remarkable precision it exhibits in the timing of developmental events following feeding. Many animals can be fed a large blood meal simultaneously and thus the growth and development of a whole population is synchronized with remarkable precision. This synchrony is maintained throughout development, probably because the insects do not feed after the initiation of development and will not feed until after ecdysis to the next instar. Feeding is known to affect developmental rates in other insects (Steel and Davey, 1985).

Further, the long length of the last larval instar (21 days) makes it possible to distinguish events that occur daily from those which occur on the longer timescale of development. The first clear evidence of a circadian rhythm in PTTH release was thus obtained in *Rhodnius*. *In vitro* incubation of *Rhodnius* brains and quantification of the amount of PTTH released by an *in vitro* bioassay (Vafopoulou *et al.*, 1996) revealed that release of PTTH occurred as a daily rhythm throughout most of larval–adult development (Vafopoulou and Steel, 1996a). Indeed, PTTH immunoreactive peptide was released with a daily rhythm as shown on dot blots (Vafopoulou and Steel, 2002). PTTH release was not confined to a short period of time at the initiation of development. The rhythm of release consisted of prominent maxima during each scotophase and minima during each photophase (Figure 6). The rhythm free-ran in DD for at least five cycles with a period length close to 24 h and was temperature compensated (Vafopoulou and Steel, 1996b); it is therefore controlled by an endogenous circadian clock. The rhythm also free-ran in LL for three to

four cycles, but with a period length somewhat shorter than that seen in DD. No rhythm was apparent after the fourth cycle in LL (Vafopoulou and Steel, 1996b). When animals were kept in LL for 30 days prior to sampling, rhythmicity in PTTH release was entirely lost (Vafopoulou and Steel, 2001); such “damping” of a circadian rhythm following chronic exposure to LL is a common phenomenon. Most interestingly, PTTH release in such animals was not just arrhythmic; it had ceased completely (Figure 7, left panels). When animals were transferred from LL to DD, the rhythmic release of PTTH was promptly reinitiated and continued to free-run in DD (Figure 7, right panels) (Vafopoulou and Steel, 2001). This clearly demonstrated that the circadian clock that regulates the rhythm of PTTH release is photosensitive. The rhythm of PTTH in the hemolymph of *Rhodnius* is synchronous with the daily rhythm of PTTH release from the brain (Figure 8a); the PTTH content of the brain also cycles during a day; it undergoes a sharp drop in the middle of each scotophase in synchrony with release of PTTH into the hemolymph

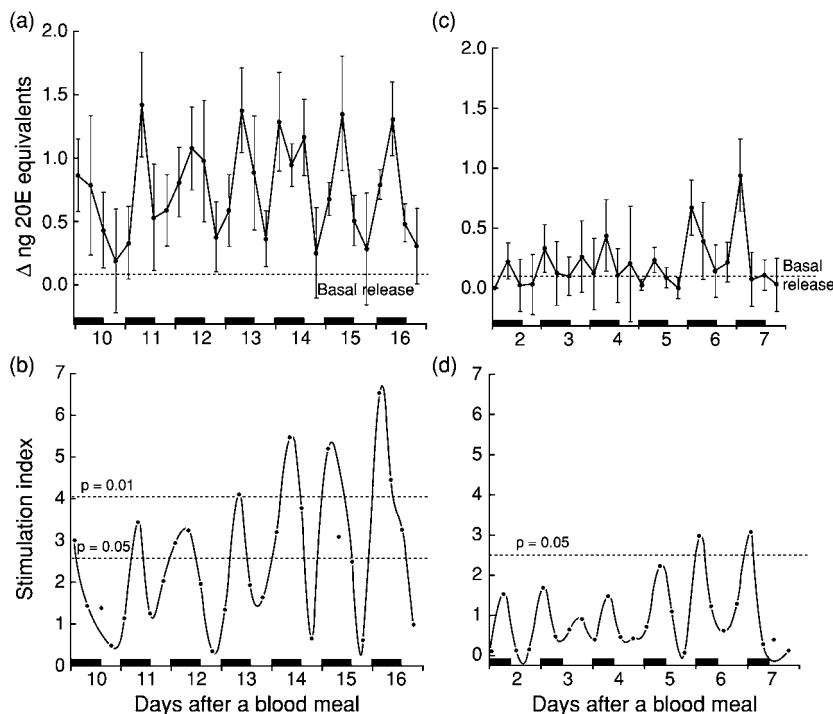


Figure 6 Circadian rhythm of prothoracicotrophic hormone (PTTH) release from the brain–retrocerebral complex during larval–adult development of *Rhodnius prolixus*. Clear peaks of PTTH release occur every scotophase throughout days 10–16 of development (a), whereas deep troughs, close to background (basal release), occur every photophase. PTTH release is close to basal level in the beginning of the instar (days 2–5) (c). At the head critical period (HCP) on day 6, PTTH release increases sharply during the scotophase and marks the onset of rhythmicity. Panels (b) and (d) show the statistical significance of the levels of PTTH release shown in panels a and b, respectively. (Reproduced with permission from Vafopoulou, X., Steel, C.G.H., 1996a. The insect neuropeptide prothoracicotrophic hormone is released with a dialy rhythm: re-evaluation of its role in development. *Proc. Natl Acad. Sci. USA* 93, 3368–3372; © National Academy of Sciences, USA.)

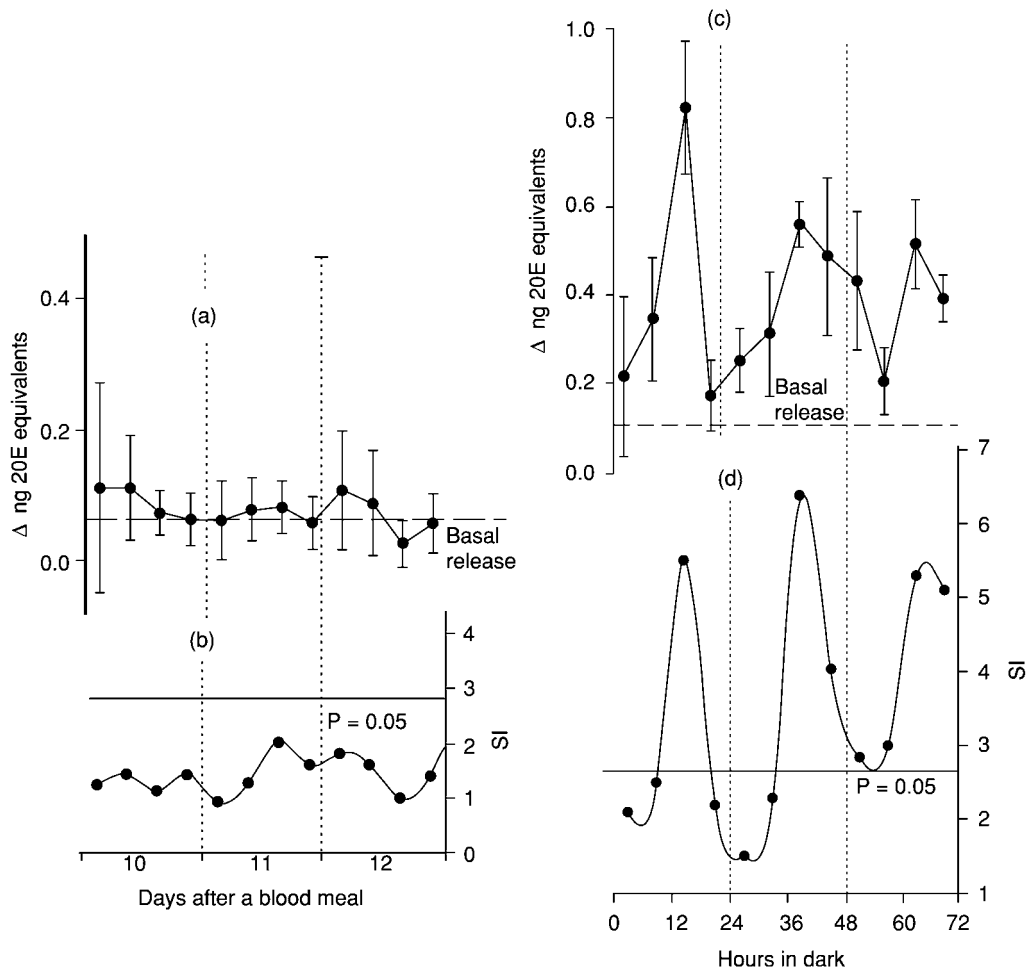


Figure 7 Induction of a daily rhythm of PTTH release from brain–retrocerebral complexes of *Rhodnius prolixus* by transfer from continuous light (LL) to continuous dark (DD). Larvae are arrhythmic following chronic exposure to LL, and there is near absence of PTTH release (a, b). Transfer to DD initiates a clear rhythm in PTTH release (c) with statistically significant peaks of roughly 24 h intervals in DD (d). (Reprinted with permission from Vafopoulou, X., Steel, C.G.H., 2001. Induction of rhythmicity in prothoracicotrophic hormone and ecdysteroids in *Rhodnius prolixus*: roles of photic and neuroendocrine Zeitgebers. *J. Insect Physiol.* 47, 935–941; © Elsevier.)

(Figure 8b). About one-third of the PTTH content of the brain is released every scotophase; the level is restored during the photophase each day, presumably by synthesis of the hormone. Thus, in *Rhodnius*, synthesis, release, and hemolymph levels of PTTH are all rhythmic events and are coupled to one another.

Synthesis of PTTH in *Rhodnius* occurs in a pair of large neurosecretory cells in the lateral region on each side of the protocerebrum (Vafopoulou *et al.*, 2004b). These PTTH cells lie adjacent to a small group of neurons immunopositive for the clock proteins PER and TIM (Figure 16) (Terry and Steel, 2004). These neurons are true clock cells since both PER and TIM exhibit circadian cycling in abundance and in migration in and out of the nucleus. The location of these clock cells in the brain is

equivalent to the lateral group of clock neurons in *Drosophila*. PTTH has also been localized to the lateral neurosecretory cell cluster in other species as in *Bombyx* (Mizoguchi *et al.*, 1990), *Manduca* (Agui *et al.*, 1979; O'Brien *et al.*, 1988), and *Samia* (Yagi *et al.*, 1995) but not in all species examined (Závodská *et al.*, 2003). A similar proximity between PER-containing neurons and PTTH cells is seen in the moth *A. pernyi*, where this arrangement is believed to regulate the release of PTTH that terminates diapause in response to long days (Sauman and Reppert, 1996b). This information raises the interesting possibility that a similar, if not the same, neuronal clock mechanism may be involved in both circadian and photoperiodic regulation of PTTH release. In another moth, *Bombyx*, the PTTH levels in the hemolymph show

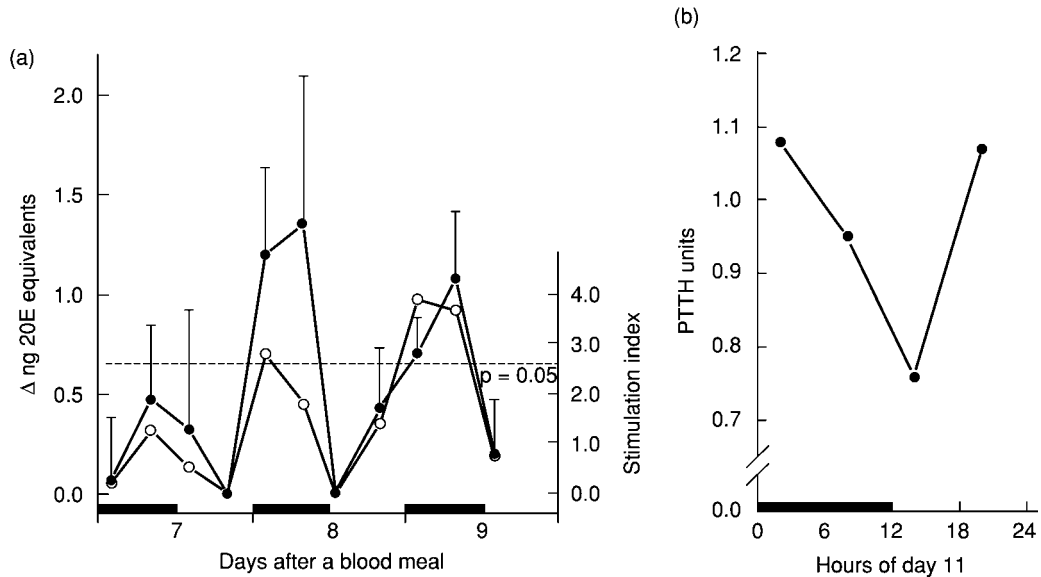


Figure 8 The daily PTTH rhythm in the hemolymph of fifth (last) larval instar of *Rhodnius prolixus* showing peaks during each scotophase (a). The pattern of the hemolymph rhythm parallels that of the PTTH release from the brain (compare with **Figure 6a**). Amounts of PTTH release (filled circles) are shown on the left axis and statistical significance (open circles) on right axis. Panel (b) shows rhythmicity in PTTH content of the *Rhodnius* brain; the brain PTTH content drops during the scotophase when the PTTH titer in the hemolymph is rising (compare a with b), suggesting release at night, whereas the brain content is replenished during the photophase, presumably by PTTH synthesis. (Reproduced with permission from Vafopoulou, X., Steel, C.G.H., 1996a. The insect neuropeptide prothoracicotropic hormone is released with a dialy rhythm: re-evaluation of its role in development. *Proc. Natl Acad. Sci. USA* 93, 3368–3372; © National Academy of Sciences, USA.)

a daily rhythmicity during larval (**Figure 9**) (Dedos and Fugo, 1999; Mizoguchi *et al.*, 2002) and pupal development (**Figure 9a**) (Mizoguchi *et al.*, 2001) suggesting clock control of rhythmicity. Rhythmic release of PTTH from the brain is also reported for *Periplaneta* (Richter, 2001). The first of the daily peaks in the fifth instar of *Bombyx* was found to be sensitive to phase shifts of the light:dark (LD) cycle (Mizoguchi *et al.*, 2002).

Rhythmicity in release of PTTH may be widespread in insects. An anatomically comparable system of clock neurons is seen in *Drosophila*. The lateral neurons are the principle circadian locus in *Drosophila* (see Section 9.3.1); their terminal arborizations in the protocerebrum overlap extensively with a pair of neurons that innervate the part of the ring gland that corresponds to the PGs (**Figure 3**) (Siegmund and Korge, 2001). This pair of neurons (PG-LP neurons in **Figure 3**) resembles the PTTH neurons seen in moths in terms of position, number of cells, the dense collateral fibers along the axon, and the contralateral projection pattern (for details, see Siegmund and Korge (2001) and Section 9.3.1), suggesting that the neuronal system for clock control of PTTH is probably also present in *Drosophila*. These presumed PTTH neurons of *Drosophila* make direct contact with the PG cells in

the ring gland (**Figure 3**) (see Section 9.3.1). Therefore, it is possible that PTTH in *Drosophila* may function as a neurotransmitter or neuromodulator rather than a hormone. Notwithstanding this difference, an essential common feature in all insects examined is the close proximity of clock neurons to the PTTH cells. Control of PTTH release by clock cells in the brains of both hemimetabolous and holometabolous insects suggests that circadian control of PTTH release appears to be conserved among insects and represents a unifying feature in the regulation of PTTH release. It should be noted that this arrangement of neuronal circadian oscillators in the brain and their control of rhythmic release of neuropeptide hormones is found in the SCN of vertebrates (see Section 9.3.2).

The continuous rhythmic release of PTTH throughout development implies that the function of PTTH is not merely to activate the PGs at the HCP. Classical studies interpreted the HCP as the only time when PTTH was released, but this view is questioned by many authors (see Section 9.4.1.1) The studies above show that PTTH is indeed released at the HCP but this release is only the first of many daily releases. In fact, the greatest quantities of PTTH are released after the HCP. As shown below (see Section 9.4.1.3), the

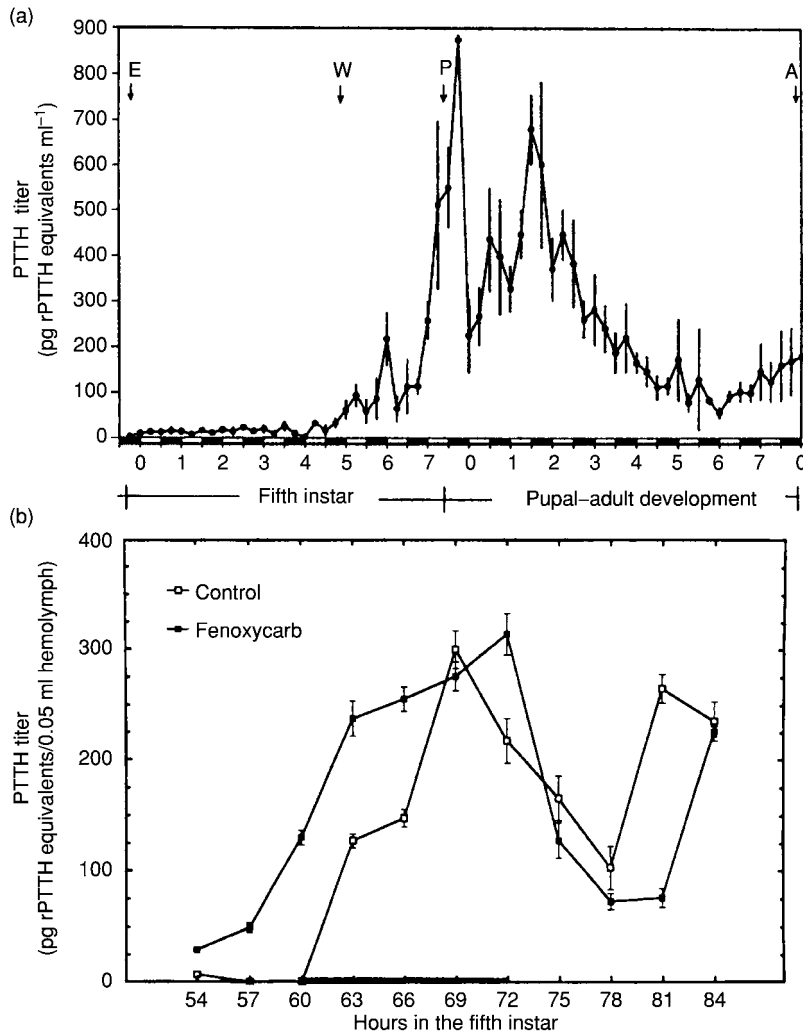


Figure 9 Daily rhythms in PTTH level in the hemolymph of *Bombyx mori*: (a) during the fifth larval instar and pupal–adult development, and (b) during a day of the fifth instar larva. Both patterns of rhythmicity show highs during the scotophase and lows during the photophase. (Reprinted with permission from (a) Mizoguchi, A., Dedos, S.G., Fugo, H., Kataoka, H., **2002**. Basic pattern of fluctuation in hemolymph PTTH titers during larval–pupal and pupal–adult development of the silkworm, *Bombyx mori*. *Gen. Comp. Endocrinol.* 127, 181–189 and (b) Dedos, S.G., Fugo, H. **1999**. Induction of dauer larvae by application of fenoxycarb early in the 5th instar of the silkworm, *Bombyx mori*. *J. Insect Physiol.* 45, 769–775; © Elsevier.)

circadian rhythm of PTTH appears to play a central role in the circadian organization of larval development and possibly also of reproductive processes in adults.

9.4.1.3. Molting hormones Ecdysteroids are major integrators of development affecting numerous physiological processes (see **Chapter 7**). Virtually all cells in the body are exposed to and respond to changes in titer of hemolymph ecdysteroids during development. Expression of genes during specific times of development is firmly understood to be a consequence of these changes in the titer of ecdysteroids. Equivalent actions of ecdysteroids appear to operate on the circadian timescale. Circadian mod-

ulation of the ecdysteroid titer appears to provide a temporal framework upon which all the ecdysteroid-responsive cells receive information concerning the time of the day. Ecdysteroids could thus aid in the organization of gene expression within a circadian cycle. Further, all tissues are exposed to the same temporal changes in ecdysteroid levels, providing a mechanism that synchronizes and coordinates gene expression in anatomically distant tissues. In this sense, ecdysteroids can act as internal Zeitgebers conveying time information to diverse developing cells and tissues. This recently formulated capacity of ecdysteroids to act as messengers of time has led to numerous studies concerning clock regulation of ecdysteroid production.

In the early 1980s, the presence of a circadian clock in the PGs of *S. cynthia* was proposed on the basis of a series of ingenious but indirect *in vivo* experiments (Mizoguchi and Ishizaki, 1982, 1984a, 1984b). The phenomenon studied was gut purge, a behavioral change at the end of the feeding, when the larva expels the contents from its gut in preparation for pupation. Gut purge is a gated phenomenon i.e., a population rhythm (Fujishita *et al.*, 1982). The gut purge rhythm free-ran in DD, was abolished in LL, and responded to light pulses (Mizoguchi and Ishizaki, 1984b). Light pulses of 15 min yielded a Type 0 phase response curve (PRC), which showed that gut purge was controlled by a circadian clock. Fujishita *et al.* (1982) associated gut purge with a small, transient increase in the hemolymph ecdysteroid titer. This ecdysteroid peak was phase shifted by light pulses in parallel with phase shifts in gut purge (Mizoguchi and Ishizaki, 1984a). Similar phase shifts in ecdysteroids were obtained in decapitated larvae, emphasizing that the brain was not required for phase-shifting. Gut purge was not monitored in these experiments because it was severely disrupted by decapitation. In the following experiments, the timing of gut purge was employed as an indicator of the timing of ecdysteroid secretion, even though it is not certain that the ecdysteroid increase is the cause of gut purge. Larvae from DD were inserted into holes in light-tight partitions, with the partition located at various different points along the larva in different experiments (Mizoguchi and Ishizaki, 1982). Thus, selected parts of the body could be exposed to 15 min light pulses, while the rest of the body remained in darkness (Figure 10). When the whole larva was illuminated, the timing of gut purge was phase-delayed. A phase delay was not observed when only the head and prothorax were illuminated, but a phase delay became evident when the mesothorax and metathorax were also illuminated. Full phase delay resulted when both the head and the whole thorax were illuminated. These experiments implicated a thoracic center (presumably the PGs) in the control of the timing of gut purge. In another experiment, three pairs of PGs were transplanted into the abdomens of intact larvae which were then transferred to DD and given 10 s light pulses to various parts of the body, i.e., anterior (brain and intrinsic PGs), posterior (implanted PGs), or whole body (Figure 10). Invariably, the time of gut purge was dictated by the implanted PGs. The authors assumed that the phase shifts in the time of gut purge were due to phase shifts in the time of the ecdysteroid rise. It was concluded that the PGs possessed a photosensitive circadian clock. However, these ingenious

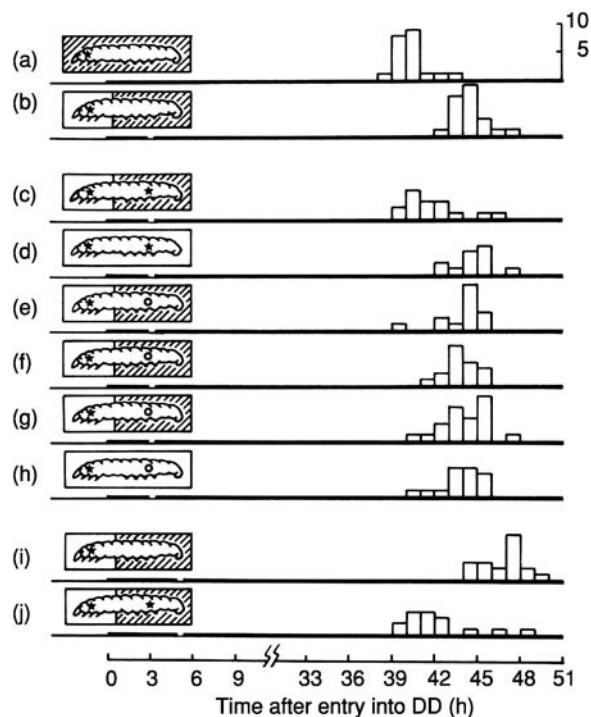


Figure 10 Effects of light pulses on the phase of gut purge of *Bombyx* larvae with and without implants of various organs in the abdomen. Larvae were transferred to DD after implantation and were given 10 s light pulses to various body parts. Pulses of light were administered 3 h (a–h) or 5 h (i and j) after transfer to DD. Clear areas on the diagrams of larvae on the left show the body areas that were illuminated. Histograms on the right show the time of gut purge. Asterisk denotes implanted prothoracic glands (PGs) and open circles other transplanted organs; (e) and (h) three brains; (f) three mesothoracic ganglia; (g) fat body. (a) Control; larvae in DD without implanted PGs and not exposed to light pulse. (b) Illumination of the head and thorax (intrinsic PGs) causes phase delay in gut purge. (c) Light pulse to anterior part of the body of a larvae with three pairs of PGs implanted (implanted PGs in dark, intrinsic PGs in light) does not phase-shift gut purge (compare c with b). (d) Whole body illumination of larvae with both sets of PGs causes phase delay. (e–h) Transplantation of various other tissues and illumination of either the anterior or the whole body of host delays gut purge as in (b). Light pulses given 5 h after transfer to DD produce similar results as those given 3 h after transfer (compare i, j with b, c). (Reproduced with permission from Mizoguchi, A., Ishizaki, H., 1982. Prothoracic glands of the saturniid moth *Samia cynthia ricini* possess a circadian clock controlling gut purge timing. *Proc. Natl Acad. Sci. USA* 79, 2726–2730; © National Academy of Sciences, USA.)

experiments are difficult to interpret. For example, both the implanted and intrinsic PGs were presumably receiving daily stimulation from PTTTH (see Section 9.4.1.2) and the four pairs of PGs would doubtlessly interact both with each other and with PTTTH from the brain in ways that cannot be predicted (Steel and Davey, 1985).

Clear evidence that a circadian clock controls ecdysteroid levels in the hemolymph was reported

for *Rhodnius* (Steel and Ampleford, 1984; Ampleford and Steel, 1985). In *Rhodnius*, the hemolymph ecdysteroid titer during the week preceding ecdysis to the adult displays a strong daily rhythm with a massive daily peak during each scotophase and a deep trough during each photophase (Figure 11). The rhythm is controlled by a circadian clock since it free-runs in aperiodic conditions (e.g., DD) with a period length that is temperature compensated. The rhythm is seen throughout larval–adult development (Vafopoulou and Steel, 1989, 1991). The daily scotophase peaks in titer are three to five times higher than the values seen in adjacent photophases. It was suggested that the daily rhythm of ecdysteroid titer probably involved both synthetic (see below) and catabolic rhythmicities. Indeed, the daily decrease in titer from the top of the scotophase peak to the bottom of the photophase trough was equal to the daily amount of ecdysteroids excreted in the feces. The removal of ecdysteroids from the hemolymph was assumed to be accomplished by a combination of conjugation, sequestration in the tissues, and excretion via the Malpighian tubules and possibly also the midgut (Steel and Ampleford, 1984).

Subsequently, rhythmicities in hemolymph ecdysteroid titers were also unraveled in various other insects. In the wax moth, *Galleria mellonella*, a circadian rhythm in the titer was seen in the last larval instar (Cymborowski *et al.*, 1989). The rhythm was also present in decapitated animals

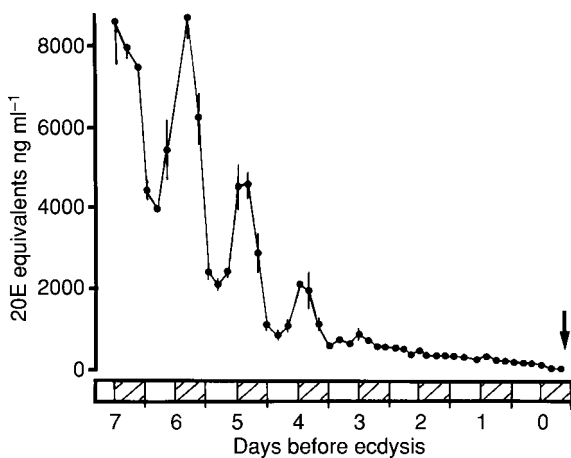


Figure 11 Rhythm of hemolymph ecdysteroid titer during larval–adult development of *Rhodnius*. Cross-hatched areas indicate scotophases and clear areas indicate photophases. Arrow marks time of ecdysis to the adult. Massive daily peaks in ecdysteroid titer occur during each scotophase. (Reprinted with permission from Ampleford, E.J., Steel, C.G.H., 1985. Circadian control of a daily rhythm in hemolymph ecdysteroid titer in the insect *Rhodnius prolixus* (Hemiptera). *Gen. Comp. Endocrinol.* 59, 453–459; © Elsevier.)

emphasizing that expression of the rhythm did not require the presence of the head (Cymborowski *et al.*, 1991). Daily rhythms in ecdysteroid titer were found in both larvae and pupae of *Bombyx* (Sakurai *et al.*, 1998; Mizoguchi *et al.*, 2001, 2002). In larvae of *Manduca*, the titer displays a series of daily “steps” (Schwartz and Truman, 1983). A daily rhythm was also seen in larvae of *Periplaneta* (Richter, 2001).

The pronounced rhythmicity in the hemolymph ecdysteroid titer of *Rhodnius* implied the existence of an underlying rhythmicity in synthesis. Subsequent experiments focused on the timed ecdysteroid synthesis by the PGs. Synthesis of ecdysteroids was studied *in vitro* following explantation of PGs every 4–5 h for many days during larval–adult development (Vafopoulou and Steel, 1991). Ecdysteroids are not stored within the PG cells, thus the rate of ecdysteroid release equals the rate of synthesis (Vafopoulou and Steel, 1989). The titer of hemolymph ecdysteroids was measured for all donors of PGs, enabling the synthesis by PGs *in vitro* to be correlated with the hemolymph titer of the same animals *in vivo*. A daily rhythm of ecdysteroid synthesis was seen that was synchronous with the hemolymph titer *in vivo*. Synthesis in the scotophase was three to five times higher than synthesis in the photophase. The synthesis rhythm free-ran in both DD and LL and was temperature compensated. Therefore, the PGs contain a circadian clock. Ecdysteroid synthesis by PGs *in vitro* showed acceleration when the PGs were transferred from LL to DD *in vitro* (Vafopoulou and Steel, 1992). This finding showed that ecdysteroid synthesis by PGs was directly photosensitive and suggested that the clock controlling synthesis might also be photosensitive. Daily rhythms in ecdysteroid synthesis have also been observed in *Galleria* (Cymborowski *et al.*, 1991), *Bombyx* (Sakurai *et al.*, 1998), and *Periplaneta* (Richter, 2001).

In *Rhodnius*, the underlying complexity of this clock began to emerge when it was found that the phase of the oscillations in synthesis differs depending on whether the animals were transferred to DD or LL (Vafopoulou and Steel, 1991). In animals that were transferred from 12:12 to DD, the rhythm of ecdysteroid synthesis by PGs free-ran with a phase similar to the entrained state. But transfer to LL resulted in abolition of the rhythm for one cycle; then the rhythm reemerged abruptly and free-ran with peaks in the subjective photophase, i.e., the rhythm in LL free-runs in antiphase to that in DD or the entrained state. The period length in LL was slightly shorter than that in the entrained state or DD. The hemolymph titer of animals transferred to

LL also undergoes the same abrupt phase inversion after a lag of one cycle. Period length and phase of a free-running rhythm are expressions of the underlying oscillator that regulates them. The differential behavior of the rhythm in DD and LL was the first indication that more than one oscillator is responsible for driving the overt rhythm of ecdysteroid synthesis. One oscillator is phase-set by lights-off, free-runs in DD, and has a period length of about 24 h. The other oscillator is phase-set by lights-on, free-runs in LL, and has a period length of less than 24 h. Apparently, the clock mechanism underlying the *Rhodnius* rhythm is complex and consists of several components. In order to dissect the components of this clock it was necessary to adopt entirely *in vitro* approaches that would eliminate hormonal influences on PGs. Rhythmic ecdysteroid synthesis persists for 42 h *in vitro* by explanted PGs in the absence of light cues (Vafopoulou and Steel, 1998). While these observations demonstrate that the PGs synthesize ecdysteroids rhythmically entirely *in vitro*, they do not exclude the possibility that this rhythm had been established by inputs received by the PGs *in vivo* and whose effects persisted for several cycles *in vitro*. Definitive demonstration that the PGs possess a photosensitive circadian oscillator came from the induction of rhythmicity in synthesis *in vitro* by light signals in PGs that were arrhythmic (Vafopoulou and Steel, 1998). Explanted PGs from arrhythmic LL animals were transferred *in vitro* to DD and ecdysteroid synthesis monitored every 3–4 h for 42 h (Figure 12). These glands responded to the transfer to DD signal with the sharp acceleration of synthesis reported in 1992 but also initiated a free-running circadian rhythm of ecdysteroid synthesis entirely *in vitro*. Therefore, the PGs possess a photosensitive circadian clock that regulates rhythmic ecdysteroid synthesis. This rhythmic synthesis drives the upward side of the hemolymph titer rhythm every day. Thus, the PGs meet all the criteria that classify them as endocrine clocks. These findings confirm and extend the PG clock model first invoked by Mizoguchi and Ishizaki (1982) in *Samia*. A PG clock was also invoked in *Galleria* where decapitations of larvae did not affect the rhythm of hemolymph ecdysteroids (Cymborowski *et al.*, 1991). In contrast, neck ligation in *Periplaneta* larvae severely depressed ecdysteroid synthesis to such an extent that no rhythm was detectable (Richter, 2001). In *Bombyx*, it is still unclear whether the rhythm of hemolymph ecdysteroids (Sakurai *et al.*, 1998; Mizoguchi *et al.*, 2001) is driven by the PTH rhythm (Mizoguchi *et al.*, 2001) or by the PG clock proposed earlier in *Samia* by Mizoguchi and Ishizaki (1982). In *Drosophila* there is good

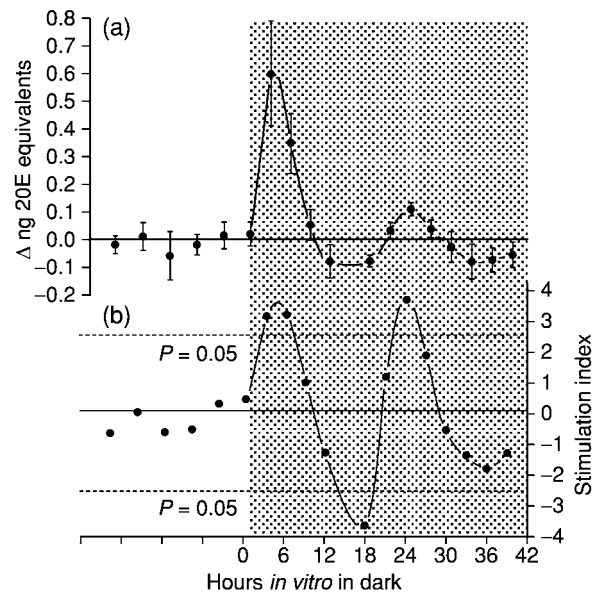


Figure 12 *In vitro* induction of rhythmic ecdysteroid synthesis by *Rhodnius* PGs following transfer from LL (clear area) to DD (stippled area). (a) Glands in LL are clearly arrhythmic (points in clear area), but become rhythmic on transfer to DD. The period length of the induced oscillations is close to 24 h. Lower panel (b) shows the statistical significance of the actual values in the upper panel (a). (Reproduced with permission from Vafopoulou, X., Steel, C.G.H. 1998. A photosensitive circadian oscillator in an insect endocrine gland: photic induction of rhythmic steroidogenesis *in vitro*. *J. Comp. Physiol. A* 182, 343–349; © Springer-Verlag.)

evidence that the PG cells of the ring gland possess an endogenous circadian clock (Emery *et al.*, 1997) but its function is unknown. Expression of the *per* gene exhibits a daily rhythm in the region of the ring gland that corresponds to the PG. The rhythm of *per* expression is entrained by light *in vitro* in incubations of the ring gland attached to the CNS. Addition of tetrodotoxin (TTX) to the incubation medium at concentrations sufficient to inhibit nerve conduction did not affect the *per* rhythm, demonstrating that the rhythm was not driven by the nervous system but was controlled locally by the PG cells. However, there is no evidence of rhythmic ecdysteroid synthesis by these cells, nor is rhythmicity evident in any of the numerous publications on *Drosophila* ecdysteroid titers (Richards, 1981). Therefore, in *Drosophila*, the function of this PG clock is unclear.

PGs comprise an ultrastructurally uniform population of about 200 cells (Beaulaton *et al.*, 1984; Dai *et al.*, 1994), which exhibit action potentials and are electrically coupled (Eusebio and Moody, 1986) by gap junctions (Dai *et al.*, 1994). Synchrony of the whole gland would therefore result from mutual coupling between its component cells. Each

PG cell, when dissociated from the whole, appears capable of synthesizing ecdysteroids (Smith *et al.*, 1986; Asahina *et al.*, 1994). The PG cells in *Rhodnius* do not receive any nerve supply (Wigglesworth, 1952) by which the cells might be synchronized, whereas those in at least some Lepidoptera are richly innervated (Lee, 1948). There is evidence that all the cells of the paired PGs of *Rhodnius* are circadian oscillators. All the cells show immunoreactivity to antibodies against the clock proteins PER and TIM (Figure 13) (Terry and Steel, 2004) and these

proteins exhibit robust circadian cycling in abundance and nuclear migration which free-runs in DD; strong immunolocalization to the nucleus occurs during the scotophase whereas the cells are almost devoid of stain during the photophase. Therefore, each PG is a group of cellular oscillators that function in unison as a result of cellular coupling. The construction of a clock from individual cellular oscillators coupled by gap junctions and local potentials is also seen in neuronal clocks in molluscs (Block *et al.*, 1993) and the mammalian SCN. Thus the PG clock, though an endocrine gland, is remarkably similar to neuronal clocks. It is of interest that PGs (at least in *Bombyx*) are, like the nervous system, of ectodermal origin (Lee, 1948).

The rhythms of both ecdysteroid synthesis and hemolymph titer *in vivo* occur in the presence of a PTH rhythm (see Section 9.4.1.2). Since the PGs are the only known targets of PTH in larvae, it is expected that PTH would affect the function of the PGs. However, as shown above, the PGs possess their own photosensitive clock and this does not support the possibility that the rhythmicity in synthesis *in vivo* is driven by rhythmic PTH. The functional relationship between the PTH clock in the brain and the PG clock was investigated by decapitation or paralysis with TTX of whole *Rhodnius* larvae in order to reveal the properties of the rhythm of ecdysteroid synthesis in the absence of rhythmic neuropeptide input (Pelc and Steel, 1997). TTX was used at a sublethal dose that caused flaccid paralysis of the whole animal and prevented release of PTH for 4 days. Rhythmicity was maintained in both ecdysteroid synthesis and the hemolymph ecdysteroid titer; the rhythms retained entrainment to LD cycles and free-ran in both DD and LL. The rhythms showed no evidence of damping or loss of entrainment, confirming that the PG clock described *in vitro* is operative *in vivo*. This finding probably explains why decapitation (after the HCP) does not arrest development; the rhythmicity in hemolymph ecdysteroids is maintained by the PG clock, which becomes directly entrained to light in headless animals. Therefore, rhythmic neuropeptide input is not required to drive rhythmic steroidogenesis by the PGs *in vivo*. This conclusion contrasts with comparable systems in vertebrates, where rhythms in steroidogenesis are regarded as passive slaves driven by rhythmic neuropeptide input (see Turek, 1994). An important finding was that in both decapitated and TTX-paralyzed animals the rhythms of synthesis and titer always adopted a phase 12 h different from those seen when PTH was present, regardless of the conditions of illumination. Therefore, the

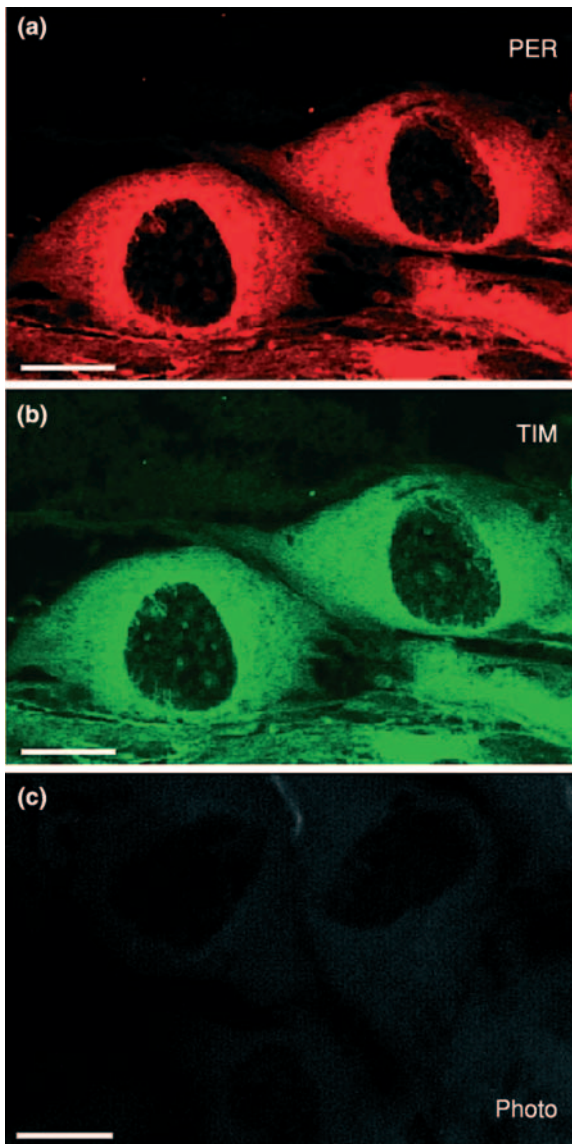


Figure 13 PER (a, red) and TIM (b, green) are colocalized in PG cells of *Rhodnius* and also show cycling. During the scotophase (a, b) immunoreactivity is high in the cytoplasm but some is also seen in the nucleus. During the photophase (c), immunoreactivity is minimal. *Rhodnius* PGs synthesize ecdysteroids during the scotophase but not during the photophase. Scale bar = 30 μm .

rhythm of steroidogenesis adopted a daytime peak when entrained to light *in vivo* in paralyzed animals (no PTH), but adopted a night-time peak when PTH was present (normal animals). It was concluded that in intact animals, PTH is the dominant entraining agent to the PG clock; photic entrainment of steroidogenesis is revealed only when rhythmic PTH input is removed. In the presence of both light and PTH the PGs entrain preferentially to PTH. Thus, both light and PTH act as Zeitgebers to the PG clock, but they contradict rather than reinforce each other. There are several examples in the literature in which circadian systems can be entrained by both light and neurochemical agents. Examples include neuropeptide Y (Yannielli and Harrington, 2000; Maywood *et al.*, 2002) and melatonin (Lewy *et al.*, 1995) actions on the mammalian circadian system and the action of serotonin on the circadian system in the eye of the mollusc *Aplysia californica* (Corrent *et al.*, 1982; Block *et al.*, 1993) and in the optic lobe of *Gryllus bimaculatus* (Saifullah and Tomioka, 2003).

The function of PTH as an entraining agent to the PG clock shows that PTH can act on the clock, not just on the pathway of steroidogenesis. But PTH action on the clock could be mediated by the same intracellular pathway by which PTH mediates stimulation of steroidogenesis. In *Manduca*, PTH regulates calcium influx into the PG cells, which leads to stimulation of calmodulin-sensitive adenylate cyclase in the PGs (Gilbert *et al.*, 2002) (see **Chapter 6**). Calcium and cAMP are known to induce phase shifts in many circadian systems; they act directly on the clock in the SCN (Tischkau *et al.*, 2003) and frequently produce PRCs which are 12 h displaced from the PRCs for light pulses (Edmunds, 1988). Therefore, this signal transduction pathway is an appropriate pathway by which PTH could regulate the PG clock.

The information above indicates that both PTH and ecdysteroids are regulated by photosensitive circadian oscillators and their circadian rhythms interact every day. It is now evident that these two hormones do not act consecutively in a “cascade” but are continuously integrated together into a daily “tango” throughout the course of development (Steel and Vafopoulou, 1999) (see Section 9.4.1.4).

9.4.1.4. Hormones as agents that couple clocks The preceding sections have documented that the circadian system of *Rhodnius*, and probably that of other insects, consists of multiple photosensitive clocks coupled to the neuroendocrine axis. Two of these clocks are found in the brain where they are located

in two symmetrical groups of neurons in the dorsal protocerebrum that control rhythmic release of PTH. Coupling of the left and right clocks in the brain to each other is probably achieved by neural connections across the midline as is the case with the paired optic lobe clocks (**Figure 2**). Each PG is also a photosensitive clock that generates rhythmicity in ecdysteroid synthesis. All cells of each PG appear to be cellular oscillators since they all exhibit circadian cycling of PER/TIM clock proteins and are coupled into a functional unit by cell junctions. Thus, the circadian system that drives the downstream rhythm of the hemolymph ecdysteroid titer consists of (at least) four anatomically discrete, photosensitive clocks. Four similar clock loci, but less fully documented, are seen in moths where the information is distributed between the three genera, *Bombyx*, *Antheraea*, and *Samia*. In *Drosophila*, the number of loci is reduced to three because the normally paired PGs are fused into one ring gland. Close association between clock cells in the brain and PTH cells is seen in the three divergent genera, *Drosophila*, *Antheraea*, and *Rhodnius*. The situation in insects therefore, is similar to the one seen in vertebrates where overt hormone rhythms express the output of multiple coupled clocks. The intricacy of functional coordination of the four (or three) circadian clocks in whole animals was studied in *Rhodnius in vivo* (Vafopoulou and Steel, 2001). PTH release ceased completely after a few cycles in animals maintained in LL; free-running rhythmic release was promptly initiated by transfer to DD, demonstrating that the circadian oscillators that control PTH rhythmicity are photosensitive. Transfer to DD also initiated a free-running rhythm of ecdysteroid synthesis in spite of the thick, brown overlying cuticle. Interestingly, the first initiated peak of steroidogenesis occurred several hours *prior* to the first peak of PTH release and was not impaired when PTH release was prevented by prior injections of TTX. These findings suggested that the initial response to a light cue involves direct response of the PG clocks to light. However, in intact animals, the second peak of steroidogenesis becomes phase-delayed until it synchronizes with the rhythm of PTH release. Thus, the PTH rhythm free-ran with a stable period and phase following its initiation, whereas the ecdysteroid rhythm became shifted by PTH after its initiation. This experiment clearly showed that both clocks in brain and PGs operate *in vivo* and can initiate rhythmicity in their respective hormones following the same light cue. However, when the PTH rhythm is present, it overrides light as a Zeitgeber to PGs and phase-sets the PG clocks. The neuropeptide therefore acts as an

entraining agent for the PG clocks in whole animals and conveys temporal information to them. Therefore, in *Rhodnius*, and probably in other insects as well, PTTH acts as a hormonal Zeitgeber to the PGs. This action synchronizes the PTTH clocks and PG clocks together into a functional unit. Therefore, the function of PTTH is integral to the circadian organization of the endocrine system.

It has been shown that the PGs are able to recognize the daily time-cues from the PTTH rhythm (Vafopoulou and Steel, 1999). Treatment of entrained PGs of *Rhodnius* with PTTH *in vitro* leads to an augmentation of ecdysteroid synthesis, but only in PGs taken from animals at certain times of the day; PGs are maximally responsive to PTTH around lights-off and insensitive to PTTH around lights-on. Therefore, PGs exhibit a daily rhythm of responsiveness to PTTH, which implies the existence of a daily up- and downregulation of PTTH receptor availability. It is interesting that the rhythm of responsiveness phase-leads that of PTTH release. Whether the responsiveness rhythm is driven by the PTTH rhythm or is endogenously controlled by the PGs themselves is not known at present. The ability of PTTH to set the phase of the PG clock (discussed above) shows that it is able to act on the clock, not just on steroidogenesis. Thus, the brain clocks communicate daily with the PG clocks.

It is probable that this communication is reciprocal, with ecdysteroids providing daily information to the brain. Feedback actions of ecdysteroids on PTTH release by the brain is cited frequently in the literature, but has not been studied from a circadian perspective. Nevertheless, it is well known that the nervous system is a major target for the action of ecdysteroids (see Chapter 4). It is possible that the rhythm of ecdysteroids acts on the clock cells in the brain or on brain neurons that provide input to the clock cells, leading to affects on PTTH release. Indeed, studies of ecdysteroid receptors (EcR) in *Rhodnius* (Vafopoulou *et al.*, 2001, 2004a) using immunohistochemistry revealed the presence of EcR in all of the 17 medial neurosecretory cells of the protocerebrum as well as the dorsal and lateral clock cells. Moreover, nuclear EcR exhibits cycling in all three locations. In the dorsal (Figure 14) as well as the lateral regions of the brain, EcR immunobinding exhibits cycling between subcellular locations within a day; immunobinding is exclusively nuclear during scotophase (Figure 14a) and exclusively cytoplasmic during photophase (Figure 14b). Therefore, there are several pathways by which ecdysteroids could provide inputs to the brain clocks. Unlike EcR in epidermal cells, which cycles

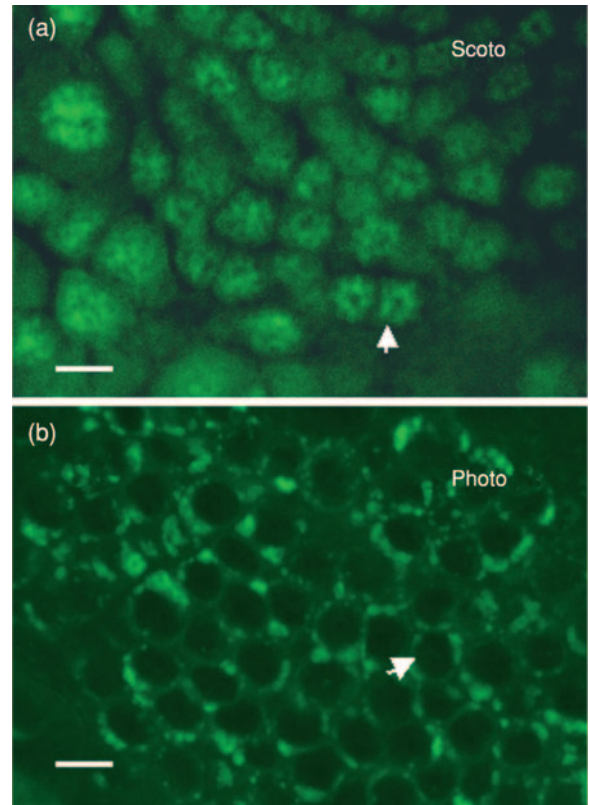


Figure 14 Ecdysteroid receptor (EcR) cycles in the dorsal clock cells (adjacent to the mushroom body) in the protocerebrum of *Rhodnius*. (a) EcR immunoreactivity is abundant and confined to nuclei during the scotophase. (b) During the photophase, EcR is confined to the cytoplasm. Arrows indicate nuclei. Scale bar = 12 μ m.

in synchrony with the hemolymph ecdysteroid rhythm, the rhythm of nuclear EcR in the brain is many hours out of synchrony with both the hemolymph ecdysteroid rhythm and the EcR rhythm in epidermal cells. These findings are consistent with the reported induction of EcR in epidermal cells by ecdysteroids, but argue against direct induction of EcR in brain neurons by ecdysteroids, raising the prospect of more complex action of ecdysteroids on the brain (Vafopoulou *et al.*, 2004a).

Yet another endocrine component of the multi-oscillator circadian system may be melatonin. Melatonin has been detected in tissue extracts of several species (Vivien-Roels and Pevet, 1993; Hardeland and Poeggeler, 2003) including hemolymph (Itoh *et al.*, 1995a, 1995b; Linn *et al.*, 1995) where changes in levels within a day have been reported. To date, true circadian studies have been conducted only in *Rhodnius* (Gorbet and Steel, 2003, 2004). A robust daily rhythm in hemolymph levels has been found, with peaks in the scotophase and minimal values in the photophase (as commonly seen in

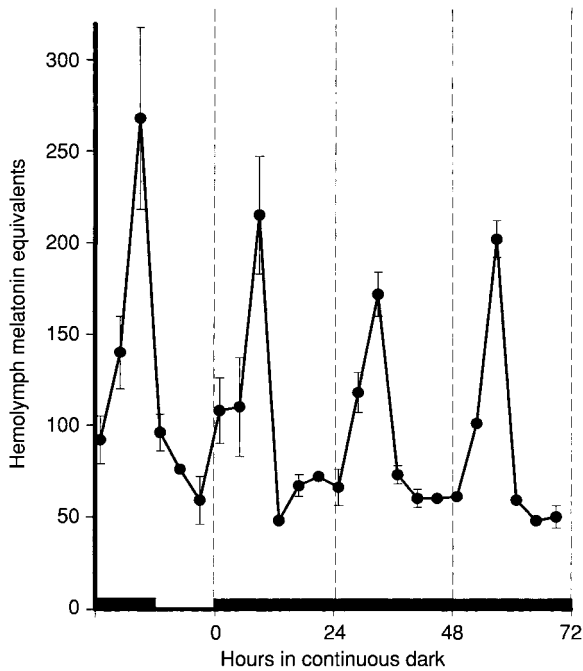


Figure 15 Rhythm of melatonin in the hemolymph of *Rhodnius*. Entrained rhythm in 12:12 (first peak) peaks in the late scotophase and free-runs in DD for at least three cycles (dark bar), illustrating circadian control of the rhythm.

vertebrates) (Figure 15). The melatonin rhythm is controlled by a circadian clock since it free-runs in DD and damps out after three cycles in LL. The rhythmic pattern and the daily peak levels of melatonin in *Rhodnius* are very similar to those seen in circulating melatonin in vertebrate blood (Gorbet and Steel, 2003). Melatonin is known to act on steroidogenic tissues in vertebrates where it influences the phase of steroid release (Pang *et al.*, 1998). Melatonin may therefore participate in the circadian regulation of the PTH–ecdysteroid neuroendocrine axis. In *Periplaneta*, no effect of melatonin on ecdysteroid synthesis by the PGs was detected, but it appears to facilitate the release of PTH from brains *in vitro* (Richter *et al.*, 2000). It is possible that rhythmic levels of melatonin in the *Rhodnius* hemolymph modulate rhythmic release of PTH by the brain by acting on some component of the PTH clock in the brain. Such an arrangement would be strikingly analogous to mammalian systems, where melatonin modulates the phase of clock neurons in the SCN (Gillette and McArthur, 1995). Another possible function of melatonin may be to modulate EcR levels in the brain. In mammals, melatonin modulates steroid hormone receptor levels (Roy and Wilson, 1981; Danforth *et al.*, 1983;

Molis *et al.*, 1994). Further, many brain regions of mammals exhibit rhythmic electrical activity. Such rhythms are driven by neuronal circadian oscillators in discrete brain loci such as the SCN; these regions can regulate the phase of rhythmic expression of steroid nuclear receptors (van Esseveldt *et al.*, 2000). The neuronal brain oscillators are characterized by the expression of PER/TIM clock proteins. Since two comparable loci of PER/TIM oscillators have been found in the lateral and dorsal regions of the *Rhodnius* brain, this raises the possibility that neuronal rhythms in the brain, and by association rhythms in EcR, could be driven from within the brain. Thus, there exist several possible mechanisms whereby the phase of EcR rhythmicity in the brain of *Rhodnius* described above may be regulated.

It is concluded that the circadian organization of developmental hormones, at least in *Rhodnius*, is achieved by four circadian clocks located in anatomically distinct sites, two in the brain and one in each PG. They are coupled into a functional unit *in vivo* by nerves within the brain and by hormonal pathways involving PTH, ecdysteroids, and possibly melatonin (Steel and Vafopoulou, 2001). It should be noted that we are portraying these hormones as agents that communicate temporal information between circadian oscillators. This portrayal is still novel for insect hormones although it is well established in mammalian systems (Reppert and Weaver, 2001; Balsalobre, 2002; Kalsbeek and Buijs, 2002).

The output of the above complex circadian system is the circadian rhythm of hemolymph ecdysteroids. Diverse cells and tissues of the developing organism are thereby exposed to a tightly regulated sequence of increases and decreases in ecdysteroid titer on a circadian timescale. Do cells respond to these daily changes in hormone levels? Apparently they do. EcR is found in many target tissues; among them, the epidermis is a primary target of ecdysteroid action and EcR in epidermal cells is well studied (see Chapter 7). In *Rhodnius*, the relative abundance of nuclear EcR immunoreactivity in epidermal cells shows a striking daily rhythm that is synchronous with the rhythm of ecdysteroids in the hemolymph (Vafopoulou *et al.*, 2001, 2004a). These close temporal relations between hormone and EcR cycling are indicative of the regulation of EcR expression by the levels of circulating ecdysteroids (see Chapter 7). Indeed when hormonally naive epidermal cells of a *Chironomus* cell line are exposed to 20E, EcR immunoreactivity disappears from the cytoplasm and increases in the nucleus (Lammerding-Köppel *et al.*, 1998). These findings further indicate that the circadian time signals embedded in the

rhythmic ecdysteroid titer are detected by the primary target cells of the hormone, i.e., the epidermis.

This complexity of the multioscillator system in *Rhodnius* and its control over the rhythmicity of hormones is shown in **Figure 16**. Since most insect cells possess ecdysteroid-responsive genes which are known to be expressed in temporally orchestrated sequences during the course of development (Riddiford *et al.*, 2000; Thummel, 2002) (see **Chapter 7**), it is possible that the rhythm in hemolymph ecdysteroids also orchestrates sequential gene expression in target cells within the circadian cycle. A case in point is the transcriptional activity of region I-18C of the salivary gland polytene chromosomes of the midge *Chironomus tentans* (Lezzi *et al.*, 1991). This chromosomal region exhibits a daily rhythm of condensation and decondensation (associated with transcriptional activity). Decondensation occurs during the scotophase and parallels increased responsiveness to exogenous 20-hydroxyecdysone (20E).

Since most cells in an organism lack mechanisms which enable them to measure time or to synchronize themselves with the environment, the circadian system above appears to be the primary mechanism that maintains internal temporal organization during development both in the insect as a whole and within cells. Since all the hormones in the circadian system persist into the adult stage (see Section 9.5.1), it is possible that they constitute a central timing system throughout life.

9.4.2. Rhythmicity in Cuticle Deposition

In many insects, new cuticle is deposited in morphologically visible “daily growth layers.” Such daily layers are found in all instars of many hemimetabolous insects and in the adults of various holometabolous species (Neville, 1983). In many species, this daily rhythm is under circadian control (Neville, 1965). These early reports demonstrate that epidermal cells exhibit circadian rhythmicity. Since the activities of epidermal cells are regulated primarily by ecdysteroids, the possibility is raised that daily growth layers in cuticle are driven by rhythms in ecdysteroids, or perhaps other hormones. The literature on daily growth layers does not address this possibility since it largely predates the discovery of rhythmicity in the endocrine system. This literature is briefly revisited below. It is suggested that daily growth layers may reflect rhythmicity in epidermal cells that is driven by ecdysteroids or other hormones (see **Chapters 11 and 12**).

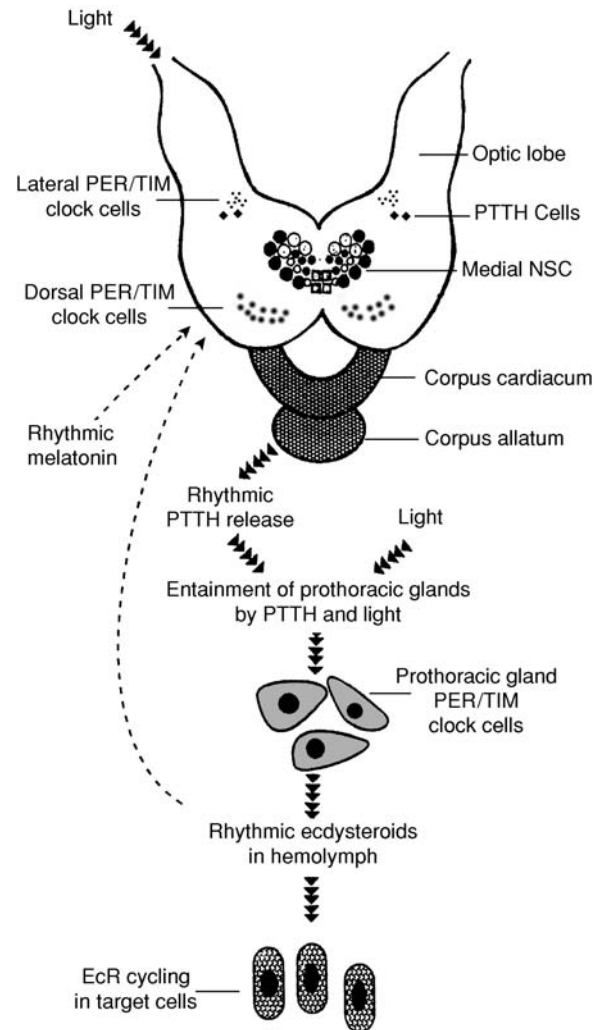


Figure 16 Schematic diagram of circadian regulation of developmental hormones in *Rhodnius*. PTH is released rhythmically from the retrocerebral complex. The PTH cells (diamonds) in the brain are adjacent to the lateral group of clock neurons. At least in *Drosophila*, these clock neurons project to the neurosecretory cells of the pars intercerebralis and also interact with the dorsal clock neurons (see **Figure 4**). PTH acts on the PGs to entrain a PER/TIM-based clock in the PG cells (**Figure 13**) that is directly photosensitive (**Figure 12**). Rhythmicity in ecdysteroid synthesis is generated by the PG clock but is entrained by both PTH and light. The resulting hemolymph titer of ecdysteroids is rhythmic, and distributes temporal information to target cells. Circadian cycling of EcR occurs in target cells, indicating rhythmic responses to ecdysteroids. The hemolymph ecdysteroid rhythm may act back on the brain (dashed arrow) via cycling EcR in the brain (**Figure 14**). The circadian rhythm of melatonin (**Figure 15**) provides a further possible input into the system. This system contains at least four circadian clocks (two in the brain, one in each PG) that are coupled by nerves and hormones (see text). Ecdysteroids are the primary controlled output of this system, distributing temporal information to all cells that possess EcR. They are potentially able to orchestrate target cell responses within a circadian cycle and to synchronize cells in distant locations with each other.

The first part of the cuticle to be deposited during a molt is a thin epicuticle composed of several layers (Hepburn, 1985). Deposition of epicuticle occurs around the middle of the molt cycle in the presence of peak titers of ecdysteroids. The bulk of the cuticle (procuticle) is deposited during decreasing titers of ecdysteroids. Frequently, by the time of ecdysis only the outer part of procuticle (the future exocuticle) has been secreted. The deposition of procuticle continues after ecdysis and may continue until the next apolysis; this region is usually termed endocuticle. Cuticle consists of chitin microfibrils which are embedded in a matrix of proteins and are laid down as plaques on the surface of the epidermal cells (Neville, 1975). At any particular depth in the cuticle, the microfibrils lie parallel to one another in the plane of the cuticle. However, when viewed from above, the orientation of the microfibrils rotates anticlockwise through successive layers within the cuticle; this arrangement of microfibrils forms a helicoidal pattern between successive levels within the procuticle. A vertical section of this type of procuticle gives the appearance of lamellae because each orientation of microfibrils is seen to repeat every 180° of the helix. Thus, each lamella consists of several layers of microfibrils whose orientation changes progressively with the depth of the cuticle. In many insects, procuticle may be lamellate throughout resulting in endocuticle and exocuticle regions that are morphologically indistinguishable. However, there are many species in which the endocuticle consists of microfibrils arranged in alternating regions of lamellate layers (with helicoidal orientation) and nonlamellate layers (with uniform orientation). In these species, the cuticle normally grows by one pair of these bands each day; for part of the day lamellate cuticle is laid down, and for the rest of the day nonlamellate cuticle is laid down. The physiological mechanism regulating the deposition of endocuticle is largely unknown (Riddiford, 1985). Nevertheless, the presence of these daily growth layers in diverse insect species (Neville, 1983) has spawned interest in the circadian control of cuticle secretion and its implications for rhythmicity in the epidermis and the hormones that regulate cuticle secretion.

There is considerable evidence that daily deposition of alternate layers of lamellate and nonlamellate cuticle is under circadian regulation in a number of insects. In adults of the locusts *Schistocerca gregaria* and *Locusta migratoria*, the daily rhythm of bands in the hind tibia exhibited all the characteristics of an endogenously controlled rhythm; the rhythm free-ran in DD, was abolished in LL, was temperature compensated, and was entrained by

light (Neville, 1965). Removal of the eyes and ocelli or painting of the head capsule black or transplantation of cylinders of tibia into the hemocoel of imaginal hosts did not affect the cuticle rhythm in locusts (Neville, 1967). These experiments demonstrated that rhythmic cuticle deposition was independent of organized photoreceptors and did not require nervous regulation. The results are consistent with the possibility that rhythmic cuticle secretion is sustained by rhythmic humoral factors in the hemolymph. However, the cuticle rhythm in cockroaches such as *Blaberus craniifer* and *L. maderae*, exhibits many of the characteristics of circadian regulation (temperature compensation, for example) (Wiedenmann *et al.*, 1986; Weber, 1995), but is not entrained by LD cycles (Wiedenmann *et al.*, 1986). When animals were transferred to aperiodic conditions such as LL for a prolonged period prior to ecdysis, rhythmicity in cuticle deposition still commenced at ecdysis throughout the animal and was maintained daily thereafter (Wiedenmann *et al.*, 1986). It was inferred that the initiation of banding in the cuticle occurs at ecdysis by some unidentified signal. This signal could be a hemolymph factor, perhaps a hormone, released around the time of ecdysis (Lukat *et al.*, 1989). This unknown signal was apparently unnecessary for the maintenance of the rhythm after its initiation, since the banding rhythm continued in leg pieces that were implanted into older animals in which cuticle rhythmicity had ceased (Lukat *et al.*, 1989). In some species, this signal may occur prior to ecdysis because several daily bands may already be present in the cuticle immediately after ecdysis (Kristensen, 1966). Together, these studies of cuticular banding suggest that rhythmicity is initiated at or before ecdysis by a humoral agent, perhaps a hormone. The rhythm appears not to require additional inputs after ecdysis. These features sustain the possibility that rhythmicity is initiated by (rhythmic) hemolymph ecdysteroids at or prior to ecdysis. Once the rhythm is initiated, continuous input seems to be unnecessary; indeed the hemolymph ecdysteroid levels seen after ecdysis are very low (Steel and Vafopoulou, 1989).

A further consideration in understanding the regulation of rhythmic cuticle deposition involves *per*-based oscillators that may reside in, or close to, the epidermis. Rhythmic *per* expression was found in several peripheral tissues in *Drosophila*, including the thorax, legs, and wings (Plautz *et al.*, 1997). In this insect, the thoracic apodemes exhibit a daily rhythm in cuticle bands (Johnston and Ellison, 1982). It is therefore possible that *per*-based peripheral oscillators may drive the cuticular rhythm in

apodemes. However, the general body cuticle of *Drosophila* does not show daily growth bands. It seems important to determine whether *per* expression is seen specifically in epidermal cells and whether the peripheral oscillators noted in appendages are entrainable by humoral signals.

9.4.3. Circadian Regulation of Ecdysis

The endocrine regulation of ecdysis is treated elsewhere. Previous reviews include Reynolds (1980, 1987), Truman and Morton (1990), Truman *et al.* (1991), Hesterlee and Morton (1996), Horodyski (1996), Ewer *et al.* (1997), Nässel (2000), Predel and Eckert (2000), Jackson *et al.* (2001), Ewer and Reynolds (2002), and Mesce and Fahrbach (2002). This section addresses only those aspects of the subject that bear on the control of the timing of ecdysis.

The term ecdysis refers to the complex of behaviors by which an insect extracts itself from an old exoskeleton. Eclosion refers specifically to the adult ecdysis of holometabolous insects. Since every particular ecdysis occurs only once in the lifetime of an insect, circadian rhythms in ecdysis can be detected only in a population of insects of mixed ages. Such circadian “gating” of ecdysis has been described in numerous species of insects. The subject has long been of particular importance to circadian biology, the conceptual foundations of which were elucidated using the eclosion rhythm of *Drosophila* (see Pittendrigh, 1993). *Drosophila* continues to be the organism of choice for genetic manipulations of both eclosion and the molecular machinery of circadian clocks in general (Stanewsky, 2002, 2003; Hall, 2003). By contrast, knowledge of the physiology and endocrinology of ecdysis derives almost exclusively from the large moths *A. pernyi*, *Hyalophora cecropia*, *M. sexta*, and *B. mori*.

Ecdysis occurs as soon as sufficient new cuticle is deposited below the old cuticle to provide sufficient structural support for both the insect as a whole and for its internal organs. A preecdysis sequence of behaviors then occurs, which serves to loosen remaining attachments between the new and old cuticles, followed by an ecdysis sequence characterized by rhythmic peristaltic contractions of the abdomen that propel the old cuticle away, revealing the new cuticle. The new cuticle is then inflated by swallowing air or water and then hardened by quinone tanning of cuticular proteins. At the time of ecdysis, the new cuticle is usually no more than half its final thickness. Cuticle secretion continues for days or weeks after ecdysis (as do many other components of the molting process, including muscle

development and remodeling of neural circuits). Ecdysis is often, but incorrectly, described as the final event in molting or as occurring at the end of the molting process.

Insects seem to have experienced strong selective pressure to undergo ecdysis as early as possible during the molting process. The presence of two cuticles around the body, one of which is loose, encumbers locomotion and makes chewing difficult. Further, the presence of two cuticles within the tracheal system impairs gas exchange. Insects seek secluded locations away from predators once new cuticle formation is under way. These considerations illustrate that the time of ecdysis must be intimately coordinated with the progress of cuticle secretion. The latter is governed primarily by systematic changes in the titer of hemolymph ecdysteroids (Steel and Vafopoulou, 1989). Thus, the time of ecdysis is coordinated with molting via ecdysteroids. There is considerable experimental support for this view, summarized below. Coordination of ecdysis with molting does not necessarily involve circadian timing. The fact that circadian timing usually fine-tunes the relationship with the timing of molting is often ascribed to the need for ecdysis to take place at a time of day when predators are inactive.

There is an additional set of reasons why circadian control is needed in the case of the adult ecdysis. Closely related species may have eclosion gates that are separated by many hours. Many species mate soon after adult eclosion. Gating of eclosion may therefore serve to ensure that mating occurs between members of a single species and therefore may represent a mechanism for reproductive isolation. A secondary consequence of the timing of mating by the timing of eclosion is that the reproductive states of males and females become synchronized with each other; this in turn creates synchrony in the processes of reproduction within the population as a whole. The gating of eclosion has classically been ascribed to the action of a brain-centered “eclosion clock” (Truman and Riddiford, 1970; Truman, 1972b). Only two inputs have been documented that influence the timing of ecdysis: ecdysteroids and an eclosion clock. Whether or not the mechanisms that control ecdysis differ between larval and adult ecdyses has been debated extensively in the literature (Truman *et al.*, 1981; Ewer and Reynolds, 2002).

The importance of ecdysteroids in timing ecdysis was first shown by Slama (1980). He found that injection of 20E into pharate adult *Tenebrio molitor* delayed ecdysis. Using *Manduca*, Truman *et al.* (1983) reported that 20E injection appeared to

affect both the pupal ecdysis and eclosion, but in different ways (Figure 17a and b). When physiological doses of 20E (1 μg or less) were injected within 8 h of ecdysis, the delay in the time of ecdysis was progressive and dose dependent in both larvae and pupae. But at supraphysiological doses (5 μg or more) the pupal ecdysis was prevented whereas eclosion was not. The high doses of 20E induced discontinuous delays in eclosion, with the animals "jumping" to progressively later eclosion gates as the dose of 20E was increased (Figure 17b). This effect was attributed to a direct circadian control over eclosion (by the brain) that was presumed to be

absent from larvae. This conclusion was consistent with earlier studies (Truman, 1972a) reporting that the ecdysis gate for larvae of *Manduca* became fixed at the HCP for decapitation (see Section 9.4.1.1) and that phase shifts of the light cycle applied after the HCP failed to generate phase shifts in the time of ecdysis. Since the HCP is a gated event, it was inferred that the gating of larval ecdyses was set by prior endocrine events that control molting. These events were presumed to involve the circadian control of PTTH release and, by association, ecdysteroid levels (Ewer and Reynolds, 2002) (see Section 9.4.1). Collectively, these experiments showed that

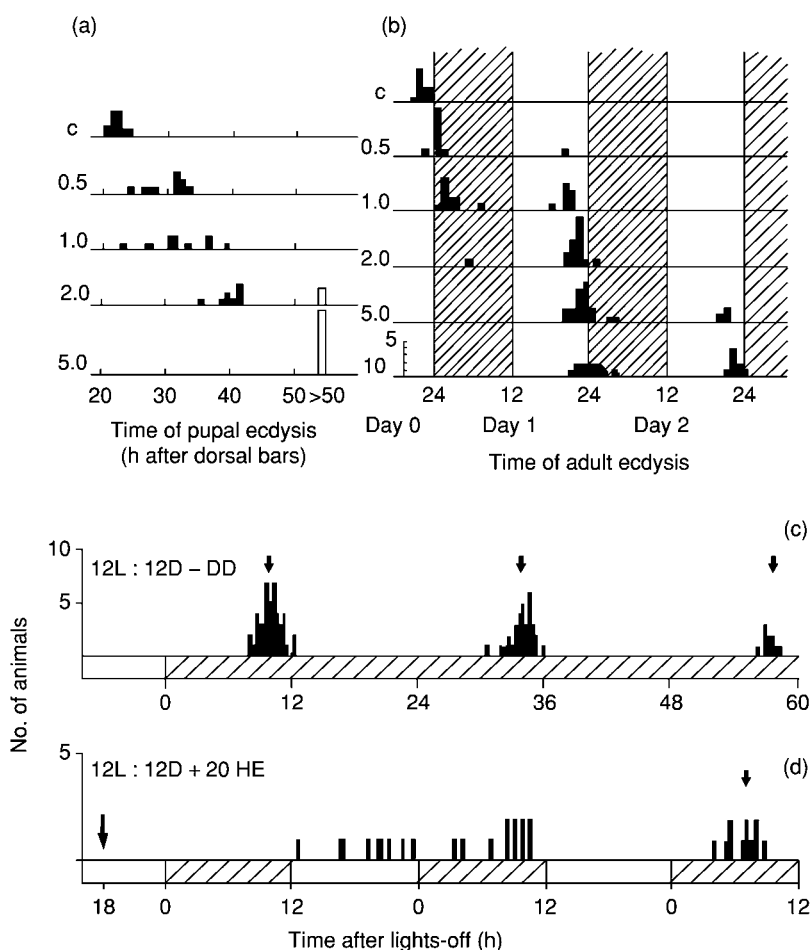


Figure 17 Upper panel: injection of increasing amounts of 20-hydroxyecdysone (20E) produces increasing delays of the time of (a) pupal ecdysis and (b) adult eclosion in *Manduca sexta*. The time of pupal ecdysis is delayed progressively as the dose of 20E increases. The same is true for eclosion for small doses of 20E; however, at higher doses of 20E the time of eclosion is displaced into the following gate. Open bars indicate animals that did not undergo ecdysis and (c) indicates the ecdysis time of control animals. Amounts of 20E injected range from 0.5 to 5.0 μg . (Reprinted with permission from Truman, J.W., Rountree, D.B., Reiss, S.E., Schwartz, L.M., 1983. Ecdysteroids regulate the release and action of eclosion hormone in the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol.* 29, 895–900; © Elsevier.) Lower panel: in *Rhodnius prolixus*, the imaginal ecdysis rhythm free-runs in DD (c). Injection of 40 ng 20E (8% of the lowest dose used in *Manduca*) at the long arrow in (d) abolishes the first gate after injection, but timing recovers in subsequent gates. The response resembles (a) rather than (b) in *Manduca*. (Reproduced with permission from Steel, C.G.H., Ampleford, E.J., 1984. Circadian control of hemolymph ecdysteroid titres and the ecdysis rhythm in *Rhodnius prolixus*. In: Porter, R., Collins, G.M. (Eds.), *Photoperiodic Regulation of Insect and Molluscan Hormones*. Pitman Press, London, pp. 150–169; © John Wiley & sons Ltd.)

ecdysis of all instars requires a decline in the titer of ecdysteroids. Such a decline is characteristic of the period of exocuticle deposition during molting (Steel and Vafopoulou, 1989), and illustrates the role of ecdysteroids in coordinating the time of ecdysis with the progress of molting. Thus, a steadily declining ecdysteroid titer would provide the ecdysis control system with information about which of the daily ecdysis gates should be employed by an individual insect. This has been the preferred model for the eclosion of moths for many years; the silkmoths *Antheraea* and *H. cecropia* appear to have a circadian clock in the brain that is specific for the control of eclosion (Truman and Riddiford, 1970; Truman, 1972b) and the hemolymph ecdysteroid titer of *Manduca* contains no information about circadian time as it exhibits no clear circadian rhythmicity (Schwartz and Truman, 1983).

In a growing number of species, the hemolymph ecdysteroid titer shows circadian oscillations (see Section 9.4.1.3). In such species, the circadian time signals conveyed by the ecdysteroid titer could regulate the circadian gating of ecdysis without the involvement of a specific ecdysis clock. For example, ecdysis in the silkmoth *Bombyx* is tightly gated (Sakurai, 1983) and the ecdysteroid titer exhibits a daily rhythm (Sakurai *et al.*, 1998; Mizoguchi *et al.*, 2001). Interestingly, the time of ecdysis is set early in molting in both *Manduca* (Truman, 1972a) and *Bombyx* (Sakurai, 1983); in *Bombyx* the time is set on the day in which rhythmicity in ecdysteroids commences (Sakurai *et al.*, 1998). It is possible that the precision with which ecdysteroid titers are timed in various species influences the precision with which ecdysis is gated.

All ecdyses in *Rhodnius* are tightly gated (Figure 17c) (Ampleford and Steel, 1982) and the circadian regulation of ecdysteroids has been extensively studied in this species (see Section 9.4.1.3). In *Rhodnius*, unlike *Bombyx*, the gate for ecdysis can be phase-shifted for many days after the onset of rhythmicity in the ecdysteroid titer (Ampleford and Steel, 1982, 1985), presumably because the rhythm in ecdysteroids is itself entrainable by light (see Section 9.4.1.3); it is not known if the same occurs in *Bombyx*. In *Rhodnius*, injection of a much smaller dose of 20E than was used with *Manduca* much longer in advance of ecdysis has a dramatic influence on the gating of ecdysis (Figure 17d). The first ecdysis gate is abolished and animals emerge outside the gate until the time of the second gate, when gating is restored. It seems that *Rhodnius* ecdysis is extremely sensitive to small perturbations of ecdysteroid levels (see discussion by Ewer and Reynolds, 2002). The intimate relation between the gating of

ecdysis and the ecdysteroid titer rhythm was revealed in *Rhodnius* by experiments showing that manipulations of the ecdysteroid titer led to predictable changes in the ecdysis rhythm (Ampleford and Steel, 1986). Thus, circadian time signals present in the circulating ecdysteroid titer provide important signals that regulate the gating of ecdysis. Nevertheless, factors other than the ecdysteroid rhythm appeared to be involved in generating the rhythm of ecdysis (Ampleford and Steel, 1986).

From the above it is apparent that the ecdysis control system reads the circulating levels of ecdysteroids to determine the developmental readiness for ecdysis, i.e., which of the daily gates will be used by an individual insect. This information is contained in the ecdysteroid titer whether or not it is under circadian control. In those species where the ecdysteroid titer also contains circadian information, this information contributes to the timing of ecdysis within a day, i.e., to the gated nature of ecdysis. Indeed, it would be strange if such circadian input to the ecdysis control system was ignored.

How and where do ecdysteroids act on the system that controls ecdysis? No clear evidence exists in the literature, but some possibilities have been raised. The ecdysis control system consists of eclosion hormone (EH) synthesized in ventromedial (VM) neurons in the protocerebrum of the brain, ecdysis triggering hormone (ETH) and preecdysis triggering hormone (PETH) synthesized in Inka cells of segmentally repeated epitracheal glands and crustacean cardioactive peptide (CCAP) synthesized in segmentally repeated neurons in the abdominal ganglia. The nature and mechanism of interactions between these peptides and their roles in coordination of ecdysis are the subjects of continuing research with many regulatory schemes in the literature. Some recent schemes are given by Žitňan and Adams (2000), Jackson *et al.* (2001), and Ewer and Reynolds (2002). EH was the first of these neuro-peptides to be discovered and has the most extensive literature (see Truman, 1992). Early evidence indicated two links between ecdysteroids and EH. The decline in the ecdysteroid titer to some critical value is necessary for both the initiation of release of EH and for the acquisition of the ability of the CNS to respond to EH (Truman *et al.*, 1983; Morton and Truman, 1988). EH synthesis is apparently not regulated by ecdysteroids, since EH mRNA is continuously present in the cells from the embryo to the adult (Riddiford *et al.*, 1994). The VM neurons of adult *Drosophila* possess a branch that terminates in the vicinity of the lateral clock neurons (Siegmund and Korge, 2001), suggesting a site for clock input at eclosion. This branch is not seen in the larval

stages of *Manduca* (Truman and Copenhaver, 1989), consistent with earlier evidence of a brain clock controlling eclosion in *Manduca*. However, the VM neurons do not seem to be necessary for either larval ecdysis or for eclosion. An expression system was used to drive transcription of cell death genes in the VM neurons of *Drosophila* (McNabb *et al.*, 1997; Baker *et al.*, 1999). These “EH knock-out” flies exhibited important defects in ecdysis behavior in most cases, but about one-third of the flies completed all ecdyses and emerged as adults. Moreover, these flies exhibited a normal circadian rhythm of eclosion (McNabb *et al.*, 1997). These findings suggest that although EH may be a normal component of the ecdysis control system, in the absence of EH both ecdysis behavior and the mechanism that times the behavior can be activated by alternate pathways. Jackson *et al.* (2001) concluded that multiple pathways must be present by which timed ecdysis can be initiated.

Ecdysteroids affect the Inka cells directly and in several ways. The rising ecdysteroid titers early in the molt induces EcR and then expression of the *eth* gene, leading to synthesis of both ETH and PETH in the Inka cells. The *eth* gene contains ecdysone response elements (Žitňan *et al.*, 1999). By contrast with EH (above), deletion of the *eth* gene prevented ecdysis in 98% of animals and death occurred at the transition from first to second instar (Park *et al.*, 2002). Ecdysteroids also suppress the development of responsiveness of the Inka cells to EH (Kingan and Adams, 2000) and are necessary for the abdominal nervous system to develop sensitivity to ETH. EH and ETH each promote the release of the other (Ewer *et al.*, 1997; Kingan *et al.*, 1997); thus, timed ecdysteroid signals to either the VM cells or the Inka cells would result in timed release of both EH and ETH. Both peptides activate the CCAP neurons in the abdominal ganglia; local release of CCAP acts as a proximate trigger of the ecdysis motor program (Gammie and Truman, 1997). The relative importance of EH (Ewer *et al.*, 1997; Gammie and Truman, 1997) and ETH (Žitňan and Adams, 2000) in activating the CCAP pathway is currently debated. The CCAP neurons themselves may be additional targets of ecdysteroid action (Žitňan and Adams, 2000; Jackson *et al.*, 2001) (see **Chapter 4**).

In summary, ecdysteroids appear to influence all the neuropeptides comprising the ecdysis control system, creating numerous potential control points for the timing of ecdysis by ecdysteroids. Operating in concert, these controls would effectively confine ecdysis behavior to a very narrow time window, even without input from a brain clock. Such may be the case in those larval ecdyses that are rather

loosely gated. If the ecdysteroid titer is itself under circadian control, this circadian timing could readily drive the ecdysis control system and result in circadian gating of ecdysis. The additional “direct” circadian timing of eclosion by the brain in some species may result from the differentiation during development of contacts between the VM neurons and the clock neurons in the brain. This additional source of timing information might increase the precision of timing with which EH is released, with consequent tighter synchrony between the release of EH and PETH/ETH and also enhanced synchrony among the segmentally repeated Inka cells. The advent of a brain-centered timing mechanism at eclosion could be associated with a deterioration in ecdysteroid-based timing associated with degeneration of the prothoracic glands at the end of larval life. As noted earlier in this section, precision of timing is especially critical at the adult ecdysis; therefore, direct circadian timing of EH release might represent a compensation for deterioration of ecdysteroid-based timing at this critical time.

9.4.4. Circadian Regulation of Egg Hatching

Egg hatching refers to the behaviors by which the first larval instar liberates itself from the confines of the egg shell, employing muscular contractions of the body and/or gnawing or chewing movements using cuticular structures known as egg bursters. Egg hatching is therefore the first manifestation in the life cycle of an insect of its ability to express complex behavioral patterns. Hatching occurs only once in the life of an insect; it is inextricably coordinated with the timed events of embryonic development. In an insect population entrained to stable LD cycles, egg hatching occurs with regularity and consistency at restricted times of the day; it is therefore a gated phenomenon. The time of hatching is not determined by the time of oviposition. Numerous studies have shown that the hatching rhythm is circadian in nature; it free-runs under continuous light conditions and is entrainable by light and/or temperature cycles (see below). Early experiments by Minis and Pittendrigh (1968), for example, showed that eggs of *Pectinophora gossypiella* raised in LD exhibited a distinct daily rhythm of hatching, whereas eggs raised in aperiodic conditions (LL) showed arrhythmic hatching. The rhythm could be initiated by transfer of eggs from LL to DD. Similarly, daily rhythms of egg hatching have been described in various Lepidoptera and Orthoptera; for example, the hatching rhythms of the southwestern corn borer *Diatraea grandiosella* (Takeda,

1983), the silkworm *Antheraea* (Sauman *et al.*, 1996), and the house cricket *Gryllus* (Tomioka *et al.*, 1991) all free-run in DD. Collectively, these studies show that egg hatching is controlled by an endogenous circadian oscillator, which differentiates and becomes functional prior to hatching. Thus investigations concerning egg hatching have focused on how hatching is coordinated with the progression of embryogenesis and when during embryonic development the clock that controls hatching becomes detectable.

Minis and Pittendrigh (1968) transferred arrhythmic eggs from LL to DD at various times throughout embryonic development. They obtained rhythmic hatching only from eggs transferred to DD at 50–60% of development or later; transfers at earlier times failed to induce rhythmicity in hatching. In a converse experimental design, when eggs of *Gryllus* were transferred to LL at 60% of embryogenesis or later, hatching remained rhythmic. But if transfer to LL occurred prior to 60% of embryonic development, then the larvae hatched arrhythmically (Tomioka *et al.*, 1991). Similar findings were reported by Sauman *et al.* (1996) for *Antheraea*. Interestingly, Itoh and Sumi (2000) suggested that the egg hatching rhythm of *Gryllus* was not strictly dependent on entrainment of the embryos to a light regime but appeared to be influenced by the mother as well; this implies the involvement of a maternal factor in the development of the circadian time keeping system in the embryo (possibly melatonin) (Figure 19). Together, these findings show that an endogenous photosensitive circadian clock that controls egg hatching becomes functional at about 50–60% of development. This suggests that some component of the timekeeping system, such as the light entrainment pathway or the clock itself, is either not differentiated or not fully functional prior to this point in embryonic development. Minis and Pittendrigh (1968) found that entrainment of hatching to temperature cycles became possible at about the same time as light entrainment became possible, indicating that both clock and entrainment pathways become functional at about the same time of embryogenesis. Further, exposure of eggs of *Pectinophora* to various wavelengths of monochromatic light during the second half of embryonic development demonstrated that the embryos were most sensitive to monochromatic light of 390–480 nm. This range of light sensitivity suggested that carotenoid-based photoreceptors could be responsible for light entrainment in these embryos. By contrast, carotenoid-depleted eggs of *Bombyx* (raised on a carotenoid-deprived diet) showed normal patterns of egg hatching rhythmicity, even

though the resulting first instar larvae exhibited massively reduced ocellar photosensitivity and suppressed phototaxis (Sakamoto and Shimizu, 1994). It is therefore possible that the light input pathway for the hatching rhythm may be different from that of other major rhythms such as the locomotion rhythm. In conclusion, the above observations show that both clock and entrainment pathways become established and functional at 50–60% of embryogenesis.

What developmental events occur in the embryo at this time and are they implicated in the physiological control of hatching? Neurogenesis begins at about 20–25% of embryonic development in *Drosophila* (Doe and Goodman, 1985), *Manduca* (Dorn *et al.*, 1987a), and grasshoppers (Jan and Jan, 1982). By 30% of development differentiated neurons, glial cells and sheath cells appear (Thompson and Siegler, 1993). In the CNS of *L. migratoria*, synapses and action potentials become detectable at about 70% of development (Leitch *et al.*, 1992). Nerve cells that are essential to both development and circadian time keeping become differentiated and functional during embryogenesis. Neurosecretory cells of *Manduca* become immunopositive for PTTH around 24–30% of development (Westbrook and Bollenbacher, 1990). At this young age, PTTH biological activity can also be extracted from embryos (Dorn *et al.*, 1987b). By 80% of development, the basic neuroarchitecture of these cells has been established, including axons that terminate in the CA (Westbrook and Bollenbacher, 1990). PTTH material active in bioassays was also extracted from *Bombyx* embryos in the second half of embryogenesis (Chen *et al.*, 1987). In *Locusta*, immunoreactivity for PTTH was detected in late embryos (Goltzene *et al.*, 1992). Differentiation of PGs begins around 40% of development in *Manduca* (Dorn *et al.*, 1987a) and *Locusta* (Lagueux *et al.*, 1979). Whether or not these young PGs are engaged in the synthesis of ecdysteroids is unclear (see below). Eggs are deposited with a rich supply of maternal ecdysteroids, mainly in the form of inactive conjugates. It is generally accepted that ecdysteroids are essential for the embryonic molts (Hoffmann and Lagueux, 1985; Kadono-Okuda *et al.*, 1994). Embryos undergo one or more embryonic molts, the number varying with the insect species. For example, embryos of *L. migratoria* deposit three embryonic cuticles prior to hatching; the first, the serosal cuticle, appears at 20% of development. It is followed by the first embryonic molt at about 35% and then by the second embryonic molt at about 45% of development. At 75–80% of development the cuticle of the first larval instar is deposited.

Peaks of ecdysteroids precede each embryonic molt (Hoffmann and Lagueux, 1985). *Manduca* undergoes two embryonic molts (Dow *et al.*, 1988). However, Diptera (including *Drosophila*) do not form embryonic cuticles prior to that of the first instar larva at about 75–80% of development. Embryonic molts closely correlate with increases in biologically active ecdysteroid (Dorn, 1983; Lanzrein *et al.*, 1985; Slinger and Isaac, 1988). Whether active ecdysteroids are synthesized *de novo* in the embryos (Beydon *et al.*, 1989; Dorn, 1983; Slinger and Isaac, 1988; Espig *et al.*, 1989; Horike and Sonobe, 1999; Warren *et al.*, 2002) or are produced by conversion of the inactive stored deposits (Sall *et al.*, 1983; Bownes *et al.*, 1988; Thompson *et al.*, 1988; Kadono-Okuda *et al.*, 1994) seems to vary among species. In *Bombyx*, Kadono-Okuda *et al.* (1994) applied an inhibitor of ecdysone synthesis to females at various stages of vitellogenesis, resulting in eggs with various amounts of stored ecdysteroids. The authors found that these eggs containing different amounts of ecdysteroids completed different degrees of embryonic development, and they concluded that ecdysteroids provided by the mother were essential for the normal progress of embryogenesis and egg hatching. Regardless of the origin of active ecdysteroids, the embryo apparently possesses the ability to count time and to determine when active ecdysteroids are needed for molting.

Several other neurohormones potentially important for the insect timekeeping system are also present in embryos. Truman *et al.* (1981) found that *Hyalophora* embryos contained material that was active in eclosion hormone bioassays. The amount of this material dropped at the times of embryonic ecdyses, but not at hatching. However, Fugo *et al.* (1985), obtained converse results in *Bombyx*: eclosion hormone activity did not fluctuate during embryonic molts but dropped abruptly following hatching. JH was also detected in embryos of several insects such as the cockroach *Nauphoeta cinerea* (Lanzrein *et al.*, 1985), the locust *Locusta* (Temin *et al.*, 1986), and *Bombyx* (Gharib and DeReggi, 1983) (review: Truman and Riddiford, 2002).

Where is the clock that controls egg hatching located in the embryo? The first detection of photic entrainment of the egg hatching rhythm in *A. pernyi* (50–60% of development) coincides with the first appearance of PER and TIM clock proteins in four pairs of cells of the embryonic brain (Sauman *et al.*, 1996). Staining in the brain was exclusively cytoplasmic and did not exhibit daily oscillations in and out of the nuclei, as is required of cells possessing a

molecular oscillator. At 60–70% of development, PER and TIM immunoreactivity was detected in the nuclei of midgut epithelial cells. In this location, staining exhibited robust daily oscillations that free-ran in DD. Treatment of embryos the day before hatching with *per* antisense oligodeoxynucleotide not only decreased PER immunoreactivity dramatically, but also abolished the egg hatching rhythm. Control embryos injected with a reverse orientation antisense oligonucleotide of *per* retained rhythmicity. Therefore, the *per* gene products are essential for the expression of egg hatching rhythm. Sauman and Reppert (1998) then examined the roles of brain and midgut in the control of the egg hatching rhythm (Figure 18). Brains from embryos 1 day before hatching were transplanted into recipient embryos, which were entrained to a light cycle that was phase delayed by 8 h relative to the donor brains. Hatching was then monitored in DD. It was observed that the implanted brain dictated the phase of hatching (Figure 18). By contrast, transplants of midgut did not affect the time of hatching. It became clear from these experiments that the brain is responsible for the regulation of the timing of hatching within a day (i.e., the hatching gate). This regulation is apparently achieved by the release of a “chronoactive” humoral factor from the brain, possibly a neurohormone. It was inferred that the PER-positive cells in the brain constitute the clock responsible for the egg hatching rhythm despite the failure of PER to oscillate in these cells. These cells appear to correspond to the PER-positive cells found in the brain of adult *Antheraea* (Sauman and Reppert, 1996a), which appear to regulate the release of PTH (see Section 9.4.1.2). The brain also regulates the circadian cycling of PER into the nuclei of midgut epithelial cells, but this regulation requires intact nervous connection of the midgut with the brain and does not depend on a diffusible chronoactive substance from the brain. The role of this midgut oscillator is unknown. It is possible that it regulates the timing of deconjugation of maternal ecdysteroids during embryonic molts. These considerations suggest another possible pathway by which the brain might regulate ecdysteroids during embryonic molts.

Melatonin is a well-established mediator of photoperiodic information in vertebrates and a crucial element of their timekeeping system. Embryos of *Gryllus* contain both melatonin (Figure 19b) and *N*-acetyltransferase (NAT) (Figure 19a), a key enzyme in the synthetic pathway of melatonin (Itoh and Sumi, 1998a, 1998b). Freshly laid eggs contain a rich supply of melatonin of maternal origin (Figure 19b) (Itoh *et al.*, 1994, 1995a, 1995b). At about 20% of embryonic development, the level of

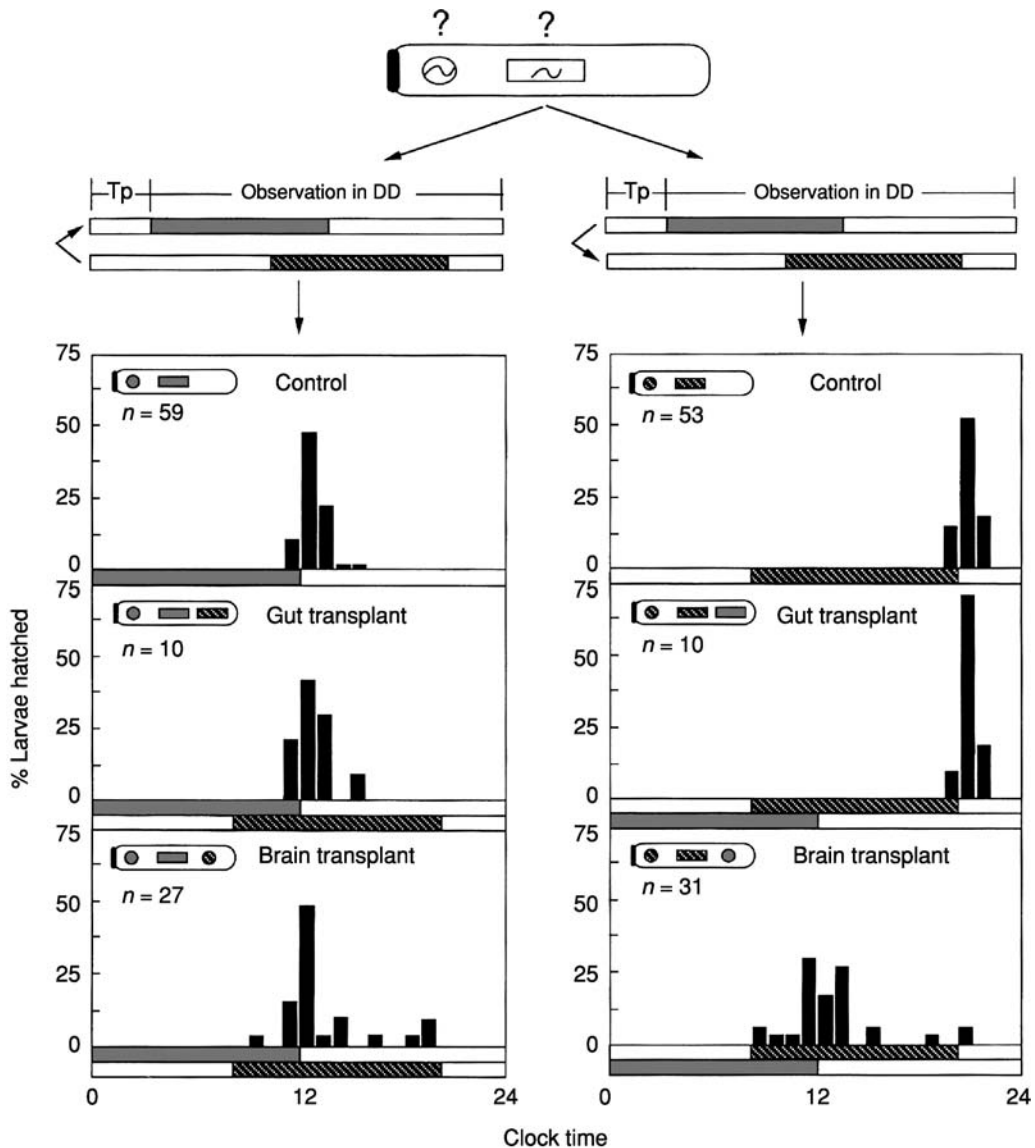


Figure 18 Experiments demonstrating a diffusible factor from the brain of developing first instar larvae of the silkworm *Antheraea pernyi* that controls the circadian gating of egg hatching behavior. Brains or midguts of embryos were transplanted (Tp) into host embryos 1 day before egg hatching. Host embryos were entrained to a light cycle that was 8 h delayed (panels on left) or 8 h advanced (panels on right) relative to the light cycle of donor embryos. The embryos were transferred to DD following surgery and allowed to hatch. The experiments on the left show that when donor guts (middle panel) or donor brains (bottom panel) were obtained from embryos that were 8 h phase delayed relative to the host, the time of hatching of host embryos occurred at the same time as that of controls (top panel). This shows that the circadian gate of hatching is determined by the host and the subsequent release of any factors from implanted tissues is redundant. Right panels show equivalent experiments using implants that were 8 h phase advanced relative to the host. Implantation of midgut did not affect the hatching time (middle panel; compare with control panel above). However, implantation of brain advanced the time of hatching to 8 h earlier than it would normally occur in controls (bottom panel). This shows that the implanted brain releases a factor that times hatching. This factor can be detected when it is released earlier than that of the intrinsic brain but not when it is released later. (Reprinted with permission from Sauman, I., Reppert, S.M., 1998. Brain control of embryonic circadian rhythms in the silkworm *Antheraea pernyi*. *Neuron* 20, 741–748; © Elsevier.)

melatonin in the eggs begins to show a clear daily rhythm; this time coincides with the first appearance of NAT in the eggs (Itoh and Sumi, 1998a, 1998b). By 40% of development, NAT was shown to free-run in DD, suggesting that clock control of NAT was operative at this time. The rhythm was also light

entrainable. These experiments show that circadian control of NAT and possibly of melatonin synthesis are initiated around mid-embryogenesis in crickets. The significance of melatonin in timekeeping in vertebrates suggests that work with melatonin in insects merits further investigation.

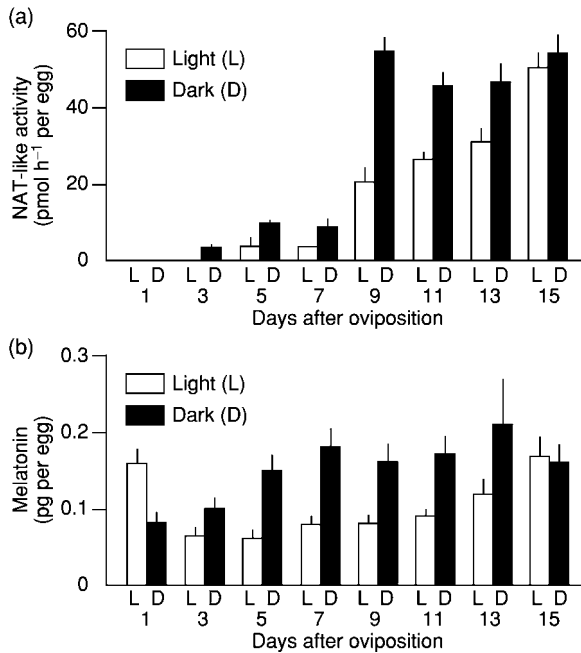


Figure 19 Changes in *N*-acetyltransferase (NAT)-like activity (a) and melatonin (b) between photophase (L) and scotophase (D) during embryogenesis of the cricket *Gryllus bimaculatus*. First instar larvae hatch around days 16–18. Newly laid eggs are devoid of NAT activity; activity is first detected at day 3 and from then on it shows daily periodic changes until hatching with highs in scotophase and lows in photophase. In contrast, melatonin is present in newly oviposited eggs. After day 3, the level of melatonin changes rhythmically with highs during scotophase and lows during photophase, suggesting intrinsic synthesis by the embryo. Photosensitivity and ability for timekeeping in the embryos of *Gryllus* is apparently established around the middle of embryogenesis. (Reprinted with permission from Itoh, M.T., Sumi, Y. 1998a. Melatonin and serotonin *N*-acetyltransferase activity in developing eggs of the cricket *Gryllus bimaculatus*. *Brain Res.* 781, 91–99; © Elsevier.)

9.5. The Adult Endocrine System

9.5.1. Circadian Regulation of the Adult Endocrine System

All the major developmental hormones (PTTH, ecdysteroids, and JH) persist in the adult. Their roles in circadian timekeeping may also persist into the adult. Rhythms in hemolymph JH levels have been reported and pathways of clock control over the CA are known. By contrast, PTTH and ecdysteroid titers have not yet been shown to be rhythmic in adults, even though there are extensive data on these rhythms in larvae. Ecdysteroids and JH have long been known to play key roles in the regulation of reproduction. There are numerous circadian rhythms in endocrine and behavioral events that are organized around and within reproductive

cycles, which raises the prospect that these hormones may also be involved in the circadian organization of reproduction.

PTTH, whose role in the circadian organization of larvae was discussed above (see Section 9.4.1.2), is also found in various adult insects. The first purification of PTTH employed heads of adult *Bombyx* (Ishizaki and Suzuki, 1994); PTTH was later identified at high levels in adult hemolymph (Mizoguchi *et al.*, 2001). Likewise, the brain of adult *Rhodnius* both contains and releases high quantities of biologically active PTTH (Vafopoulou *et al.*, 1996); further, the PTTH cells in the larval brain and their associated clock neurons persist in the adult brain (Terry and Steel, 2004). Similarly, the PTTH cells and the clock neurons that regulate PTTH release are also present in the brain of adult *Antheraea* (Sauman and Reppert, 1996b). There is therefore considerable evidence that the machinery for circadian regulation of PTTH persists in the adult. Even though the function of PTTH in the adult is currently unknown (see Chapter 6), it is conceivable that PTTH is as important to the circadian organization of the adult as it is in larvae. It is tempting to speculate that PTTH of adults continues to function in the regulation of ecdysteroids.

Ecdysteroids are present in the adult, where they are involved in various aspects of egg and sperm production and, along with JH, could be considered as the gonadotropins of insects (De Loof *et al.*, 2001). The primary source of ecdysteroids in larvae, the PGs, degenerate soon after ecdysis to the adult, but production of ecdysteroids continues in the adult by other tissues, such as the ovaries and testes. The hemolymph levels of ecdysteroids are much lower in adults than in larvae and consequently rhythmicity in ecdysteroid levels in adult insects has not yet been detected.

Synthesis and release of JH by the CA continues in the adult stage (see Chapter 8) and there is recent evidence suggesting that it may play an important role in the circadian organization of the adult. In some instances, JH synthesis is under the control of clock cells in the brain, the hemolymph level of JH is rhythmic, and JH is intimately involved in the expression of many circadian rhythms in the adult. Some of this evidence is discussed below.

JH synthesis by the CA is regulated by nerves from the brain and involves stimulatory (allatotropin) and inhibitory (allatostatin) neuropeptide inputs (see Chapter 8). The pattern of distribution of allatotropin/allatostatin immunopositive neurons in the lateral region of the protocerebrum that project to the retrocerebral complex is quite similar among different groups of insects. In *Manduca*, for

example, lateral allatostatin neurons are organized into two groups; one neuron group, the Ib cells, project immunopositive ipsilateral axons directly to the CA (Žitňan *et al.*, 1995b). Similar situations of lateral allatostatin-positive neurons that arborize terminally in the CA were found in cockroaches (Stay *et al.*, 1994), crickets (Stay *et al.*, 1994), locusts (Veelaert *et al.*, 1995), several other lepidopterans (Jeon and Lee, 1999; Audsley *et al.*, 2000), and in the ring gland of *Drosophila* (Žitňan *et al.*, 1992). Allatotropin-immunopositive lateral neurons in the brains of both *Manduca* (Žitňan *et al.*, 1995a) and *Bombyx* (Park *et al.*, 2001) also possess axons that terminate in the CA. Thus, the regulation of JH synthesis by these compounds could occur locally in the CA. In addition, these peptides may be released into the hemolymph where they may exert other regulatory functions. There is neuroanatomical evidence of connections of the circadian system to the CA by direct connection with clock cells in the brain or by indirect connection of these clock cells with the allatotropin/allatostatin cells in the brain. In adults of *Antheraea*, four PER-immunopositive cells were localized in each brain hemisphere in the protocerebrum that form a neural network; the axons of these cells coalesce to form one neural tract that projects to the CA (Sauman and Reppert, 1996a). Therefore, there is a neural pathway in *Antheraea* between clock cells and the CA that could provide circadian input directly into the CA. The situation in *Drosophila* is slightly different. In *Drosophila*, two groups of neurosecretory neurons in the protocerebrum terminate at synaptic endings within the CA (Figure 3); both these groups of neurons have dendritic fields that synapse with, and presumably receive input from, the lateral clock neurons that are PDF-positive and show cycling of *per* and *tim* (Siegmund and Korge, 2001). Thus, clock neurons are connected to the CA in both *Antheraea* and *Drosophila*, potentially providing circadian input to the gland.

In contrast to the above evidence of allatotropins as targets of clock control, there is evidence that allatotropins may provide an input to the clock. In *L. maderae*, allatotropin-immunoreactive neurons are present in the accessory medulla (Petri *et al.*, 1995), adjacent to the optic lobe clock (see Section 9.3.1). Injections of allatotropin in the vicinity of the accessory medulla resulted in stable phase shifts in the rhythm of locomotor activity (Petri *et al.*, 2002). The phase-response curves produced by injection of allatotropin were closely similar to those produced by light pulses (Figure 20). In other words, pulses of allatotropin have a similar action on the clock as do pulses of light. The authors suggested

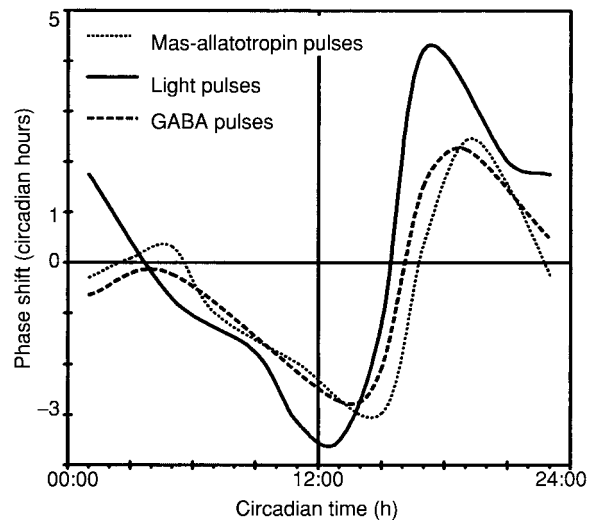


Figure 20 Phase-response curves of locomotor activity of the cockroach *Leucophaea maderae* obtained in response to injections of *Manduca* allatotropin (Mas-allatotropin), γ -aminobutyric acid (GABA), and 6 h light pulses. The close similarities of all phase-response curves suggests that both compounds may be released in response to light. (Reproduced with permission from Petri, B., Homberg, U., Loesel, R., Stengl, M., 2002. Evidence for the role of GABA and Mas-allatotropin in photic entrainment of the circadian clock of the cockroach *Leucophaea maderae*. *J. Exp. Biol.* 205, 1459–1469; © The company of Biologists Ltd.)

that allatotropin-like compounds are either a functional unit of the clock itself or modulators of photic input to the optic lobe clock in cockroaches.

Another mechanism that may be involved in the regulation of rhythmicity in the circulating levels of JH is clock-controlled availability of JH-binding proteins (JHBP) in the hemolymph (see Chapter 8). In *Drosophila*, the transcripts of the clock-controlled gene *take-out* (*to*) undergo daily cycling and free-run in DD; the circadian rhythm of *to* transcription is regulated by the *clock* and *cycle* clock genes (So *et al.*, 2000). Phylogenetic analysis of TO classifies it as a member of an insect protein superfamily that includes JHBPs from the hemolymph of *Manduca* and *Heliothis virescens*, a nuclear JHBP from epidermal cells of *Manduca* (Touhara and Prestwich, 1992; Touhara *et al.*, 1993; Wojtasek and Prestwich, 1995) and a putative protein from the head epidermis of *Manduca* that may be involved in JH binding (Du *et al.*, 2003). TO is more closely related to the hemolymph JHBPs than to the nuclear JHBPs. These observations suggest that the genes for JHBPs might also be clock-controlled. Though it is not known at present if TO binds the same ligands (if any) as JHBPs, these findings raise the exciting possibility that

JH transport and/or cell action may be regulated by the circadian availability of JHBPs.

Further indication of circadian regulation of JH comes from its involvement in rhythms of sensory responsiveness. JH plays an important role in the central nervous processing of sex pheromone olfactory input in males of *Agrotis ipsilon*. Males of this moth exhibit a daily rhythmicity of responsiveness to female sex pheromone that is synchronous with the daily rhythms of calling and pheromone release by females (Gemeno and Haynes, 2000) (see Section 9.5.4.1). Olfactory information is detected by antennal receptor neurons and then integrated centrally by interneurons in the antennal lobe (AL) of the brain (Anton and Gadenne, 1999; Gadenne *et al.*, 2001; Greiner *et al.*, 2002). Allatectomy of young males abolished behavioral responsiveness to sex pheromone (Gadenne *et al.*, 1993; Duportets *et al.*, 1996), even though the antennal olfactory neurons remained sensitive to pheromone signal (Gadenne *et al.*, 1993). Intracellular recording from the AL interneurons revealed that allatectomy significantly reduced their sensitivity to pheromone; moreover, injection of JH restored their sensitivity (Anton and Gadenne, 1999; Gadenne and Anton, 2000). Further, the proportion of AL interneurons that show sensitivity to pheromone stimulation was significantly lower in allatectomized males, but could be restored to normal levels with JH injections (Gadenne and Anton, 2000). The sensitivity to pheromones of antennal lobe interneurons increased with age and JH, whereas their sensitivity to plant volatiles was unaffected (Greiner *et al.*, 2002). Therefore, it appears that JH acts specifically on the central neural processing of sex pheromone input in males of *Agrotis*. Gadenne *et al.* (2001) showed that *Agrotis* males mate once every scotophase. Newly mated males exhibited a marked reduction in sensitivity in the antennal lobe neurons when exposed to female sex pheromones, whereas the peripheral olfactory neurons in the antennae remained highly sensitive. Sensitivity of the antennal lobe neurons recovered by the next scotophase, when the males were able to mate again. These findings show that the central neural processing system for sex pheromones undergoes daily fluctuations in sensitivity. Since this sensitivity is JH dependent, the possibility is raised that a daily rhythm of JH levels might underlie these rhythms of sensitivity.

Another example demonstrating that JH mediates rhythmicity in sensory interneurons is reported in the house cricket, *Acheta domesticus*. The males of *Acheta* exhibit a circadian rhythm of stridulation (Rence *et al.*, 1988) (see

Section 9.5.4.2). Females recognize and respond to this acoustic sexual signal by exhibiting positive phonotactic behavior. Phonotactic behavior in females is regulated primarily by JH (Koudele *et al.*, 1987; Atkins and Stout, 1994; Stout *et al.*, 1998). Allatectomy of females exhibiting phonotaxis resulted in decline in the directionality of behavior and sexual responsiveness, whereas topical JH application (Koudele *et al.*, 1987; Atkins and Stout, 1994) or CA transplantations (Stout *et al.*, 1976) restored them. It was found that phonotactic thresholds were regulated by changes in the level of JH, apparently by JH influencing the thresholds for firing action potentials in the L1 auditory interneuron; the L1 interneuron is a prothoracic ascending neuron that is essential for phonotactic responses to low-intensity calling songs. Intracellular and extracellular recordings of the firing of action potentials of the L1 interneuron revealed that allatectomy increased the phonotactic thresholds (Stout *et al.*, 1998). Topical application of JH onto the prothoracic ganglion or JH injection directly into the ganglion reduced both the firing thresholds of the L1 interneurons to nearly normal levels and the loudness of a mock calling song required to elicit phonotaxis (Stout *et al.*, 1998). The effects of applied JH were rapid and seen within 2 h. JH applications to other ganglia were ineffective (Stout *et al.*, 1991). Moreover, a daily rhythm in the threshold levels for phonotaxis was detected in day 3 females (Stout *et al.*, 1998), suggesting that a daily rhythm in JH levels may be the underlying cause for the observed changes in thresholds.

Evidence of daily rhythms in JH titers in hemolymph is accumulating. A clear daily rhythm is seen in *Gryllus firmus* (Zhao and Zera, 2004) (Figure 21), and day–night differences in JH titers are seen in *Apis mellifera* (Elekonich *et al.*, 2001) and *H. virescens* (Shu *et al.*, 1998). In the case of *Gryllus*, a clear JH rhythm is seen in the flight-capable (long-winged) morph, but only a weak rhythm is seen in the flightless (short-winged) morph; this finding again indicates a relationship between JH rhythmicity and behavior. In the case of honeybees, the transition from behavioral arrhythmicity of nursing bees to the rhythmic behavior of foraging bees was tentatively linked to the expression of the clock gene *per* in the brain (Toma *et al.*, 2000). *per* expression in foragers cycles in a circadian fashion and free-runs in DD. Cycling of *per* is evident in both nurses and foragers. However, the relative levels of *per* mRNA and the amplitude of cycling increased significantly with the transition from nursing to foraging, consistent with the pathways

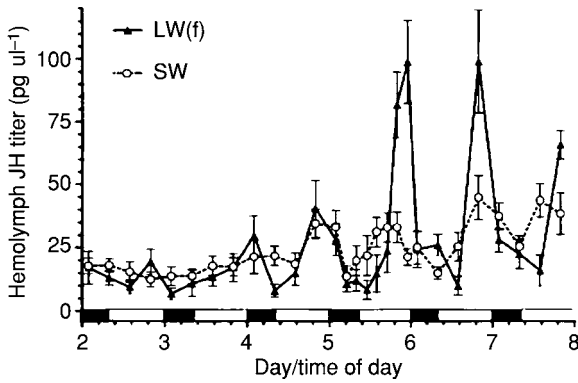


Figure 21 Daily rhythm in the hemolymph JH titer of the flight-capable (LW, long-winged) female morphs of *Gryllus firmus* (solid line). Note the daily peaks during day to night transitions. Flightless, short-winged (SW) females (dashed line) show only a weak daily rhythm in JH titer. (Reproduced with permission from Zhao, Z., Zera, A.J., 2004. The hemolymph JH titer exhibits a large-amplitude, morph-dependent, diurnal cycle in the wing-polymorphic cricket, *Gryllus firmus*. *J. Insect Physiol.* 50, 93–102.)

discussed above for control of JH by clock cells in the brain. In the honeybee *Apis cerana japonica*, *per* transcripts also oscillate daily in the brains of foragers; no comparisons were made with *per* transcripts in the brains of nurses (Shimizu *et al.*, 2001). These findings show that rhythmicity in JH titer does occur and that it may be driven by the brain.

A relationship between JH and behavioral activity rhythms is also seen in *Blattella germanica*. Males of this cockroach exhibit a bimodal circadian rhythm of locomotor activity that persists throughout their life cycle (Leppla *et al.*, 1989). Alternatively, females show a more complex pattern of locomotor activity that is clearly synchronized with the timing of each gonadotropic cycle. Immediately after emergence, the females exhibit a daily rhythm of locomotor activity. However, when they become sexually receptive during the first gonadotropic cycle, rhythmicity in locomotion is lost and the females become active throughout the day (Lee and Wu, 1994). Relative inactivity follows the expulsion of the first ootheca and then the females again become rhythmic for several days until the second gonadotropic cycle begins. Thus, rhythmic locomotion is confined to periods of expected high JH titer synchronized with gonadotropic cycles. Ovariectomy of sexually receptive females resulted in a stable circadian rhythm of locomotor activity, which was disrupted and reduced in amplitude by allatectomy (Lin and Lee, 1998). Therefore, factors associated with reproduction (perhaps ovarian factors) seem to mask the underlying circadian rhythm of locomotor activity. Topical application of JH increased the overall level of locomotor activity but it did not

restore the behavioral circadian rhythm. However, JH applied topically enters the hemolymph continuously and over many days; this method of application does not provide a rhythmic supply of JH. Therefore, it is necessary to determine if JH synthesis is rhythmic in the cockroach in order to define its role in behavioral rhythms.

JH also appears to play a role in the regulation of rhythmic pheromone production in moths (see Section 9.5.3.1). In the females of the moth *A. ipsilon*, the pheromone level in the gland (Gemeno and Haynes, 2000), pheromone production by the gland (Picimbon *et al.*, 1997), and calling behavior (Gemeno and Haynes, 2000) all exhibit synchronous daily rhythmicities. Allatectomy of newly emerged females inhibited calling behavior; operated females neither contained nor released pheromone, even though their brain–SEG complexes contained high levels of activity of pheromone biosynthesis activating neuropeptide (PBAN) (Picimbon *et al.*, 1995). Apparently, the inhibitory effect of allatectomy on pheromone production was not solely due to disruptive effects on the gonadotropic cycle. Injections of PBAN or JH restored pheromone production but not rhythmicity in allatectomized females, suggesting that allatectomy prevented PBAN release. In contrast, JH III injections into decapitated females lacking a brain–SEG complex were ineffective in inducing pheromone production, suggesting that JH did not act directly upon the pheromone gland. Rather, JH appears to be necessary for the release of PBAN and it may do so by promoting release at “precisely gated times” (Picimbon *et al.*, 1995). Similarly, in *Helicoverpa armigera*, both pheromone production and PBAN levels are rhythmic (Rafaeli *et al.*, 1991). In this moth, JH treatment induced precocious pheromone production in newly emerged females (Fan *et al.*, 1999). However, if the animals were decapitated immediately after emergence, injections of neither JH nor PBAN were effective, whereas simultaneous injection with both compounds was stimulatory. Likewise, *in vitro* treatment with both JH and PBAN, but not either alone, induced pheromone production in inactive pheromone glands from pharate adults. These findings were interpreted as evidence that the role of JH is to prime the PBAN system. The possibility should be acknowledged that JH may play a permissive role in the above systems, i.e., its presence is required to allow these rhythmic phenomena to occur as opposed to actually controlling these rhythmicities. Elucidation of the role of JH in the regulation of daily pheromone release requires experiments on animals that

have commenced pheromone release and calling behavior.

Some general conclusions can be drawn from all the above. First, it appears that JH rhythmicity may be a widespread phenomenon among adult insects. Second, rhythmicity in JH could be responsible for the coordination of numerous circadian rhythms related to reproduction. Third, PTTH and ecdysteroids, which are known to be controlled by a circadian system in larvae, as well as the neuronal pathways that mediate their circadian control, all persist in the adult. Therefore, it is conceivable that circadian regulation of all three hormones, PTTH, ecdysteroids, and JH, participate in the regulation of rhythmic reproductive processes. Therefore, the putative central timing system in larvae that was discussed in the previous sections, may well continue functioning in the adult through circadian control of the same hormones. Unfortunately, evidence of a central circadian mechanism coordinating reproductive processes is currently fragmentary.

9.5.2. Circadian Regulation of Gamete Production

The existence of circadian rhythms in gametogenesis has received scant direct experimental attention. However, accumulating evidence of rhythmicity in the hormones that regulate gametogenesis, combined with substantial evidence that the output of mature gametes (at oviposition or mating) is also under circadian control, indicate that rhythmicity pervades the entire process of gamete production. Evidence of this view will be summarized prior to an examination of detailed rhythmic mechanisms in males and females below. There are three lines of evidence that are collectively compelling.

First, the evidence of circadian control of reproductive hormones is growing rapidly (see Section 9.5.1). The primary function of these hormones is regulation of gametogenesis. It would be surprising if rhythmicity in the secretion of regulatory hormones was not accompanied by rhythmicity in gametogenesis itself. The photoperiodic regulation of reproductive diapause is a clear illustration that clock control of reproductive hormones is employed to regulate gametogenesis. It is firmly established that both JH and ecdysteroids exert a huge variety of actions on reproductive processes in both sexes (Hagedorn, 1985, 1989; Koeppe *et al.*, 1985; Wyatt and Davey, 1996; De Loof *et al.*, 2001); therefore, there are numerous potential pathways by which rhythms in these hormones could evoke rhythmicity in egg or sperm production. For example, JH regulates vitellogenin production by the fat body and its uptake by the ovary, and in males it is involved

in the regulation of accessory gland functions and affects reproductive behavior and spermatogenesis. Ecdysteroids are produced by both testes and ovaries (Gillott, 1995) and are regarded as possible insect sex steroid hormones (De Loof *et al.*, 2001). In females, ecdysteroids promote cell growth in egg follicles and in Diptera they regulate vitellogenin synthesis by both fat body and ovary. In males, ecdysteroids promote cell divisions in spermatocytes. Rhythmicity in any of these hormonal control pathways would likely lead to rhythmicity in gamete production.

Second, the discharge of mature eggs or sperm from the animal is frequently under circadian control. For example, the oviposition of mature eggs is often regulated by a circadian clock as is the mating activity of males (see Section 9.5.4.3). These behavioral rhythms require the presence of mature gametes at appropriate times of the day. The necessary coordination between these behavioral rhythms and gametogenesis could be achieved most directly by circadian control of gametogenesis.

Third, cyclic expression of the clock gene *per* has been reported in both ovaries and testes. In both ovarian follicle cells (Hardin, 1994; Hall, 1998) (see Section 9.5.2.1) and vas deferens epithelial cells (Gvakharia *et al.*, 2000) (see Section 9.5.2.2), *per* is expressed in a cycling fashion in LD but fails to free-run in DD or LL. These experiments are described in detail later. In other words, *per* cycles but not with the circadian properties that are required of a true clock. Such behavior of *per* is characteristic of oscillators that require a driving input (see Section 9.3.3). This requirement for a driving input to sustain *per* cycling in the reproductive system is further evidence of daily, rhythmic inputs to the system, most probably from rhythms in reproductive hormones. Hormones are well known as agents that entrain oscillators in vertebrates (review: Balsalobre, 2002) (see Section 9.3.3), and also in insect development (Steel and Vafopoulou, 1999) (see Section 9.4.1.4).

The above considerations lead to a testable model for the circadian regulation of gametogenesis. We suggest that a central timing system (in the brain?) controls the rhythmic release of neuropeptides that regulate rhythms in JH and/or ecdysteroids. JH and/or ecdysteroids provide the driving input to a *per*-based oscillator in the reproductive system, leading to rhythmic formation of gametes. Such a rhythm of gamete formation would be synchronized with rhythmic transport of eggs or sperm through the ducts of the reproductive system. These rhythms serve to make mature eggs and sperm available rhythmically for oviposition and mating behaviors,

respectively. These behaviors are themselves frequently rhythmic and under circadian control. This synchrony with behaviors implies action of JH and/or ecdysteroids on the nervous system, where they could act as modulators of the neural pattern generators for reproductive behaviors. Synchrony with behavioral rhythms could also be achieved centrally, by coupling of the putative neuroendocrine clock with the locomotor clock in the brain. Circadian rhythmicity potentially pervades reproductive mechanisms at all levels and in both sexes. Some of the known mechanisms are described below.

9.5.2.1. Oogenesis and egg transport Early studies with *Drosophila* by Allemand (1976a, 1976b) raised the possibility that oogenesis was rhythmic. Daily variations were reported in the frequency of egg chambers in various stages of development. These observations were interpreted as a daily rhythm in vitellogenesis, specifically in the time of onset of vitellogenic stages 8–10. The rhythm free-ran in DD for at least 5 days, implying that vitellogenesis (or at least some aspect of it), was under circadian control. Supporting evidence of such an “ovarian clock” derives from the localization of *per* transcripts and PER protein in the follicle cells surrounding small previtellogenic oocytes of *Drosophila*. However, this ovarian *per* transcript did not cycle (Hardin, 1994) and the PER protein was exclusively cytoplasmic, showing no cyclic migration to the nucleus (Liu *et al.*, 1988; Saez and Young, 1988). This unclocklike behavior of *per* argues against the presence of a true clock involving *per* in the follicle cells (Hall, 1998). However, the notion of an oscillator residing within the follicle cells merits further exploration partly because it is these cells that synthesize ecdysteroids in many adult insects (Hagedorn, 1985, 1989). In addition, follicle cells are important targets of both JH (Wyatt and Davey, 1996) and ecdysteroids (Carney and Bender, 2000). Possibly, follicle cell oscillators are entrained by (rhythmic) hormonal signals and regulate rhythmicity in vitellogenesis by the oocyte that they surround. In other words, a follicle cell oscillator could be part of a pathway by which rhythmic endocrine signals lead to a rhythm in vitellogenesis.

Rhythmic mating can also lead to rhythmic egg development, again through endocrine pathways. As in many insects (see Section 9.5.4.2), mating in *Drosophila* is rhythmic and under circadian control (Sakai and Ishida, 2001). Mating accelerates egg maturation in *Drosophila*. After mating, there is an acceleration of yolk protein accumulation in the developing eggs, so that a large number of mature eggs is available for insemination. The sex peptide that

is transferred to females by males at mating is responsible for enhanced transcription of yolk proteins in the ovary, for enhanced yolk protein uptake by the ovary (Soller *et al.*, 1997), and also for ovulation (Ottiger *et al.*, 2000). These effects of sex peptide can be simulated by methoprene application (Soller *et al.*, 1999). Sex peptide has also been shown to stimulate JH biosynthesis (Moshitzky *et al.*, 1996). Mating also affects ecdysteroid levels, which affect vitellogenin synthesis by both ovary and fat body (Harshman *et al.*, 1999; Soller *et al.*, 1999). Therefore, rhythmic mating activity could readily lead to rhythmic changes in both JH and ecdysteroid levels and consequential rhythmic stimulation of vitellogenesis.

In many species, oviposition occurs with a circadian rhythm (see Section 9.5.4.3). The time interval between ovulation and oviposition is often both constant and quite brief, implying that rhythmic oviposition may be synchronized with, or may even be a consequence of, rhythmic ovulation. The rhythmic removal of eggs from the reproductive system requires that mature eggs are available for oviposition at circadian intervals. The simplest and most efficient mechanism by which this could be achieved would be by circadian regulation of egg maturation.

The control of ovulation and oviposition involves a multiplicity of hormones acting in species-variable patterns (Nijhout, 1994). The factors that regulate contractions of the oviducts have been extensively studied in *L. migratoria* (Donini *et al.*, 2001). In this insect, transverse nerves, the caudal sympathetic system, and local neurons innervate the oviducts. Numerous hormones and neurotransmitters such as CCAP, FMRF-related peptides, proctolin, glutamate, and octopamine are all involved in regulation of the oviduct contractions. The orchestration of these various factors into the observed circadian rhythm of oviposition has not yet been examined in any insect.

9.5.2.2. Spermatogenesis and sperm transport In the male reproductive system, circadian involvement has been demonstrated clearly in the duct system that transports sperm, but not in spermatogenesis itself. The absence of data does not imply that rhythmicity is absent; it could readily result from rhythmicity in the endocrine factors that regulate spermatogenesis. Both JHs and ecdysteroids regulate spermatogenesis in a complex and apparently species-variable manner (Dumser, 1980; Hagedorn, 1985; Koeppe *et al.*, 1985; Hardie, 1995; Wyatt and Davey, 1996). For example, it has been shown that both hormones act directly on the testis where they regulate rates of cell division at specific stages

of spermatogenesis. In *B. mori*, JH affects the rate of division in spermatocytes (Kajiura *et al.*, 1993). Ecdysteroids stimulate mitotic divisions in spermatogonia of *Rhodnius* (Dumser, 1980) and meiotic divisions in both *Manduca* (Friedländer and Reynolds, 1988) and the European corn-borer, *Ostrinia nubilalis* (Gelman *et al.*, 1988). Thus, rhythms in ecdysteroids and/or JH (see Section 9.5.1) could result in rhythms of cell division in the testes. Also, other rhythmic inputs, such as mating, could impose rhythmicity on these endocrine control pathways and result in rhythmicity in spermatogenesis.

In several Lepidoptera, the movement of the sperm through the reproductive system is under circadian control. In stark contrast to the female system, it is alleged that neither nerves nor hormones are involved in the regulation of this movement (Giebultowicz, 2000). In Lepidoptera, sperm that are released from the testis are transported along the vas deferens by muscular contractions as aggregates known as bundles. These bundles may remain intact until after mating. In whole animals or isolated abdomens of *P. gossypiella* and *Ephestia (Anagasta) kuehniella* and *Spodoptera litura*, the sperm bundles move along the ducts in a series of distinct steps that is repeated as a daily rhythm (Riemann and Thorson, 1971; Riemann *et al.*, 1974; LaChance *et al.*, 1977; Thorson and Riemann, 1977; Seth *et al.*, 2002). This rhythmic movement of sperm bundles was also found in *Spodoptera littoralis* and *Lymantria dispar*, using *in vitro* incubations of organ complexes which contained the testis and upper vas deferens (UVD) and in some instances the seminal vesicle (SV) as well (Giebultowicz *et al.*, 1988; Bebas *et al.*, 2001). This rhythm of bundle transport free-ran in DD and was phase-shifted by light cues *in vitro* showing that a clock that controls the rhythm of transport resides within the reproductive system. A rhythm of sperm transport is also seen in *E. kuehniella* and *Cydia pomonella in vivo*, but fails to persist *in vitro* (Bebas *et al.*, 2001); in these species it appears that rhythmicity in sperm transport is driven by factors outside the reproductive system.

Thus, in some moths, the reproductive system contains a photosensitive circadian clock that regulates rhythmic transport of sperm. The literature refers to this as a rhythm in “sperm release,” in which the liberation of sperm from the testis and their subsequent movement by the ducts are considered as two component steps of a single rhythmic event. If correct, this view requires that liberation of sperm from the testis is also rhythmic, a phenomenon that suggests rhythmicity in spermatogenesis

itself. However, studies of the “sperm release rhythm” generally involve only studies of transport and not of release itself. Consequently, the contribution of rhythmic sperm release from the testis to the observed rhythmic movement of sperm along the ducts is unknown. However, these two processes are influenced differentially by ecdysteroids (see below), suggesting that they should be regarded as distinct events.

As mentioned above, translocation of sperm from the testis along the ducts results from muscular contractions of the wall of the UVD. The UVD of *Ephestia* consists of layers of longitudinal and circular muscle and a single cell layer of inner epithelium (Riemann and Thorson, 1976). The nerve supply to the vas deferens is abundant and evident in the lower part, and sparse (but not absent) in the UVD (Riemann and Thorson, 1976; Thorson and Riemann, 1977). In *Lymantria*, structures resembling axons were seen between the circular muscle and the basement membrane of the UVD epithelium (Giebultowicz *et al.*, 1996). Evidence of a local nerve supply is derived from analysis of the complex patterns of muscular contractions exhibited *in vitro* by the testis–UVD–SV complex (Giebultowicz *et al.*, 1996). Several distinct patterns of contractions occur in the UVD, each of which requires coordination of contraction of the circular and longitudinal muscle layers. Further, abrupt switches between these patterns also occur. One of these contraction patterns was responsible for the mass transfer of sperm bundles down the UVD to the SV (Giebultowicz *et al.*, 1996). The onset of this pattern could occur *in vitro*, showing that it was not driven directly from the CNS. This pattern commenced at the normal time of the day for sperm transfer *in vivo*, implying that the clock may control the daily time of switching between motor patterns in the UVD. The overall complexity of the contraction patterns as well as the abrupt switching between them both strongly imply the presence of local neural regulation. By contrast, the literature states that the musculature of the UVD appears to receive no innervation and that the changes in contractile pattern occur autonomously (Giebultowicz *et al.*, 1996). The functional requirements for the transport of sperm bundles along the male ducts are basically the same as those for transport of eggs along the female ducts. The importance of the local nerve supply employing a multitude of neurotransmitters has been well documented for control of the movement of eggs along oviducts (Orchard and Lange, 1988; Donini *et al.*, 2001) (see Section 9.5.2.1). Therefore, we suggest that transport of sperm bundles is regulated by a

currently unidentified local nerve supply. Viewed in this light, the mechanisms of gamete transport in the male system become more comparable to those of the female system than the current literature suggests.

The location of the clock that controls the rhythmicity of sperm transport is unclear. In *C. pomonella* both *per* transcripts and PER were localized to epithelial cells lining the UVD wall. Both transcript and protein cycled in LD, but the *per* transcript showed “statistically insignificant” fluctuations in DD and the rhythm was “disrupted” in LL (Gvakharia *et al.*, 2000). It should be recalled that this behavior of *per* is very similar to that seen in the ovaries, where it was dismissed as unclocklike (Hall, 1998) (see Section 9.5.2.1). Such *per* oscillations that are not self-sustaining are characteristic of peripheral oscillators, that are driven by rhythmic inputs such as hormones (see Section 9.3.3), as has been argued for the ovarian *per* oscillator (see Section 9.5.2.1). The epithelial UVD oscillator seems unlikely to represent the clock that controls sperm transport since (1) the oscillator is not self-sustaining (i.e., does not free-run), whereas the rhythm in sperm transport does free-run, and (2) there is no known pathway or mechanism by which epithelial cells could regulate patterns of contraction by the overlying layers of muscles. We conclude that the epithelial oscillator likely regulates activities of the epithelial cells themselves but is not the clock that regulates rhythmic sperm movement. Indeed, these epithelial cells exhibit rhythmic secretion into the duct lumen (Riemann and Giebultowicz, 1991; Giebultowicz *et al.*, 1994) and regulate the rhythmic acidification of the lumen (Bebas *et al.*, 2002), which contributes to the maturation of sperm in the UVD (Riemann and Thorson, 1976).

The organ system used above *in vitro* to study rhythmicity in sperm movement consisted of most of the reproductive system including the testis, which is the largest endocrine organ in adult males. The testis is the only confirmed site of ecdysteroid synthesis in adult male moths (Loeb *et al.*, 1984, 1988). Sperm movement from the testis into the vas deferens was temporarily inhibited by the injection of 20E into adults of *Anagasta* (Thorson and Riemann, 1982) or infusion of 20E into pharate adults of *Lymantria* (Giebultowicz *et al.*, 1990), both in a dose-dependent manner; higher doses of 20E produced greater inhibition. The effectiveness of injections of 20E into pharate adults of *Lymantria* decreased as development proceeded, suggesting that a decline in the ecdysteroid titer was necessary for the initiation of sperm transport. However, Thorson and Reimann (1982) had shown earlier

that 20E injection into isolated abdomens of adults of *Anagasta* also inhibited sperm transport, even though the normal decline in ecdysteroids titer was complete in these animals. These authors reported that the inhibition of sperm release by injected 20E did not appear to be due to inhibition of spermatogenesis because there was an accumulation of unreleased eupyrene sperm cysts at the junction of the testis and UVD in animals injected with 20E. Interestingly, the release of apyrene sperm was unaffected. Further, the rhythm of transport of sperm down the UVD was also unaffected by 20E (Figure 22). These differential actions of 20E on sperm release and sperm transport suggest that these two processes may represent separate distinct rhythms. It is possible that at least some of the reported events occurring in the testis–UVD–SV complex *in vitro* could be affected, or even caused, by ecdysteroids released from the testis *in vitro*. It seems important to examine the moth testis for rhythmicity in ecdysteroid synthesis, since this could drive rhythmicity in various events associated with sperm release and transport. This organ system is also potentially the source of a plethora of neurotransmitters, neuromodulators, and hormones from unidentified tissue adhering to the reproductive system, as is the case with the female system. It is therefore likely that a local nerve supply, various neuropeptides, neuromodulators,

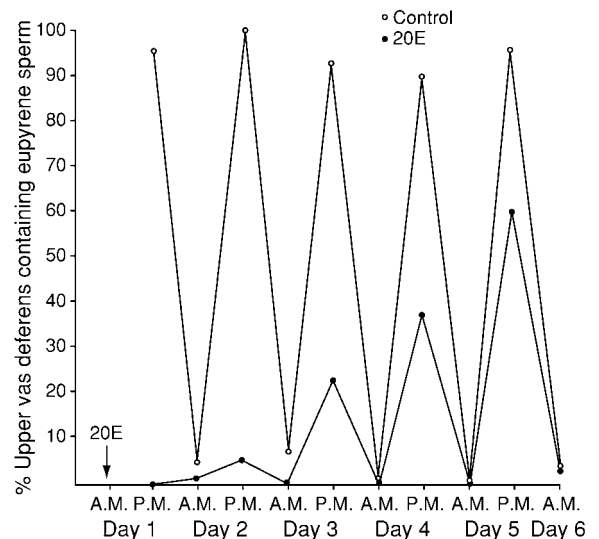


Figure 22 Injection of 20E into isolated abdomens of *Anagasta kuehniella* temporarily reduced the number of eupyrene sperm released into the upper vas deferens, but did not appear to affect the daily rhythm of sperm release. (Reprinted with permission from Thorson, B.J., Riemann, J.G., 1982. Effects of 20-hydroxyecdysone on sperm release from the testes of the Mediterranean flour moth, *Anagasta kuehniella* (Zeller). *J. Insect Physiol.* 28, 1013–1019; © Elsevier.)

and ecdysteroids all interact to regulate the timing of sperm movement.

The time during adult development at which the clock that controls rhythmic sperm transport becomes operational was examined in *Lymantria* (Giebultowicz and Joy, 1992). At various times during adult development, pupae were transferred from LD to DD, or given a light pulse in DD. Pupal day 6 was found to be the earliest day on which a light cue could initiate rhythmicity in sperm release, i.e., 2 days before sperm transport normally commences. However, this is also the time of peak ecdysteroids in the hemolymph (Giebultowicz *et al.*, 1990) as well as the testis (Loeb *et al.*, 1984, 1988), suggesting that the clock becomes functional in the presence of ecdysteroids. In an attempt to determine if 20E interacted with the clock in the reproductive system 10–20 µg 20E were injected into animals that were maintained in LD (Giebultowicz *et al.*, 1996). Appearance of sperm in the UVD was abolished for 1 day but then resumed, as previously found in isolated abdomens of adult *Anagasta* by Thorson and Reimann (1982). It was inferred from this experiment that 20E does not affect the phase of sperm release. However, it is unlikely that any phase shift could be produced by 20E in this experiment due to the presence of simultaneous but conflicting light entrainment of the rhythm.

Some general conclusions can be drawn from the above studies with the male system. Sperm bundles are transported through the male duct with a circadian rhythm under the control of a photosensitive clock that probably acts on the muscles via a local nerve supply. Sperm release from the testis may also be rhythmic and possibly regulated by ecdysteroids, even *in vitro*. The *per*-based oscillator in the UVD epithelium regulates rhythmic secretion by these cells and seems to be a driven oscillator, possibly entrained by ecdysteroids. This system offers valuable opportunities for the study of interactions between nerves and hormones in the regulation of peripheral oscillators. The shortage of information regarding nervous and hormonal regulation is not an evidence that the clock is autonomous.

9.5.3. Rhythmic Neuroendocrine Control of the Sex Pheromone Signaling System

Communication between the sexes prior to mating may be performed by visual, auditory, or chemical (pheromonal) signals. The use of sex pheromones is widespread among insects. Sex pheromones are a heterogeneous collection of small, volatile chemical compounds, which may be released by either sex (usually by the female) depending on the species, to advertise sexual receptivity and availability. Sex

pheromones vary greatly among species and there are extensive studies of their chemical composition, pathways and sites of biosynthesis in various insect groups. A common site of pheromone synthesis is the pheromone gland, but there are notable exceptions (e.g., in Diptera). Release of sex pheromones is achieved when the advertiser displays a characteristic pattern of sexual behavior called “calling,” which involves a repertoire of body movements that expose the gland to the air and facilitate dispersal of pheromone in the environment.

In almost all insect groups with a long adult life, including cockroaches, beetles, Diptera, and moths, both the behavior of calling and the accompanied release of sex pheromones occur with a daily rhythm. The females of the brown-banded cockroach, *Supella longipalpa*, for example, engage in nightly rhythmic calling behavior during which they release volatile sex pheromone; the calling rhythm is truly circadian since it free-runs in both DD and LL and is phase-set by light (Smith and Schal, 1991). Release of pheromone with a daily periodicity has also been shown in several female beetles including the scarab beetle *Anomala albopilosa* (Leal *et al.*, 1996) and the bruchid beetle *Callosobruchus maculatus* (Shu *et al.*, 1996). In some flies, the males release sex pheromones and the females respond to them. Sex pheromones in Diptera are synthesized by abdominal oenocytes and then are deposited onto the surface of the cuticle following transportation via the hemolymph to epidermal cells (Tillman *et al.*, 1999). In males of the Caribbean fruit fly, *Anastrepha suspensa*, a daily rhythm in the amount of sex pheromone circulating in the hemolymph was observed, which suggested possible daily changes in synthesis (Teal *et al.*, 1999b). These daily variations in the level of sex pheromone in the hemolymph correspond closely with the daily rhythms of calling and pheromone release (Nation, 1990; Epsky and Heath, 1993). The response of females to the release of sex pheromones by males is also rhythmic in other Diptera, such as *Rhagoletis ceraci* (Katsoyannos, 1982), *Culex quinquefasciatus* (Jones and Gubbins, 1979), and *Musca domestica* (Sybchev *et al.*, 1986) and this is usually taken to signify the occurrence of rhythmic pheromone release by the males.

Moths are the most widely studied group for rhythmicities in both calling behavior and pheromone production/release. In moths, the pheromone glands are usually located beneath the intersegmental membrane between the 8th and 9th posterior abdominal segments and they often form extrusible sacs (Bjostad *et al.*, 1987). These sacs do not usually have reservoirs for pheromone storage, so that release follows synthesis quickly. Release usually

occurs as a result of calling behavior, which facilitates the exposure of the pheromone gland. However, in some Lepidoptera such as *Bombyx*, reversal of the heartbeat causes changes in the hemolymph pressure, which results in eversion of the gland and pheromone dispersal (Ichikawa and Ito, 1999). In moths calling occurs rhythmically every day until mating. Mating then inhibits calling behavior either temporarily or permanently. Numerous studies have documented daily rhythms in calling and regulation of the rhythm by an endogenous circadian clock. In *Lymantria*, calling occurs with a daily rhythm which is abolished in LL but reinstated by transfer back to DD (Webster and Yin, 1997). In the tiger moth, *Holomelina lamae*, the calling rhythm free-runs in both DD and LL, but damps out quickly in LL. Reintroduction of LD reentrains the rhythm (Schal and Cardé, 1986) (Figure 23). Rhythmicity in calling was abolished when these moths were reared in

DD from the second instar onwards. When these DD moths were grouped according to the time of eclosion, the authors were able to detect a clear rhythm. This suggests that the clock that controls the calling rhythm may be phase-set at ecdysis. Temperature compensation of the calling rhythm was seen in *Congether punctiferalis* (Kaneko, 1986). Circadian rhythms in calling behavior were also seen in *Heliothis armigera* (Kou and Chow, 1987), *Pseudaletia unipuncta* (Delisle and McNeil, 1987), *Helicoverpa assulta* (Kamimura and Tatsuki, 1993, 1994), and *Manduca* (Itagaki and Conner, 1988). It therefore appears that in moths rhythmicity in the behavior of calling is a widespread, if not universal, phenomenon which is governed by an endogenous circadian clock mechanism.

The striking periodicity of calling behavior in moths implies that pheromone production/release is also rhythmic. Indeed, many studies have shown

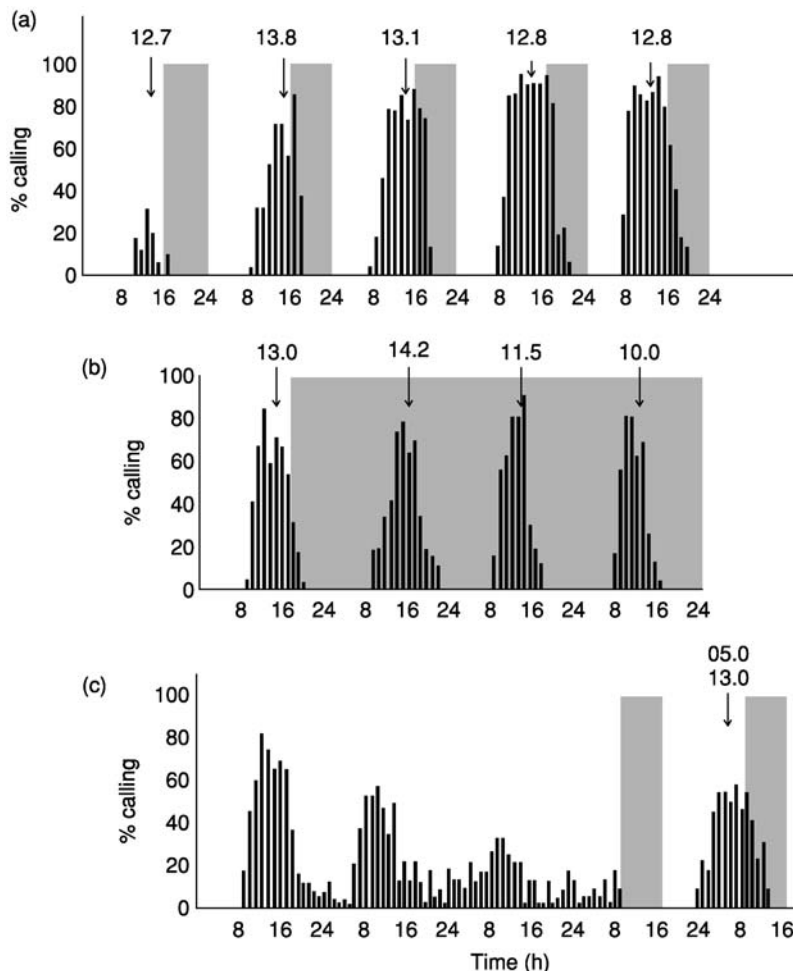


Figure 23 Entrained rhythm of calling behavior of females of the tiger moth, *Holomelina lamae* (Freeman) (a). The rhythm free-runs in DD (b) and damps out after a few cycles in LL (c). Reintroduction of the light cycle reestablishes the rhythm (right end of c). (Reproduced with permission from Schal, C., Cardé, R.T., 1986. Effects of temperature and light on calling in the tiger moth *Holomelina lamae* (Freeman) (Lepidoptera: Arctiidae). *Physiol. Entomol.* 11, 75–87; © Blackwell Publishing Ltd.)

clear, circadian rhythmicities in pheromone content of the gland and/or pheromone release. Frequently, a close temporal relationship is found between daily changes in content of the gland, release of pheromone, and calling. For example, in the diurnal moth *L. dispar*, the pheromone content of the gland (Tang *et al.*, 1992), the rate of pheromone emission from the gland (Charlton and Cardé, 1982), and the calling behavior (Webster and Yin, 1997) all exhibit synchronous daily rhythms with maxima in photophase and minima in scotophase. In the nocturnal moth *H. virescens*, the daily rhythmic changes in content of the gland (Rafaeli and Soroker, 1989; Mbata and Ramaswamy, 1990) and emission from the gland (Pope *et al.*, 1982) are also synchronous, but occur with peaks in the scotophase and troughs in the photophase. In *H. assulta*, the quantities of all five individual components of the moth's pheromone cocktail in the gland fluctuated rhythmically during the day (Choi *et al.*, 1998a) (Figure 24a) in synchrony with the calling rhythm (Kamimura and Tatsuki, 1993). Circadian control of rhythmicity was also shown in *Pseudaletia* (Delisle and McNeil, 1987), *Adoxophyes* spp. (Kou, 1992), *Agrotis segetum* (Löfstedt *et al.*, 1982), *Bombyx* (Kuwahara *et al.*, 1983; Sasaki *et al.*, 1984), *Choristoneura fumiferana* (Ramaswamy and Cardé, 1984), *Mamestra brassicae* (Noldus and Potting, 1990; Iglesias *et al.*, 1999), *Phthorimaea poerculella* (Ono *et al.*, 1990), *Platynota sultana* (Webster and Cardé, 1982), *Sesamia nonagrioides* (Babilis and Mazomenos, 1992), and *S. littoralis* (Dunkelblum *et al.*, 1987). In *Epiphyas postvittana* (Foster, 2000), rhythmicity in the pheromone content of a gland with no reservoir appears to result solely from periodic changes in pheromone synthesis, which is tightly coupled to release; there is no evidence of rhythmic degradation.

Thus, rhythmicity in the release of sex pheromone seems to be regulated directly at the level of synthesis. This fact implies that the factors that regulate synthesis may also be rhythmic (see Sections 9.5.3.1 and 9.5.3.2). However, there are some notable exceptions; in *Trichoplusia ni* release of pheromone occurs rhythmically (Sower *et al.*, 1970), but pheromone production and release are not synchronous (Hunt and Haynes, 1990). Rather, synthesis of pheromone appears to be continuous and the daily rhythmicity in content of the gland results from rhythmic release caused by rhythmicity in abdominal movements during calling. Thus, circadian regulation of pheromone release in *Trichoplusia* may be driven by a clock governing behavior rather than a clock that regulates pheromone synthesis.

The synchrony of the pheromone release rhythm with the behavioral rhythm and their common responses to light manipulations suggest that they share common components in the underlying circadian clock mechanism. In other words, the pheromone clock and the behavioral clock may be closely linked. The clock for the calling rhythm and possibly that for the pheromone rhythm appear to be entrained by a brain photoreception center in *Andevidia peponis* (Sasaki *et al.*, 1987). Surgical removal of both compound eyes and ocelli did not interfere with the ability of the calling rhythm to respond to phase shifts of the LD cycle. Localized illumination of various regions in the brain with 100 μm fiber optic light guides revealed that such phase shifts were induced by illumination of the mediodorsal protocerebrum near the medial neurosecretory cells. Illumination of other brain areas, including the optic lobes, was ineffective.

9.5.3.1. Rhythmicity in hormonal regulation of pheromone production The head is required for both pheromone production and calling behavior. Decapitation of females of the nocturnal moth *S. littoralis* either at the onset of a scotophase prior to the nocturnal peak of pheromone production or at the preceding photophase abolished both the production of pheromone and calling behavior (Martinez and Camps, 1988). Injection of an extract from the brain-SEG-retrocerebral complex restored both pheromone production and calling behavior equally well when administered either in the scotophase or photophase, but did not restore rhythmicity to either process. These findings imply that the expression of the pheromone release rhythm by the gland is dependent on the continuous presence of the head. Apparently the pheromone rhythm depends on circadian input from the head. The obvious implication was that the head contained a factor(s) that was essential for driving the oscillations of the overt pheromone rhythm.

A head factor with pheromonotropic activity (PBAN) was eventually identified in many moths (Raina and Klun, 1984; Martinez and Camps, 1988; Rafaeli and Soroker, 1989; Raina *et al.*, 1989; Ma and Roelofs, 1995; Zhu *et al.*, 1995; Zhao *et al.*, 2002) and was subsequently isolated and characterized as a small, 33–34 amino acid neuropeptide with an amidated C-terminus. PBAN has been sequenced in *Bombyx* (Kitamura *et al.*, 1989), *Helicoverpa zea* (Raina *et al.*, 1989), and *Lymantria* (Masler *et al.*, 1994) and deduced from cDNA sequences in *A. ipsilon* (Duportets *et al.*, 1998), *H. assulta* (Choi *et al.*, 1998b), *Mamestra* (Jacquin-Joly *et al.*, 1998), and *S. littoralis* (Iglesias

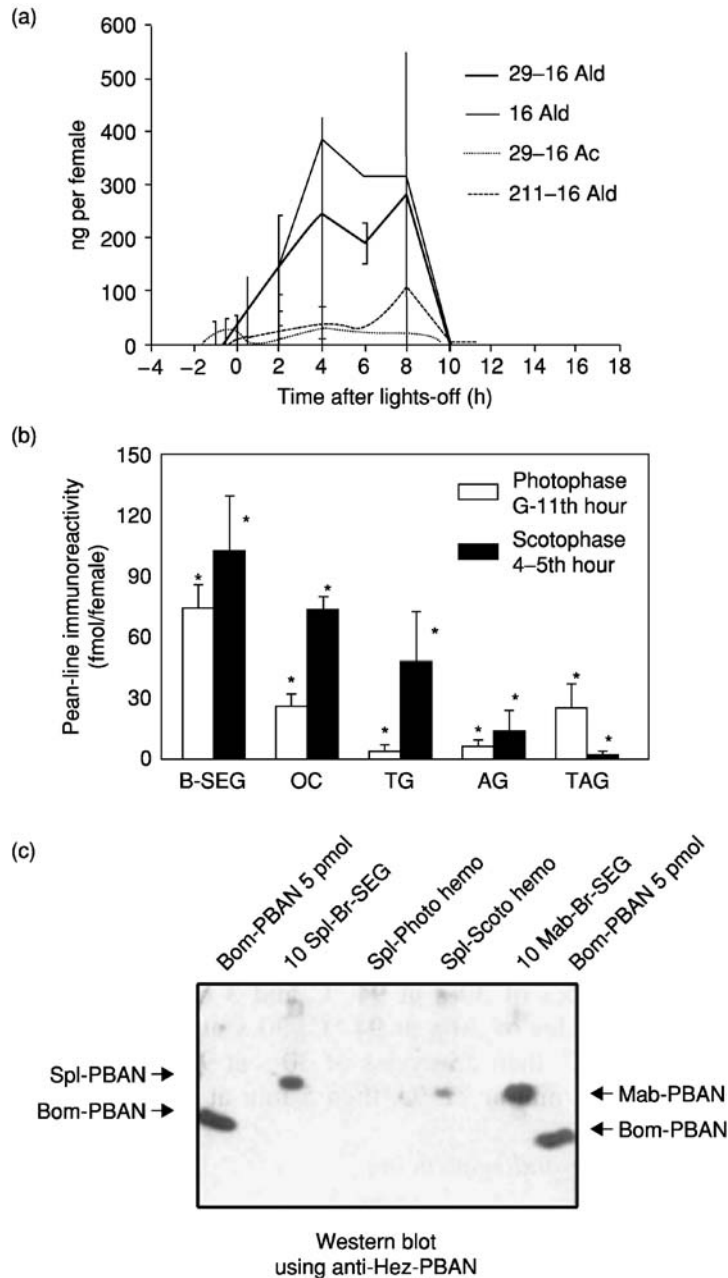


Figure 24 Daily rhythm of the five pheromone components in the pheromone gland of day 1 females of the tobacco budworm, *Helicoverpa assulta* in a 15h light:9h dark cycle. The dark bar on the abscissa indicates scotophase (a). (Reprinted with permission from Choi, M.Y., Tatsuki, S., Boo, K.S., **1998a**. Regulation of sex pheromone biosynthesis in the Oriental tobacco budworm, *Helicoverpa assulta* (Lepidoptera: Noctuidae). *J. Insect Physiol.* 44, 653–658; © Elsevier.) (b) Day and night differences in the level of PBAN-like immunoreactivity in several neural tissues of females of *Helicoverpa armigera*. B-SEG indicates brain–subesophageal ganglia complex; CC, corpus cardiacum; TG, prothoracic ganglia; AG, abdominal ganglia, excluding the terminal abdominal ganglion (TAG). (Rafaeli, A., Hirsch, J., Soroker, V., Kamensky, B., Raina, A.K., **1991**. Spatial and temporal distribution of pheromone biosynthesis-activating neuropeptide in *Helicoverpa* (*Heliothis*) *armigera* using RIA and *in vitro* bioassay. *Arch. Insect Biochem. Physiol.* 18, 119–129; © Wiley. Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.) (c) Western blot showing the presence of a single immunoreactive peptide to antibody against PBAN of *Helicoverpa zea* in the hemolymph of scotophase (Spl-Scoto hemo) but not photophase (Spl-Photo hemo) females of *Spodoptera littoralis*. The peptide is also present in brain–subesophageal ganglia complexes (Spl-Br-SEG) of *Spodoptera*. Controls, synthetic PBAN-I from *Bombyx mori* (Bom-PBAN) and Br-SEG extracts from *Mamestra brassicae* (Mab-Br-SOG). (Reprinted with permission from Iglesias, F., Marco, P., François, M.-C., Camps, F., Fabriàs, F., *et al.*, **2002**. A new member of the PBAN family in *Spodoptera littoralis*: molecular cloning and immunovisualization in scotophase hemolymph. *Insect Biochem. Mol. Biol.* 32, 901–908; © Elsevier.)

et al., 2002). All PBANs share a high degree of sequence homology, the C-terminal pentapeptide being the most conserved. In *Bombyx*, PBAN is synthesized as a preprohormone that is trimmed to several peptides posttranslationally; one of these peptides bears striking similarity to the *Bombyx* embryonic diapause hormone (Sato *et al.*, 1993), possibly suggesting common regulation for both diapause peptide and PBAN.

In two species of *Helicoverpa* and in *Ostrinia*, immunohistochemistry and assays for both biological activity and expression of the PBAN gene have identified small groups of nerve cells in the SEG as the primary (but not necessarily the only) (see Section 9.5.3.2) source of PBAN (Ma and Roelofs, 1995; Choi *et al.*, 1998b; Ma *et al.*, 1998, 2000). Mapping of the axonal pathways from the PBAN-immunopositive cells in SEG in *Bombyx* revealed connections to the corpus cardiacum (CC), which suggested that the material produced in these PBAN cells in SEG may be transported to the CC for release (Ichikawa *et al.*, 1995). PBAN activity in the CC was detected in several moths. In *Ostrinia*, removal of the retrocerebral complex (CC and CA) significantly reduced the production of pheromone by the gland (Ma and Roelofs, 1995). The CC also immunostained intensely for PBAN and CC extracts exhibited strong pheromonotropic activity in both *Helicoverpa* spp. (Rafaeli *et al.*, 1991) and *Ostrinia* (Ma and Roelofs, 1995). These findings implied that the CC is probably the site of release of PBAN into the hemolymph.

Clear daily fluctuations in the PBAN content of the CC were found using a radioimmunoassay (RIA)

for PBAN and assays for biological activity. In *Helicoverpa armigera*, the level of PBAN in the CC was significantly higher in scotophase than in photophase suggesting that transport to CC and/or release of PBAN from the CC were rhythmic (Rafaeli *et al.*, 1991; Rafaeli, 1994) (Figure 24b). In contrast, the level of PBAN in brain-SEG extracts did not change during the course of a day, suggesting that PBAN synthesis may be continuous (Rafaeli *et al.*, 1991; Rafaeli, 1994). Similar observations were also made in *A. segetum* in which PBAN biological activity in the brain-SEG did not show daily changes (Rosén, 2002). The view that PBAN release from CC is rhythmic was strengthened by extracellular recordings of the action potentials in the NCC-V nerve of *Bombyx*; this nerve contains the axonal projections from the PBAN-producing cells in SEG to CC (Ichikawa, 1998). A clear daily rhythm of bursting firing activity was found. This pattern of firing is associated with the release of neuropeptide in many other systems. The rhythm of bursting firing was synchronous with both the daily rhythm of calling behavior and with the pheromone content of the gland. The bursting activity rhythm free-ran in both DD (Figure 25a) and LL (Figure 25b), revealing endogenous circadian control (Tawata and Ichikawa, 2001). Collectively, these findings indicate that release of PBAN into the hemolymph occurs with a daily rhythm that is under circadian control.

Daily cycling in PBAN-immunoreactivity and/or biological activity in the hemolymph has also been detected in several moths. In the hemolymph of calling females of *Heliothis zea*, a peptide fraction with PBAN activity was detected in scotophase

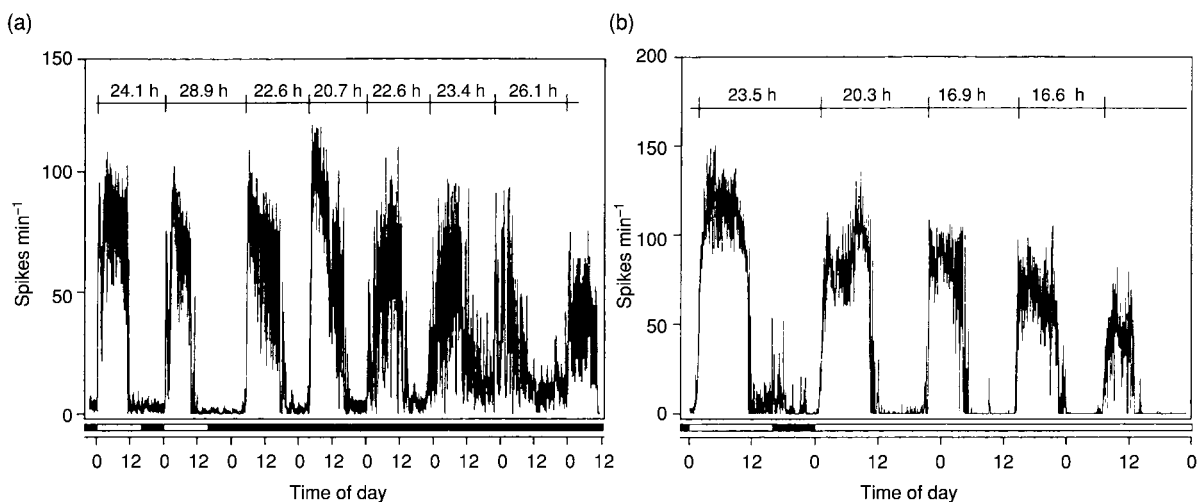


Figure 25 Entrained rhythm of the firing activity of the PBAN-producing cells, recorded from the nervus corporis cardiacum (NCC-V) of virgin females of the silkworm, *Bombyx mori* (a). The rhythm free-runs in both DD (dark bar in a) and LL (light bar in b). (Reproduced with permission from Tawata, M., Ichikawa, T., 2001. Circadian firing of neurosecretory cells releasing pheromonotropic neuropeptides in the silkworm, *Bombyx mori*. *Zool. Sci.* 18, 645–649; © The Zoological Society of Japan.)

(Ramaswamy *et al.*, 1995) but not in photophase. Daily cycling of PBAN-like immunoreactivity was also detected in the hemolymph of females of *Mamestra*. This rhythm was also synchronous with the daily rhythms of pheromone production in the pheromone gland and calling behavior (Iglesias *et al.*, 1999). Similarly, in *S. littoralis*, Western blot analysis of hemolymph peptides of virgin females revealed the presence of PBAN-like compounds during the scotophase but not during the photophase (Iglesias *et al.*, 2002) (**Figure 24c**). In another moth, *A. segetum*, a bioassay for PBAN revealed that the hemolymph of females contained high PBAN activity in the scotophase but low in the photophase (Rosén, 2002). All these findings taken together support the view that at least in some moths, PBAN is released in the hemolymph where it exhibits a strong daily cycling. In the hemolymph, PBAN appears to function as a classical hormone, its principal target being the pheromone gland. Indeed, isolated pheromone glands were successfully stimulated by PBAN *in vitro* (Rafaeli and Soroker, 1989; Jurenka *et al.*, 1991; Matsumoto *et al.*, 1995; Zhao *et al.*, 2002). *In vitro* assays showed that PBAN acts on pheromone glands via a Ca^{2+} -calmodulin-dependent cyclase (review: Ramaswamy *et al.*, 1994), implying the presence of PBAN receptors on the surface of the pheromone gland cells. A putative cell membrane receptor for PBAN was identified recently in pheromone glands of *H. armigera* (Rafaeli and Gileadi, 1997; Altstein *et al.*, 1999, 2001). These findings imply that rhythmicity in the level of PBAN in the hemolymph drives rhythmicity in the production of pheromone by the pheromone gland.

However, there is a wide distribution of PBAN in the nervous system, and in some species the pheromone gland is directly innervated by the terminal abdominal ganglion (TAG). For example, the pheromone glands of two *Heliothis* species (Teal *et al.*, 1989, 1999a; Christensen *et al.*, 1991) and *Manduca* (Christensen *et al.*, 1994) are innervated from the TAG, whereas those in *Ostrinia* are not (Ma and Roelofs, 1995). PBAN immunoreactivity, gene expression (Rafaeli *et al.*, 1991; Ma and Roelofs, 1995; Ma *et al.*, 1998), and biological activity (Rosén, 2002) have been localized not only to the brain-SEG complexes and CC, but also to the thoracic and abdominal ganglia. In *H. armigera*, both PBAN immunoreactivity and biological activity cycled during a day in the thoracic and abdominal ganglia (Rafaeli *et al.*, 1991) (**Figure 24b**), suggesting possibly novel functional significance and circadian control. PBAN was also found in other neural sites. In *H. zea*, processes from the PBAN-positive

cells in the SEG project into the ventral nerve cord (Kingan *et al.*, 1990). In *A. segetum*, PBAN activity was localized in the ventral nerve cord (VNC) where it fluctuated daily (Rosén, 2002). All the above raise the interesting possibility that there may be novel sites of synthesis and/or release of PBAN in the nervous system. Furthermore, the findings that PBAN and/or PBAN gene expression is found in males (Rafaeli *et al.*, 1993; Zhu *et al.*, 1995; Choi *et al.*, 1998b) and that putative PBAN receptors were located in thoracic muscles and the CNS (Elliot *et al.*, 1997) imply novel sites of action and functions of PBAN in addition to regulation of pheromone production in the pheromone gland. The significance of this information is at the moment unclear.

JH is also involved in the regulation of pheromone production as shown in the armyworm moth, *Pseudaletia* (Cusson and McNeil, 1989; Cusson *et al.*, 1994) and the black cutworm, *A. ipsilon* (Picimbon *et al.*, 1995) (see Section 9.5.1). Allatectomy at emergence inhibited pheromone production; injections of JH or CA extracts restored it. When females of the above species or of *H. armigera* (Fan *et al.*, 1999) were decapitated at emergence, injections of JH alone were ineffective in restoring pheromone production; however, injection of JH together with PBAN (or brain extract) restored it. This strongly suggested that the presence of JH was necessary for rhythmic, PBAN-dependent, pheromone production. The role of JH could be to regulate the production/release of PBAN by the brain-SEG (Cusson *et al.*, 1994; Picimbon *et al.*, 1995), or JH could be needed to prime the pheromone glands for the action of PBAN (Fan *et al.*, 1999). The latter may be achieved by upregulation of putative PBAN receptors in pheromone glands by JH (Rafaeli *et al.*, 2003). Either of these possibilities raises yet again the prospect that JH synthesis may be under circadian control. A fruitful line of enquiry would be the examination of JH levels in relation to rhythms of PBAN release and/or pheromone production.

9.5.3.2. Regulation of pheromone glands by rhythms in octopamine In moths where the pheromone gland is innervated, there is considerable evidence that neural control from the VNC is essential for the regulation of pheromone production. Octopamine release by efferent neurons from the TAG to the pheromone gland was implicated as exerting stimulatory and/or inhibitory control over the gland and, by implication, regulating the daily rhythm of pheromone production. Transection of the nerves from the TAG to the gland inhibited pheromone production in *Heliothis zea*, *H. virescens*

(Teal *et al.*, 1989, 1999a; Christensen *et al.*, 1991, 1992, 1994), and *A. segetum* (Rosén, 2002). Electrical stimulation of the nerve connectives anterior to the TAG induced pheromone synthesis in females in photophase, a time when they are not normally engaged in pheromone production (Christensen *et al.*, 1991, 1992). In these moths, intact nerve connections to the pheromone gland are essential for pheromone production. Octopamine induced pheromone production by the gland when it was injected into intact females in photophase (no pheromone synthesis) or isolated abdomens of females in scotophase with transected nerve connectives (inhibition of pheromone synthesis) (Christensen *et al.*, 1991, 1992). In *H. zea*, the level of octopamine in the TAG exhibited daily cycling, which occurred in antiphase to the daily cycle of the octopamine level in the pheromone gland (Figure 26). In mid-photophase, the TAG contained more octopamine than the pheromone gland. However, a few hours prior to lights-off, the octopamine levels in these two locations changed swiftly; the level of octopamine in the TAG decreased sharply, whereas the level in the gland increased significantly. About 1–2 h after lights-off the octopamine levels in both the TAG and the pheromone gland returned to the mid-photophase levels. The increase in octopamine level

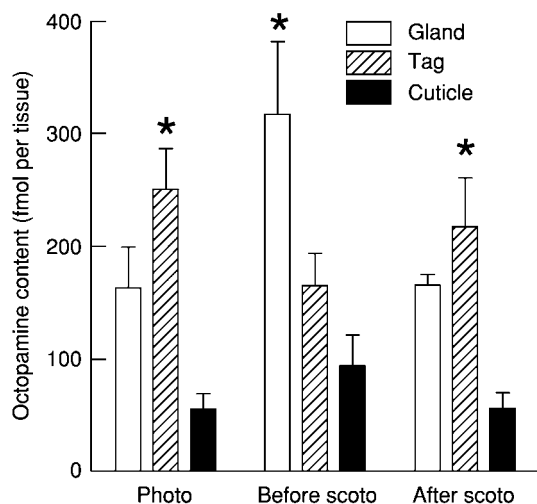


Figure 26 Day and night differences in octopamine levels in the pheromone gland, terminal abdominal ganglion (TAG), and abdominal cuticle (control) in females of *Heliothis zea*. “Photo” indicates mid-photophase; “Before scoto” indicates 0–2 h prior to lights-off; “After scoto” indicates 0–2 h after lights-off. Asterisks indicate statistically significant differences from controls. (Reprinted with permission from Christensen, T.A., Lehman, H.K., Teal, P.E.A., Itagaki, H., Tumlinson, J.H., *et al.*, 1992. Diel changes in the presence and physiological actions of octopamine in the female sex-pheromone glands of *Heliothis* moths. *Insect Biochem. Mol. Physiol.* 22, 841–849; © Elsevier.)

in the pheromone gland occurred at the time of pheromone emission and calling behavior. These findings provide strong circumstantial evidence that a rhythm in octopamine is involved in rhythmic pheromone synthesis. Experiments with administration of both octopamine and PBAN led the authors to suggest that the role of PBAN in the regulation of pheromone synthesis may be indirect. It was proposed that PBAN is transported to the TAG via the VNC, where it stimulates the octopaminergic innervation of the pheromone gland. However, Jurenka *et al.* (1991) and Ramaswamy *et al.* (1995) were unable to reproduce the above findings using the same moth and with the same experimental approach. It has also been proposed that octopamine may inhibit pheromone production. In *H. armigera*, octopamine or its agonist clonidine inhibited the pheromonotropic activity of PBAN using *in vitro* preparations of the pheromone gland (Rafaeli and Gileadi, 1997; Rafaeli *et al.*, 1997), or *in vivo* in photophase animals that were injected with PBAN (Rafaeli *et al.*, 1997). *In vivo*, the inhibition by octopamine or its agonist was reversed by injection of adrenergic antagonists only if the females were decapitated. Clonidine also inhibited normal pheromone increase in scotophase animals (Rafaeli *et al.*, 1997). These findings led to the hypothesis that the daily declines during photophase in pheromone content of the gland may be attributable to daily inhibition of PBAN action mediated by octopaminergic receptors of the α_2 -type.

9.5.4. Integration of Rhythmic Reproductive Behaviors with Hormones

The sequence of physiological processes that lead to the development of mature eggs and sperm is under complex endocrine control. Evidence of circadian regulation of these hormones is discussed above (see Section 9.5.1). Successful reproduction involves detecting and attracting a mate, copulation, and subsequent oviposition of fertilized eggs. In the context of hormones and circadian systems, these behaviors are significant in two ways, that may not be as distinct as the literature describes them. First, each of these behaviors occurs only at a temporally very precise part of the reproductive cycle, different behaviors being confined to different parts of the cycle. This point illustrates that the onset and termination of each behavior is intimately coordinated with the progress of egg and sperm development and therefore implies that the behaviors are modulated by signals from reproductive hormones. It further implies that different behaviors are executed in the presence of (and potentially influenced by)

different hormonal milieus. Second, all of these many distinct behaviors are performed rhythmically and are under circadian control. This point indicates integration between the reproductive hormones and the circadian system. The nature of this integration has received little experimental attention. The evidence summarized below indicates that it is unlikely that hormones directly drive reproductive behaviors. Rather, it appears that the clocks that drive behaviors are subject to sophisticated modulation by the endocrine milieu. For example, stridulation, courtship, mating, and oviposition all appear to be influenced by the locomotor clock (in the optic lobe) but each behavior takes place at a different time in the reproductive process. A model is envisaged in which the locomotor clock drives rhythmicity in the pattern generators for these behaviors, but which pattern generator becomes activated at any particular moment is influenced by the endocrine milieu. JH, octopamine, and various neuropeptides have been invoked as modulators of these behaviors, but much remains to be learned about how and where they act.

9.5.4.1. Rhythms in olfactory responses In many species, olfactory responses to odors occur with a daily rhythm which is regulated by a circadian clock. The presence of a daily periodicity in olfactory responses is most apparent in the detection and response to sex pheromones. Successful mating depends on the daily coordination of the activities of males and females and it relies on temporal synchrony between the daily rhythm of release of sex pheromone by one sex (see Section 9.5.3) and the daily rhythm of responsiveness by the other. Daily rhythmicity in the behavioral rhythm of responsiveness to sex pheromones has been seen in a variety of insect groups including cockroaches (Liang and Schal, 1990), beetles (Shu *et al.*, 1996), flies (Katsoyannos, 1982; Pivnic, 1993), and moths (Shorey and Gaston, 1965; Castrovillo and Cardé, 1979; Linn and Roelofs, 1992). The responsiveness rhythm is entrained by light cues in *Trichoplusia* (Shorey, 1966; Linn and Roelofs, 1992) and *Lymantria* (Linn *et al.*, 1992), and free-runs in DD in *L. dispar* (Linn *et al.*, 1992), *Trichoplusia* (Shorey, 1966; Linn and Roelofs, 1992), *Laspeyresia pomonella* (Castrovillo and Cardé, 1979), and the cockroach *Supella* (Liang and Schal, 1990), thus showing that it is controlled by an underlying circadian clock.

Circadian changes in responsiveness to sex pheromones could theoretically derive from circadian control over changes in olfactory sensitivity in the antennal sense organs or equally in the central

processing of the olfactory input. Evidence suggests that both control mechanisms may in fact be operational in different insects. Explanted antennae of *Drosophila* incubated *in vitro* exhibited a daily rhythmicity in the expression of the *per* gene, which free-ran in DD for many cycles (Plautz *et al.*, 1997); the rhythm was entrained by light *in vitro*. Electroantennogram responses of *Drosophila* to odorants such as ethyl acetate or benzaldehyde revealed a daily rhythm of responsiveness, which free-ran in DD and damped out quickly in LL (Krishnan *et al.*, 1999). A *per* transgenic line (Krishnan *et al.*, 1999) and *cry* mutants (Krishnan *et al.*, 2001) confirmed that olfactory responses in *Drosophila* are controlled by a circadian clock residing in the antennae (Figure 5) (see Section 9.3.3). This antennal clock may be involved in the regulation of the rhythm of responsiveness to sex pheromones via regulation of the sensory neurons or binding proteins to pheromones that have been localized in the antennae of several insects.

A central mechanism in the antennal lobes appears to control the daily rhythmicity in pheromone sensitivity in the moth *A. ipsilon*, which is modulated by JH (Gadenne *et al.*, 2001) (see Section 9.5.1). In addition, injection of octopamine affects many aspects of the responsiveness of male moths to sex pheromones, including the circadian rhythm of responsiveness (Linn *et al.*, 1996; Linn, 1997). The complexity of octopamine effects on behavior suggests that octopamine may exert its influence on the male responsiveness by acting on the CNS. Experiments with phase advances and phase delays of the responsiveness rhythms showed that octopamine injections were effective only when administered within a narrow time window in the daily cycle prior to the daily peaks of responsiveness, suggesting a circadian component to the regulation of responsiveness by octopamine. In support of this notion is the finding that the levels of octopamine in the brain of *Trichoplusia* males exhibited a daily periodicity that was synchronous with the daily rhythm of responsiveness (Linn *et al.*, 1996); the octopamine rhythm in the brain free-ran in DD and damped out rapidly in LL. All this implies that the mechanism of control of the responsiveness rhythm is centrally located and possibly related to the locomotor clock (Linn *et al.*, 1996), whose output to the tritocerebrum may be modulated by circadian changes in octopamine.

There is also evidence that octopamine acts peripherally by modulating the sensitivity of the sensory neurons that detect sex pheromone in the antennae of males of *Antheraea polyphemus* (Pophof, 2000), *Mamestra* (Grosmaître *et al.*, 2001), and *Bombyx*

(Pophof, 2002). Octopamine receptors were cloned from the antennae of *Bombyx* and *H. virescens* and localized near the base of the olfactory hairs in *Heliothis* (Nickisch-Rosenegk *et al.*, 1996). The effects of octopamine on pheromone perception may thus be mediated by these antennal receptors; the modulatory action of octopamine may occur either at the level of generation of action potentials in the antennal receptor neurons (Pophof, 2002) or by acting on the accessory cells of the sensillum (Dolzer *et al.*, 2001). The olfactory receptor neurons in the antennae project axons directly into the antennal lobes, from whence projection neurons connect the antennal lobes with the lateral protocerebrum either directly or indirectly via the mushroom bodies (Heimbeck *et al.*, 2001; Hansson, 2002). *Drosophila* transgenic flies in which the function of these projection neurons was disrupted by transgenic expression of tetanus toxin show defects in both odor detection and courtship behavior of males (Heimbeck *et al.*, 2001). There is therefore a neural pathway that connects olfactory inputs with rhythmic behavioral outputs. In general, it appears that octopamine modulates the antennal olfactory clock whose output is integrated with the central locomotor clock. In turn, the outputs of the locomotor clock are responsible for driving various rhythmic behaviors such as upwind flight of the males, courtship, and mating. Further, JH appears to be involved in the modulation of the central neural pathways of the above rhythmic behaviors (see Section 9.5.1).

9.5.4.2. Bioluminescence, courtship song, and stridulation Several species, primarily Coleoptera, use bioluminescence for sexual attraction. The best-studied examples are the nightly displays of flashes of light by fireflies as a means of photic dialogue between the sexes. During flashing the animals are capable of emitting, receiving, and interpreting visual signals from the other sex. One sex, usually the male, emits a characteristic rhythmic pattern of light flashes during flight that is recognized by conspecific females; the females respond with an equally stereotyped pattern of flashes. In some firefly species, flashing by males may become synchronized in a local population of fireflies. In these synchronously flashing populations of males, the rhythmic pattern of flashing is self-sustained, runs with a stable free-running period, is entrained and phase-shifted to periodic pulses of light; therefore, it exhibits all the characteristics of an ultradian rhythm that is endogenously controlled (Buck, 1988). Flashing occurs with a circadian rhythm, within which there is a strong ultradian rhythm (Buck, 1988).

Both rhythms are interrelated in terms of formal clock properties and physiological control (see below).

The efficacy of flashing as a mating signal depends also on the ability of females to receive and interpret rhythmic photic signals from the males. A daily rhythm in visual responsiveness to light flashes was observed in *Photuris versicolor* that free-runs in DD and is synchronized with the rhythm of flying and flashing (Lall, 1993). Therefore, the synchronous occurrence of all three rhythmic phenomena – bioluminescence, visual responsiveness, and flight – suggests that they may all be regulated by a common circadian clock. Indeed, it has been suggested that the ultradian flashing oscillator is associated with the locomotor clock in the brain (Bagnoli *et al.*, 1976; Case, 1984).

The regulation of courtship behavior in *Drosophila* provides another example of a behavior that has both circadian and ultradian properties. In wild-type flies of *Drosophila*, mating is preceded by courtship during which the male orients himself and directs auditory signals (the courtship song) towards the female by extension and vibration of the wings. One of the components of this song is a train of pulses with an interpulse interval that oscillates sinusoidally with a mean value of 55 s. This ultradian rhythm free-runs in LL and is temperature compensated; it is therefore regulated endogenously (Kyriakou and Hall, 1980). Mutations in *per* altered both this ultradian rhythm and the circadian locomotor rhythm. Construction of genetic mosaic flies for *per* showed that song phenotype was determined by expression of the *per* allele in the thoracic ganglia but not in the brain (Konopka *et al.*, 1996). The only cells that express *per* in the thoracic ganglia were glial cells (Ewer *et al.*, 1992). A general conclusion from both fireflies and *Drosophila* is that the oscillators for ultradian and circadian rhythms appear to share common components at both the cellular and anatomical levels.

Many insects also communicate using rhythmic acoustic signals produced by specialized structures. The best-known example is stridulation in crickets. In numerous species, stridulation is under circadian control. For example, in *Teleogryllus commodus*, stridulation occurs with a strong daily rhythm that free-runs in both DD and LL (Sokolove and Loher, 1975); it is, therefore, controlled by an endogenous circadian clock. In these species, removal of the eyes or severance of the eyes from the optic lobes results in a free-running rhythm of stridulation in either LD or LL, but with a period length characteristic of DD (Sokolove and Loher, 1975). These experiments are consistent with the view that the

stridulation rhythm is controlled by the same clock that controls the locomotor rhythm. Subsequent experiments showed that the stridulation rhythm could be uncoupled from the locomotor rhythm, but the clocks controlling both rhythms were both located in the optic lobes (Wiedenmann, 1983; Wiedenmann and Loher, 1984; Wiedenmann *et al.*, 1988). It was suggested that each optic lobe contains two circadian clocks (one for each rhythm) that are normally coupled together.

9.5.4.3. Mating and oviposition Daily periodicities in mating behavior have been identified in all major insect groups. It is generally accepted that an endogenous circadian oscillator controls rhythmicity in mating, although there have been only a few true circadian studies. Endogenous circadian control of mating has been observed in beetles (Walker, 1979; Boucher and Pierre, 1988), fruit flies (Loher and Zervas, 1979; Sakai and Ishida, 2001), and moths (Han *et al.*, 2000). In *Drosophila*, mating occurs with a circadian rhythm that free-runs in DD and is abolished in *per* and *tim* null mutants (Sakai and Ishida, 2001). Further, in *disconnected* mutants which lack some of the lateral clock neurons that control locomotion and have severe defects in the optic lobes, rhythmicity in mating was also abolished. Taken together, these observations showed that rhythmicity in mating behavior may be controlled by the locomotor clock. Thus, the *Drosophila* mating repertoire becomes an illustration of the fine coordination of clock-controlled mating activities between the sexes. Successful mating contact depends on olfactory responses of males to the release of female sex pheromones; these responses are controlled by a *per*-based olfactory clock in the antennae (Krishnan *et al.*, 1999) (see Section 9.5.4.1). Males respond to the female chemical signal by performing the courtship song, which is also *per* controlled (Kyriakou and Hall, 1982) (see Section 9.5.4.2). Courtship song, in turn, affects female receptivity (Kyriakou and Hall, 1982; Hall, 2002). Thus it appears probable, as Sakai and Ishida (2001) suggested, that the mating rhythm in females may be a consequence of circadian control over pheromone release and/or responsiveness to male courtship song.

Oviposition behavior is also under circadian control in numerous insects, such as bugs (Ampleford and Davey, 1989), crickets (Sefiani, 1987), moths (Riedl and Loher, 1980; Bell, 1981; Yamaoka and Hirao, 1981; Shearer *et al.*, 1995), flies and mosquitoes (Allemand, 1983; Joshi, 1996). The physiological links between mating and oviposition are both complex and species-variable. Peptides transferred

to the female at mating have been shown to enhance fertility, promote oviposition, or inhibit further mating by the female (Kubli, 1992; Stanley-Samuelson, 1994). Further, proteinaceous material transferred to the female at mating can be utilized as nutrient to accelerate oogenesis; amino acids and proteins from the spermatophore have been detected in the eggs of many species (Huignard, 1983; Mullins *et al.*, 1992). In some insects such as the tettigonids and crickets, the female feasts on the spermatophore whose peptide components can be found later in eggs (Simmons and Gwynne, 1993). Thus, the male contributes to both completion of egg development by the female and to the behavioral changes that follow mating. These points emphasize the close integration between the mechanisms that control egg and sperm development with those that control rhythmic behavior.

The circadian control of oviposition behavior must be synchronized with the presence and movement of eggs along the oviducts. Complex neuroendocrine mechanisms control the process of egg movement, extensively studied in orthopterans. There are pattern generators in the TAG for both digging (Thompson *et al.*, 1999) and regulation of oviducal contractions (Kalogianni and Theophilidis, 1993), and contractions are influenced by proctolin, octopamine, and FMRFamide-related peptides (Noronha and Lange, 1997; Orchard *et al.*, 1997). Despite extensive physiological analysis, the origin of circadian control of this system is unexplored.

References

- Agui, N., Granger, N.A., Gilbert, L.I., Bollenbacher, W.E., 1979. Cellular localization of the insect prothoracicotrophic hormone: *in vitro* assay of a single neurosecretory cell. *Proc. Natl Acad. Sci. USA* 76, 5694–5698.
- Allemand, R., 1976a. Les rythmes de vitellogenèse et d'ovulation en photopériode LD 12:12 de *Drosophila melanogaster*. *J. Insect Physiol.* 22, 1031–1035.
- Allemand, R., 1976b. Influence de modifications des conditions lumineuses sur les rythmes circadiens de vitellogenèse et d'ovulation chez *Drosophila melanogaster*. *J. Insect Physiol.* 22, 1075–1080.
- Allemand, R., 1983. The circadian oviposition rhythm of *Drosophila melanogaster*. 2. Influence of biotic factors. *Biol. Behav.* 8, 273–288.
- Allen, G., Rappe, J., Earnest, D.J., Cassone, V.M., 2001. Oscillating on borrowed time: diffusible signals from immortalized suprachiasmatic nucleus cells regulate circadian rhythmicity in cultured fibroblasts. *J. Neurosci.* 21, 7937–7943.
- Altstein, M., Ben-Aziz, O., Daniel, S., Zeltser, I., Gilon, C., 2001. Pyrokinin/PBAN radioreceptor assay: development and application for the characterization of a

- putative receptor from the pheromone gland of *Heliothis peltigera*. *Peptides* 22, 1379–1389.
- Altstein, M., Gabay, T., Ben-Aziz, O., Daniel, S., Zeltser, I., *et al.*, 1999. Characterization of a putative pheromone biosynthesis activating neuropeptide (PBAN) receptor from the pheromone gland of *Heliothis peltigera*. *Invert. Neurosci.* 4, 33–40.
- Ampleford, E.J., Davey, K.G., 1989. Egg laying in the insect *Rhodnius prolixus* is timed in a circadian fashion. *J. Insect Physiol.* 35, 183–188.
- Ampleford, E.J., Steel, C.G.H., 1982. Circadian control of ecdysis in *Rhodnius prolixus* (Hemiptera). *J. Comp. Physiol. A* 147, 281–286.
- Ampleford, E.J., Steel, C.G.H., 1985. Circadian control of a daily rhythm in hemolymph ecdysteroid titer in the insect *Rhodnius prolixus* (Hemiptera). *Gen. Comp. Endocrinol.* 59, 453–459.
- Ampleford, E.J., Steel, C.G.H., 1986. Induction of rhythmic modulation of hemolymph ecdysteroids in the insect *Rhodnius prolixus* by treatments which elicit rhythmic ecdysis. *Gen. Comp. Endocrinol.* 63, 353–361.
- Andreatic, R., Chaney, S., Hirsh, J., 1999. Requirement of circadian genes for cocaine sensitization in *Drosophila*. *Science* 285, 1066–1068.
- Anton, S., Gadenne, C., 1999. Effect of juvenile hormone on the central nervous processing of sex pheromone in an insect. *Proc. Natl Acad. Sci. USA* 96, 5764–5767.
- Asahina, M., Fugo, H., Takeda, S., 1994. Ecdysteroid synthesis in dissociated cells of the prothoracic gland of the silkworm, *Bombyx mori*. *Zool. Sci.* 11, 107–111.
- Atkins, G., Stout, J., 1994. Processing of song signals in the cricket and its hormonal control. *Am. Zool.* 34, 655–669.
- Audsley, N., Duve, H., Thorpe, A., Weaver, R.J., 2000. Morphological and physiological comparisons of two types of allatostatin in the brain and retrocerebral complex of the tomato moth, *Lacanobia oleracea* (Lepidoptera: Noctuidae). *J. Comp. Neurol.* 424, 27–46.
- Babilis, N.A., Mazomenos, B.E., 1992. Pheromone production in *Sesamia nonagrioides*: diel periodicity and effect of age and mating. *J. Insect Physiol.* 38, 561–564.
- Bagnoli, P., Brunelli, M., Magni, F., Musumeci, D., 1976. Neural mechanisms underlying spontaneous flashing and its modulation in the firefly *Luciola lusitanica*. *J. Comp. Physiol.* 108, 133–156.
- Baker, J.D., McNabb, S.L., Truman, J.W., 1999. The hormonal coordination of behavior and physiology at adult ecdysis in *Drosophila melanogaster*. *J. Exp. Biol.* 202, 3037–3048.
- Balsalobre, A., 2002. Clock genes in mammalian peripheral tissues. *Cell Tissue Res.* 309, 193–199.
- Beaulaton, J., Porcheron, P., Gras, R., Cassier, P., 1984. Cytophysiological correlations between prothoracic gland activity and hemolymph ecdysteroid concentration in *Rhodnius prolixus* during the fifth larval instar: further studies in normal and decapitated larvae. *Gen. Comp. Endocrinol.* 53, 1–16.
- Bebas, P., Cymborowski, B., Giebultowicz, J.M., 2001. Circadian rhythm of sperm release in males of the cotton leafworm, *Spodoptera littoralis*: *in vivo* and *in vitro* studies. *J. Insect Physiol.* 47, 859–866.
- Bebas, P., Cymborowski, B., Giebultowicz, J.M., 2002. Circadian rhythm of acidification in insect vas deferens regulated by rhythmic expression of vacuolar H⁺-ATPase. *J. Exp. Biol.* 205, 37–44.
- Bell, C.H., 1981. The influence of light cycle and circadian rhythm on oviposition in 5 pyralid moth pests of stored products. *Physiol. Entomol.* 6, 231–240.
- Beydon, P., Permana, A.D., Colardeau, J., Moriniere, M., Lafont, R., 1989. Ecdysteroids from developing eggs of *Pieris brassicae*. *Arch. Insect Biochem. Physiol.* 11, 1–12.
- Bjostad, L.B., Wolf, W.A., Roelofs, W.L., 1987. Pheromone biosynthesis in lepidopterans: desaturation and chain shortening. In: Blomquist, G.J., Prestwich, G.D. (Eds.), *Pheromone Biochemistry*. Academic Press, San Diego, CA, pp. 77–120.
- Block, G.D., Khalsa, S.B.S., McMahan, D.G., Michel, S., Guesz, M., 1993. Biological clocks in the retina cellular mechanisms of biological timekeeping. *Int. Rev. Cytol.* 146, 83–144.
- Bollenbacher, W.E., Granger, N.A., Katahira, E.J., O'Brien, M.A., 1987. Developmental endocrinology of larval molting in the tobacco hornworm, *Manduca sexta*. *J. Exp. Biol.* 128, 175–192.
- Boucher, L., Pierre, D., 1988. Mating rhythm of *Caryedon serratus* (Coleoptera: Bruchidae) in laboratory and natural conditions. *Ann. Soc. Entomol. France* 24, 151–160.
- Bowen, M.F., Bollenbacher, W.E., Gilbert, L.I., 1984. *In vitro* studies on the role of the brain and prothoracic glands in the pupal diapause of *Manduca sexta*. *J. Exp. Biol.* 108, 9–24.
- Bownes, M., Shirras, A., Blair, M., Collins, J., Coulson, A., 1988. Evidence that insect embryogenesis is regulated by ecdysteroids released from yolk proteins. *Proc. Natl Acad. Sci. USA* 85, 1554–1557.
- Buck, J., 1988. Synchronous rhythmic flashing of fireflies. 2. *Q. Rev. Biol.* 63, 265–289.
- Carney, G.E., Bender, M., 2000. The *Drosophila ecdysone receptor (EcR)* gene is required maternally for normal oogenesis. *Genetics* 154, 1203–1211.
- Case, J.F., 1984. Vision in mating behavior of fireflies. *Symp. Roy. Entomol. Soc. London* 12, 195–222.
- Castrovillos, P.J., Cardé, R.T., 1979. Environmental regulation of female calling and male pheromone response periodicities in the codling moth (*Laspeyresia pomonella*). *J. Insect Physiol.* 25, 659–668.
- Charlton, R.E., Cardé, R.T., 1982. Rate and diel periodicity of pheromone emission from female gypsy moths (*Lymantria dispar*), determined with a glass-adsorption collection system. *J. Insect Physiol.* 28, 423–430.
- Chen, J.H., Fugo, H., Nakajima, M., Nagasawa, H., Suzuki, A., 1987. Neurohormones in developing embryos of the silkworm, *Bombyx mori*: the presence and characteristics of prothoracicotropic hormone, B. *J. Insect Physiol.* 33, 407–411.

- Choi, M.Y., Tanaka, M., Kataoka, H., Boo, K.S., Tatsuki, S., 1998b. Isolation and identification of the cDNA encoding the pheromone biosynthesis activating neuropeptide and additional neuropeptides in the Oriental tobacco budworm, *Helicoverpa assulta* (Lepidoptera: Noctuidae). *Insect Biochem. Mol. Biol.* 28, 759–766.
- Choi, M.Y., Tatsuki, S., Boo, K.S., 1998a. Regulation of sex pheromone biosynthesis in the Oriental tobacco budworm, *Helicoverpa assulta* (Lepidoptera: Noctuidae). *J. Insect Physiol.* 44, 653–658.
- Christensen, T.A., Itagaki, H., Teal, P.E.A., Jasensky, R.D., Tumlinson, J.H., et al., 1991. Innervation and neural regulation of the sex pheromone gland in female *Heliothis* moths. *Proc. Natl Acad. Sci. USA* 88, 4971–4975.
- Christensen, T.A., Lashbrook, J.M., Hildebrand, J.G., 1994. Neural activation of the sex-pheromone gland in the moth *Manduca sexta*: real-time measurement of pheromone release. *Physiol. Entomol.* 19, 265–270.
- Christensen, T.A., Lehman, H.K., Teal, P.E.A., Itagaki, H., Tumlinson, J.H., et al., 1992. Diel changes in the presence and physiological actions of octopamine in the female sex-pheromone glands of *Heliothis* moths. *Insect Biochem. Mol. Physiol.* 22, 841–849.
- Colwell, C.S., Page, T.L., 1990. A circadian rhythm in neural activity can be recorded from the central nervous system of the cockroach. *J. Comp. Physiol. A* 166, 643–649.
- Corrent, G., Eskin, A., Kay, I., 1982. Entrainment of the circadian rhythm from the eye of *Aplysia*: role of serotonin. *Am. J. Physiol.* 242, R326–R332.
- Cusson, M., McNeil, J.N., 1989. Involvement of juvenile hormone in the regulation of pheromone release activities in a moth. *Science* 243, 210–212.
- Cusson, M., Tobe, S.S., McNeil, J.N., 1994. Juvenile hormones: their role in the regulation of the pheromonal communication system of the armyworm, *Pseudaletia unipuncta*. *Arch. Insect Biochem. Physiol.* 25, 329–345.
- Cymborowski, B., Muszynska-Pytel, M., Porcheron, P., Cassier, P., 1991. Hemolymph ecdysteroid titers controlled by a circadian clock mechanism in larvae of the wax moth, *Galleria mellonella*. *J. Insect Physiol.* 37, 35–40.
- Cymborowski, B., Smietanko, A., Delbecq, J.P., 1989. Circadian modulation of ecdysteroid titer in *Galleria mellonella* larvae. *Comp. Biochem. Physiol. A* 94, 431–438.
- Dai, J.-D., Costello, M.J., Gilbert, L.I., 1994. The prothoracic glands of *Manduca sexta*: a microscopic analysis of gap junctions and intercellular bridges. *Invert. Reprod. Devel.* 25, 93–110.
- Dai, J.-D., Mizoguchi, A., Satake, S., Ishizaki, H., Gilbert, L.I., 1995. Developmental changes in the prothoracicotrophic hormone content of the *Bombyx mori* brain-retrocerebral complex and hemolymph: analysis by immunogold electron microscopy, quantitative image analysis, and time-resolved fluoroimmunoassay. *Devel. Biol.* 171, 212–223.
- Danforth, D.N., Jr., Tamarkin, L., Lippman, M.E., 1983. Melatonin-induced increase in cytoplasmic estrogen receptor activity in hamster uteri. *Endocrinology* 113, 81–85.
- Dedos, S.G., Fugo, H., 1999. Induction of dauer larvae by application of fenoxycarb early in the 5th instar of the silkworm, *Bombyx mori*. *J. Insect Physiol.* 45, 769–775.
- Delisle, J., McNeil, J.N., 1987. Calling behavior and pheromone titer of the true armyworm *Pseudaletia unipuncta* (Haw.) (Lepidoptera: Noctuidae) under different temperature and photoperiodic conditions. *J. Insect Physiol.* 33, 315–324.
- De Loof, A., Baggerman, G., Breuer, M., Claeys, I., Cerstiaens, A., et al., 2001. Gonadotropins in insects: an overview. *Arch. Insect Biochem. Physiol.* 47, 129–138.
- Doe, C.Q., Goodman, C.S., 1985. Early events in insect neurogenesis. 1. Development and segmental differences in the pattern of neuronal precursor cells. *Devel. Biol.* 111, 193–205.
- Dolzer, J., Krannich, S., Fischer, K., Stengl, M., 2001. Oscillations of the transepithelial potential of moth olfactory sensilla are influenced by octopamine and serotonin. *J. Exp. Biol.* 204, 2781–2794.
- Donini, A., Agricola, H.-J., Lange, A.B., 2001. Crustacean cardioactive peptide is a modulator of oviduct contractions in *Locusta migratoria*. *J. Insect Physiol.* 47, 277–285.
- Dorn, A., 1983. Hormones during embryogenesis of the milkweed bug *Oncopeltus fasciatus* (Heteroptera: Lygaeida). *Entomol. Gen.* 8, 193–214.
- Dorn, A., Bishoff, S.T., Gilbert, L.I., 1987a. An incremental analysis of the embryonic development of the tobacco hornworm, *Manduca sexta*. *Int. J. Invert. Reprod. Devel.* 11, 137–158.
- Dorn, A., Gilbert, L.I., Bollenbacher, W.E., 1987b. Prothoracicotrophic hormone activity in the embryonic brain of the tobacco hornworm, *Manduca sexta*. *J. Comp. Physiol. B* 157, 279–283.
- Dow, R.C., Carlson, S.D., Goodman, W.D., 1988. A scanning electron microscope study of the developing embryo of *Manduca sexta* (L.) (Lepidoptera: Sphingidae). *Int. J. Insect Morphol. Embryol.* 17, 231–242.
- Du, J., Hiruma, K., Riddiford, L.M., 2003. A novel gene in the *takeout* gene family is regulated by hormones and nutrients in *Manduca* larval epidermis. *Insect Biochem. Mol. Biol.* 33, 803–814.
- Dumser, J.B., 1980. The regulation of spermatogenesis in insects. *Annu. Rev. Entomol.* 25, 341–369.
- Dunkelblum, E., Kehat, M., Harel, M., Gordon, D., 1987. Sexual behavior and pheromone titer of the *Spodoptera littoralis* female moth. *Entomol. Exp. Applic.* 44, 241–248.
- Duportets, L., Dufour, M.C., Bécard, J.M., Gadenne, C., Couillaud, F., 1996. Inhibition of male corpora allata activity and sexual pheromone responsiveness in the black cutworm, *Agrotis ipsilon*, by the hypocholesterolemic agent, fluvastatin. *Arch. Insect Biochem. Physiol.* 32, 601–611.

- Duportets, L., Gadenne, C., Dufour, M.C., Couillaud, F., 1998. The pheromone biosynthesis activating neuro-peptide (PBAN) of the black cutworm moth, *Agrotis ipsilon*: immunohistochemistry, molecular characterization and bioassay of its peptide sequence. *Insect Biochem. Mol. Biol.* 28, 591–599.
- Edmunds, L.N., Jr., 1988. Cellular and Molecular Bases of Biological Clocks: Models and Mechanisms for Circadian Timekeeping. Springer, Berlin.
- Elekonich, M.M., Schultz, D.J., Bloch, G., Robinson, G.E., 2001. Juvenile hormone levels in honey bee (*Apis mellifera* L.) foragers: foraging experience and diurnal variation. *J. Insect Physiol.* 47, 1119–1125.
- Elliot, J.T., Jurenka, R.A., Prestwich, G.D., Roelofs, W.L., 1997. Identification of soluble proteins for an insect neuropeptide. *Biochem. Biophys. Res. Commun.* 238, 925–930.
- Emery, I.F., Noveral, J.M., Jamison, C.F., Siwicki, K.K., 1997. Rhythms of *Drosophila period* gene expression in culture. *Proc. Natl Acad. Sci. USA* 94, 4092–4096.
- Epsky, N.D., Heath, R.R., 1993. Pheromone production by male *Anastrepha suspensa* (Diptera: Tephritidae) under natural light cycles in greenhouse studies. *Environ. Entomol.* 22, 464–469.
- Espig, W., Thiry, E., Hoffmann, K.H., 1989. Ecdysteroids during ovarian development and embryogenesis in the cricket *Gryllus bimaculatus* de Geer. *Invert. Reprod. Devel.* 15, 143–154.
- Eusebio, E.J., Moody, W.J., 1986. Calcium-dependent action potentials in the prothoracic gland of *Manduca sexta*. *J. Exp. Biol.* 126, 531–636.
- Ewer, J., Frisch, B., Hamblen-Coyle, M.J., Rosbach, M., Hall, J.C., 1992. Expression of the *period* gene within different cell types in the brain of *Drosophila* adults and mosaic analysis of these cells' influence on circadian behavioral rhythms. *J. Neurosci.* 12, 3321–3349.
- Ewer, J., Gammie, S.C., Truman, J.W., 1997. Control of insect ecdysis by a positive-feedback endocrine system: roles of eclosion hormone and ecdysis triggering hormone. *J. Exp. Biol.* 200, 869–881.
- Ewer, J., Reynolds, S., 2002. Neuropeptide control of molting in insects. In: Pfaff, D., Arnold, A., Etgen, A., Fahrbach, S., Rubin, A.R. (Eds.), *Hormones, Brain and Behavior*, vol. 3. Academic Press, New York, pp. 1–92.
- Fan, Y., Rafaeli, A., Gileadi, C., Applebaum, S.W., 1999. Juvenile hormone induction of pheromone gland PBAN-responsiveness in *Helicoverpa armigera* females. *Insect Biochem. Mol. Biol.* 29, 635–641.
- Foster, S.P., 2000. Periodicity of sex pheromone biosynthesis, release and degradation in the lightbrown apple moth *Epiphyas postvittana* (Walker). *Arch. Insect Biochem. Physiol.* 43, 125–136.
- Friedländer, M., Reynolds, S.E., 1988. Meiotic metaphases are induced by 20-hydroxyecdysone during spermatogenesis of the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol.* 34, 1013–1019.
- Frisch, B., Hardin, P.E., Hamblen-Coyle, M.J., Rosbach, M., Hall, J.C., 1994. A promotorless *period* gene mediates behavioral rhythmicity and cyclical *per* expression in a restricted subset of the *Drosophila* nervous system. *Neuron* 12, 555–570.
- Fugo, H., Saito, H., Nagasawa, H., Suzuki, A., 1985. Eclosion hormone activity in developing embryos of the silkworm, *Bombyx mori*. *J. Insect Physiol.* 31, 293–298.
- Fujishita, M., Ishizaki, H., 1981. Circadian clock and prothoracicotropic hormone secretion in relation to the larval–larval ecdysis rhythm of the saturniid *Samia cynthia ricini*. *J. Insect Physiol.* 27, 121–128.
- Fujishita, M., Ishizaki, H., 1982. Temporal organization of circadian events in relation to the circadian clock during larval–pupal development in *Samia cynthia ricini*. *J. Insect Physiol.* 28, 77–84.
- Fujishita, M., Ohnishi, E., Ishizaki, H., 1982. The role of ecdysteroids in the determination of gut-purge timing in the saturniid, *Samia cynthia ricini*. *J. Insect Physiol.* 28, 961–968.
- Gadenne, C., Anton, S., 2000. Central processing of sex pheromone stimuli is differentially regulated by juvenile hormone in a male moth. *J. Insect Physiol.* 46, 1195–1206.
- Gadenne, C., Dufour, M.C., Anton, S., 2001. Transient post-mating inhibition of behavioral and central responses to sex pheromones in an insect. *Proc. R. Soc. London B* 268, 1631–1635.
- Gadenne, C., Renou, M., Sreng, L., 1993. Hormonal control of pheromone responsiveness in the male black cutworm, *Agrotis ipsilon*. *Experientia* 49, 721–724.
- Gammie, S.C., Truman, J.W., 1997. Neuropeptide hierarchies and the activation of sequential motor behaviors in the hawkmoth, *Manduca sexta*. *J. Neurosci.* 17, 4389–4397.
- Gelman, D.B., Woods, C.W., Borkovec, A.B., 1988. Effects of ecdysone and 20-hydroxyecdysone on apyrene spermiogenesis in European corn borer, *Ostrinia nubilalis*. *J. Insect Physiol.* 34, 733–738.
- Gemeno, C., Haynes, K.F., 2000. Periodical and age-related variation in chemical communication system of black cutworm moth, *Agrotis ipsilon*. *J. Chem. Ecol.* 26, 329–342.
- Gharib, B., DeReggi, M., 1983. Changes in ecdysteroid and juvenile hormone levels in developing eggs of *Bombyx mori*. *J. Insect Physiol.* 29, 871–876.
- Giebultowicz, J.M., 2000. Molecular mechanism and cellular distribution of insect circadian clocks. *Annu. Rev. Entomol.* 45, 769–793.
- Giebultowicz, J.M., Blackburn, M.B., Thomas-Laemont, P.A., Weyda, F., Raina, A.K., 1996. Daily rhythm in myogenic contractions of vas deferens associated with sperm release cycle in a moth. *J. Comp. Physiol. A* 178, 629–636.
- Giebultowicz, J.M., Feldlaufer, M., Gelman, D.B., 1990. Role of ecdysteroids in the regulation of sperm release from the testis of the gypsy moth, *Lymantria dispar*. *J. Insect Physiol.* 36, 567–571.
- Giebultowicz, J.M., Joy, J.E., 1992. Ontogeny of the circadian system controlling release of sperm from the insect testis. *J. Biol. Rhythms* 7, 203–212.

- Giebultowicz, J.M., Joy, J.E., Riemann, J.G., Raina, A.K., 1994. Changes in protein patterns in sperm and vas deferens during the daily rhythm of sperm release in the gypsy moth. *Arch. Insect Biochem. Physiol.* 27, 65–75.
- Giebultowicz, J.M., Riemann, J.G., Raina, A.K., Ridgway, R.L., 1988. Circadian system controlling release of sperm in the insect testes. *Science* 245, 1098–1099.
- Giebultowicz, J.M., Stanewsky, R., Hall, J.C., Hege, D.M., 2000. Transplanted *Drosophila* excretory tubules maintain circadian clock cycling out of phase with the host. *Curr. Biol.* 10, 107–110.
- Gilbert, L.I., Rybczynski, R., Warren, J.T., 2002. Control and biochemical nature of the ecdysteroidogenic pathway. *Annu. Rev. Entomol.* 47, 883–916.
- Gillette, M.U., McArthur, A.J., 1995. Circadian actions of melatonin at the suprachiasmatic nucleus. *Behav. Brain Res.* 73, 135–139.
- Gillette, M.U., Mitchell, J.W., 2002. Signalling in the suprachiasmatic nucleus: selectively responsive and integrative. *Cell Tissue Res.* 309, 99–107.
- Gillott, C., 1995. Insect male mating systems. In: Leather, S.R., Hardie, J. (Eds.), *Insect Reproduction*. CRC Press, Boca Raton, FL, pp. 33–55.
- Goltzene, F., Holder, F., Charlet, M., Meister, M., Oka, T., 1992. Immunocytochemical localization of *Bombyx*-PTTH-like molecules in neurosecretory cells of the brain of the migratory locust *Locusta migratoria*: a comparison with neuroparsin and insulin-related peptide. *Cell Tissue Res.* 269, 133–140.
- Gorbet, D.J., Steel, C.G.H., 2003. A miniature radioimmunoassay for melatonin for use with small samples from invertebrates. *Gen. Comp. Endocrinol.* 134, 193–197.
- Gorbet, D.J., Steel, C.G.H., 2004. Circadian regulation of a daily rhythm in the level of melatonin in the hemolymph of the insect *Rhodnius prolixus*. *J. Insect Physiol.* (in press).
- Greiner, B., Gadenne, C., Anton, S., 2002. Central processing of plant volatiles in *Agrotis ipsilon* males is age-independent in contrast to sex pheromone processing. *Chem. Senses* 27, 45–48.
- Grosmaître, X., Marion-Poll, F., Renou, M., 2001. Biogenic amines modulate olfactory receptor neurons firing activity in *Mamestra brassicae*. *Chem. Senses* 26, 653–661.
- Gvakharia, B.O., Kilgore, J.A., Bebas, P., Giebultowicz, J.M., 2000. Temporal and spatial expression of the *period* gene in the reproductive system of the codling moth. *J. Biol. Rhythms* 15, 4–12.
- Hagedorn, H.H., 1985. The role of ecdysteroids in reproduction. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 8. Pergamon, Oxford, pp. 205–262.
- Hagedorn, H.H., 1989. Physiological roles of hemolymph ecdysteroids in the adult insect. In: Koolman, J. (Ed.), *Ecdysone*. Georg Thieme Verlag, Stuttgart, pp. 279–289.
- Hall, J.C., 1998. Genetics of biological rhythms in *Drosophila*. *Adv. Genet.* 38, 135–184.
- Hall, J.C., 2002. Courtship lite: a personal history of reproductive behavioral neurogenetics in *Drosophila*. *J. Neurogenet.* 16, 135–163.
- Hall, J.C., 2003. *Genetics and Molecular Biology of Rhythms in Drosophila and Other Insects*. Academic Press, New York.
- Han, G., Du, J., Li, J., 2000. Mating behavioral ecology of *Ancylis sativa* adult. *Yingyong Shengtai Xuebao* 11, 99–102.
- Hansson, B.S., 2002. A bug's smell-research into insect olfaction. *Trends Neurosci.* 25, 270–274.
- Hardeland, R., Poeggeler, B., 2003. Non-vertebrate melatonin. *J. Pineal Res.* 34, 233–241.
- Hardie, J., 1995. Hormones and reproduction. In: Leather, S.R., Hardie, J. (Eds.), *Insect Reproduction*. CRC Press, Boca Raton, FL, pp. 95–108.
- Hardin, P.E., 1994. Analysis of *period* mRNA cycling in *Drosophila* head and body tissues indicates that body oscillators behave differently from head oscillators. *Mol. Cell Biol.* 14, 7211–7218.
- Harshman, L.G., Loeb, A.M., Johnson, B.A., 1999. Ecdysteroid titers in mated and unmated *Drosophila melanogaster* females. *J. Insect Physiol.* 45, 571–577.
- Hege, D.M., Stanewsky, R., Hall, J.C., Giebultowicz, J.M., 1997. Rhythmic expression of a *per*-reporter in the Malpighian tubules of decapitated *Drosophila*: evidence for a brain independent circadian clock. *J. Biol. Rhythms* 12, 300–308.
- Heimbeck, G., Bugnon, V., Gendre, N., Keller, A., Stocker, R.F., 2001. A central neural circuit for experience-independent olfactory and courtship behavior in *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA* 98, 15336–15341.
- Helfrich-Förster, C., 1995. The *period* clock gene is expressed in central nervous system, which also produces a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA* 92, 612–616.
- Helfrich-Förster, C., 1997. Development of pigment-dispersing hormone-immunoreactive neurons in the nervous system of *Drosophila melanogaster*. *J. Comp. Neurol.* 380, 335–354.
- Helfrich-Förster, C., 2003. The neuroarchitecture of the circadian clock in the brain of *Drosophila melanogaster*. *Microsc. Res. Tech.* 62, 94–102.
- Helfrich-Förster, C., Stengl, M., Homberg, U., 1998. Organization of the circadian system in insects. *Chronobiol. Internat.* 15, 567–597.
- Hepburn, H.R., 1985. Structure of the integument. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 3. Pergamon, Oxford, pp. 1–58.
- Herzog, E.D., Tosini, G., 2001. The mammalian circadian clock shop. *Seminars Cell Devel. Biol.* 12, 295–303.
- Hesterlee, S., Morton, D.B., 1996. Insect physiology: the emerging story of ecdysis. *Curr. Biol.* 6, 648–650.

- Hoffmann, J.A., Lagueux, M., 1985. Endocrine aspects of embryonic development in insects. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 1. Pergamon, Oxford, pp. 435–460.
- Homberg, U., Davis, N.T., Hildebrand, J.G., 1991b. Peptide immunocytochemistry of neurosecretory cells in the brain and retrocerebral complex of the sphinx moth *Manduca sexta*. *J. Comp. Neurol.* 303, 35–52.
- Homberg, U., Würden, S., Dircksen, H., Rao, K.R., 1991a. Comparative anatomy of pigment-dispersing hormone-immunoreactive neurons in the brain of orthopteroid insects. *Cell Tissue Res.* 266, 343–357.
- Horike, N., Sonobe, H., 1999. Ecdysone 20-monooxygenase in eggs of the silkworm, *Bombyx mori*: enzymatic properties and developmental changes. *Arch. Insect Biochem. Physiol.* 41, 9–17.
- Horodyski, F.M., 1996. Neuroendocrine control of insect ecdysis by eclosion hormone. *J. Insect Physiol.* 42, 917–924.
- Huignard, J., 1983. Transfer and fate of male secretions deposited in the spermatophore of females of *Acanthoscelides obtectus* Say. *J. Insect Physiol.* 29, 55–63.
- Hunt, R.E., Haynes, K.F., 1990. Periodicity in the quantity and blend ratios of pheromone components in glands and volatile emissions of mutant and normal cabbage looper moths, *Trichoplusia ni*. *J. Insect Physiol.* 36, 769–774.
- Hurst, W.J., Earnest, D., Gillette, M.U., 2002. Immortalized suprachiasmatic nucleus cells express components of multiple circadian regulatory pathways. *Biochem. Biophys. Res. Commun.* 292, 20–30.
- Ichikawa, T., 1998. Activity patterns of neurosecretory cells releasing pheromonotropic neuropeptides in the moth *Bombyx mori*. *Proc. Natl Acad. Sci. USA* 95, 4055–4060.
- Ichikawa, T., Hasegawa, K., Shimizu, I., Katsuno, K., Kataoka, H., et al., 1995. Structure of neurosecretory cells with immunoreactive diapause hormone and pheromone biosynthesis activating neuropeptide in the *Bombyx mori*. *Zool. Sci.* 12, 703–712.
- Ichikawa, T., Ito, K., 1999. Calling behavior modulates heartbeat reversal rhythm in the silkworm, *Bombyx mori*. *Zool. Sci.* 16, 203–209.
- Iglesias, F., Jacquin-Joly, E., Marco, M.-P., Camps, F., Fabriàs, G., 1999. Temporal distribution of PBAN-like immunoreactivity in the hemolymph of *Mamestra brassicae* females in relation to sex pheromone production and calling behavior. *Arch. Insect Biochem. Physiol.* 40, 80–87.
- Iglesias, F., Marco, P., François, M.-C., Camps, F., Fabriàs, F., et al., 2002. A new member of the PBAN family in *Spodoptera littoralis*: molecular cloning and immunovisualization in scotophase hemolymph. *Insect Biochem. Mol. Biol.* 32, 901–908.
- Ikonomov, O.C., Stoynev, A.G., Shisheva, A.C., 1998. Integrative coordination of circadian mammalian diversity: neuronal networks and peripheral clocks. *Prog. Neurobiol.* 54, 87–97.
- Ishizaki, H., Suzuki, A., 1994. The brain secretory peptides that control molting and metamorphosis of the silkworm, *Bombyx mori*. *Int. J. Devel. Biol.* 38, 301–310.
- Itagaki, H., Conner, W.E., 1988. Calling behavior of *Manduca sexta* (L.) (Lepidoptera: Sphingidae) with notes on the morphology of the female sex pheromone gland. *Ann. Entomol. Soc. America* 81, 795–807.
- Itoh, M.T., Hatori, A., Sumi, Y., Suzuki, T., 1994. Identification of melatonin in different organs of the cricket, *Gryllus bimaculatus*. *Zool. Sci.* 11, 577–581.
- Itoh, M.T., Hatori, A., Sumi, Y., Suzuki, T., 1995a. Day-night changes in melatonin levels in different organs of the cricket (*Gryllus bimaculatus*). *J. Pineal Res.* 18, 165–169.
- Itoh, M.T., Nomura, T., Sumi, Y., Suzuki, T., 1995b. Melatonin and arylalkylamine N-acetyltransferase activity in the silkworm, *Bombyx mori*. *Mol. Cell. Endocrinol.* 115, 59–64.
- Itoh, M.T., Sumi, Y., 1998a. Melatonin and serotonin N-acetyltransferase activity in developing eggs of the cricket *Gryllus bimaculatus*. *Brain Res.* 781, 91–99.
- Itoh, M.T., Sumi, Y., 1998b. Circadian clock controlling arylalkylamine N-acetyltransferase-like activity in the cricket (*Gryllus bimaculatus*) eggs. *Brain Res.* 799, 172–175.
- Itoh, M.T., Sumi, Y., 2000. Circadian clock controlling egg hatching in the cricket (*Gryllus bimaculatus*). *J. Biol. Rhythms* 121, 241–245.
- Jackson, R.R., Schroeder, A.J., Roberts, M.A., McNeil, G.P., Kume, K., et al., 2001. Cellular and molecular mechanisms of circadian control in insects. *J. Insect Physiol.* 47, 833–842.
- Jacquin-Joly, E., Burnet, M., François, M.C., Amma, D., Nagnan-LeMeillour, P., et al., 1998. cDNA cloning and sequence determination of the pheromone biosynthesis activating neuropeptide of *Mamestra brassicae*: a new member of the PBAN family. *Insect Biochem. Mol. Biol.* 28, 251–258.
- Jan, L.Y., Jan, Y.N., 1982. Antibodies to horseradish peroxidase as specific neuronal markers in *Drosophila* and grasshopper embryos. *Proc. Natl Acad. Sci. USA* 79, 2700–2704.
- Jeon, S.K., Lee, B.H., 1999. Immunocytochemical identification of allatostatin producing neurons in developing central nervous system of the silk moth, *Bombyx mori*. *Korean J. Entomol.* 29, 233–238.
- Johnston, J.S., Ellison, J.R., 1982. Exact age determination in laboratory and field-caught *Drosophila*. *J. Insect Physiol.* 28, 773–779.
- Jones, M.D.R., Gubbins, S.J., 1979. Modification of female circadian flight-activity by a male accessory gland pheromone in the mosquito, *Culex pipiens quinquefasciatus*. *Physiol. Entomol.* 4, 345–352.
- Joshi, D.S., 1996. Psi-mutation affects phase angle difference, free-running period and phase shifts in *Aedes krombeini* (Stegomyia). *Biol. Rhythm Res.* 27, 421–430.
- Jurenka, R.A., Jacquin, E., Roelofs, W.L., 1991. Stimulation of pheromone biosynthesis in the moth

- Helicoverpa zea*: action of a brain hormone on pheromone glands involves Ca^{2+} and cAMP as second messengers. *Proc. Natl Acad. Sci. USA* 88, 8621–8625.
- Kadono-Okuda, K., Amornsak, W., Yamashita, O., 1994. Controlled ecdysteroid accumulation in eggs of the silkworm, *Bombyx mori*, by an imidazole compound (KK-42) and embryogenesis in these eggs. *Arch. Insect Biochem. Physiol.* 25, 121–135.
- Kajiura, Z., Nakagaki, M., Takei, R., 1993. Spermiogenesis of the testes of juvenile hormone-treated silkworm larvae, *Bombyx mori*. *Comp. Biochem. Physiol. A* 106, 495–499.
- Kalogianni, E., Theophilidis, G., 1993. Centrally generated rhythmic activity and modulatory function of the oviductal dorsal unpaired median (DUM) neurons in two orthopteran species (*Calliptamus* sp. and *Decticus albifrons*). *J. Exp. Biol.* 174, 123–138.
- Kalsbeek, A., Buijs, R.M., 2002. Output pathways of the mammalian suprachiasmatic nucleus: coding circadian time by transmitter selection and specific targeting. *Cell Tissue Res.* 309, 109–118.
- Kamimura, M., Tatsuki, S., 1993. Diel rhythms of calling behavior and pheromone, production of Oriental tobacco budworm moth, *Helicoverpa assulta* (Lepidoptera: Noctuidae). *J. Chem. Ecol.* 19, 2953–2963.
- Kamimura, M., Tatsuki, S., 1994. Effects of photoperiodic changes on calling behavior and pheromone production in the Oriental tobacco budworm moth, *Helicoverpa assulta* (Lepidoptera: Noctuidae). *J. Insect Physiol.* 40, 731–734.
- Kaneko, J., 1986. Effect of temperature on the timing of calling of the yellow peach moth, *Congethes punctiferalis* (Guenee) (Lepidoptera: Pyralidae). *Jap. J. Appl. Entomol. Zool.* 30, 239–246.
- Kaneko, M., Hall, J.C., 2000. Neuroanatomy of cells expressing clock genes in *Drosophila*: transgenic manipulation of the *period* and *timeless* genes to mark the perikarya of circadian pacemaker neurons and their projections. *J. Comp. Neurol.* 422, 66–94.
- Kaneko, M., Helfrich-Förster, C., Hall, J.C., 1997. Spatial and temporal expression of the *period* and *timeless* genes in the developing nervous system of *Drosophila*: newly identified pacemaker candidates and novel features of clock gene product cycling. *J. Neurosci.* 17, 6745–6760.
- Katsoyannos, B.I., 1982. Male sex pheromone of *Rhagoletis ceraci* (Diptera: Tephritidae): factors affecting release and response and its role in the mating behavior. *Z. Angew. Entomol.* 94, 187–198.
- Kingan, T.G., Adams, M.E., 2000. Ecdysteroids regulate secretory competence in Inka cells. *J. Exp. Biol.* 203, 3011–3018.
- Kingan, T.G., Gray, W., Žitnan, D., Adams, M.E., 1997. Regulation of ecdysis-triggering hormone secretion by eclosion hormone. *J. Exp. Biol.* 200, 3245–3256.
- Kingan, T.G., Raina, A.K., Blackburn, M., Ma, M., 1990. Distribution of PBAN-like immunoreactivity in the CNS of the corn earworm, *Heliothis zea*. *Neurosci. Abstr.* 16, 856.
- Kitamura, A., Nagasawa, H., Kataoka, H., Inoue, T., Matsumoto, S., et al., 1989. Amino acid sequence of pheromone biosynthesis-activating-neuropeptide (PBAN) of the silkworm, *Bombyx mori*. *Biochem. Biophys. Res. Commun.* 163, 520–526.
- Knobloch, C.A., Steel, C.G.H., 1987. Effects of decapitation at the head critical period for molting on hemolymph ecdysteroid titers in final-instar male and female *Rhodnius prolixus* (Hemiptera). *J. Insect Physiol.* 33, 967–972.
- Koepe, J.K., Fuchs, M., Chen, T.T., Hunt, L.-M., Kovalick, G.E., et al., 1985. The role of juvenile hormone in reproduction. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 8. Pergamon, Oxford, pp. 165–203.
- Konopka, R.J., Kyriakou, C.P., Hall, J.C., 1996. Mosaic analysis in the *Drosophila* CNS of circadian and courtship-song rhythms affected by a *period* clock mutation. *J. Neurogen.* 11, 117–139.
- Kopec, S., 1922. Studies on the necessity of the brain for the inception of insect metamorphosis. *Biol. Bull.* 42, 323–342.
- Kou, R., 1992. Calling behavior and pheromone titer in the smaller tea tortrix moth, *Adoxophyes* spp. (Lepidoptera: Tortricidae). *J. Chem. Ecol.* 18, 855–861.
- Kou, R., Chow, Y.S., 1987. Calling behavior of the cotton bollworm, *Heliothis armigera* (Lepidoptera: Noctuidae). *Ann. Entomol. Soc. America* 80, 490–493.
- Koudele, K., Stout, J.F., Reichert, D., 1987. Factors which influence female crickets' (*Acheta domesticus*) phonotactic and sexual responsiveness to males. *Physiol. Entomol.* 12, 67–80.
- Krishnan, B., Dryer, S.E., Hardin, P.E., 1999. Circadian rhythms in olfactory responses of *Drosophila melanogaster*. *Nature* 400, 375–378.
- Krishnan, B., Levine, J.D., Lynch, K.S., Dowse, H.B., Funes, P., et al., 2001. A novel role for cryptochrome in a *Drosophila* circadian oscillator. *Nature* 411, 313–317.
- Kristensen, B.I., 1966. Incorporation of tyrosine into the rubber-like cuticle of locusts studied by autoradiography. *J. Insect Physiol.* 12, 173–177.
- Kubli, E., 1992. The sex peptide. *BioEssays* 14, 779–784.
- Kuwahara, Y., Adachi, S., Tsuchida, N., 1983. Bombykol content in female silkworm moth, *Bombyx mori* (Lepidoptera: Bombycidae): effect of age, mating and body weight. *Appl. Entomol. Zool.* 18, 182–190.
- Kyriakou, C.P., Hall, J.C., 1980. Circadian rhythm mutations in *Drosophila melanogaster* affect short-term fluctuations in the male's courtship song. *Proc. Natl Acad. Sci. USA* 77, 6729–6733.
- Kyriakou, C.P., Hall, J.C., 1982. The function of courtship song rhythms in *Drosophila*. *Anim. Behav.* 30, 794–801.
- LaChance, L.E., Richard, R.D., Ruud, R.L., 1977. Movement of eupyrene sperm bundles from the testis and storage in the ductus ejaculatoris duplex of the male

- pink bollworm: effects of age, strain, irradiation, and light. *Ann. Entomol. Soc. America* 70, 647–651.
- Lagueux, M., Hetru, C., Goltzene, E., Kappler, C., Hoffmann, J.A., 1979. Ecdysone titer and metabolism in relation to cuticulogenesis in embryos of *Locusta migratoria*. *J. Insect Physiol.* 25, 709–723.
- Lall, A.B., 1993. Nightly increase in visual sensitivity correlated with bioluminescent flashing activity in the firefly *Photuris versicolor* (Coleoptera: Lampyridae). *J. Exp. Zool.* 265, 609–612.
- Lammerding-Köppel, M., Spindler-Barth, M., Steiner, E., Lezzi, M., Drews, U., et al., 1998. Immunohistochemical localization of ecdysteroid receptor and ultraspiracle in the epithelial cell line from *Chironomus tentans* (Insecta, Diptera). *Tissue Cell* 30, 187–194.
- Lanzrein, B., Gentinetta, V., Abegglen, H., Baker, F.C., Miller, C.A., et al., 1985. Titrers of ecdysone, 20-hydroxyecdysone and juvenile hormone III throughout the life cycle of a hemimetabolous insect, the ovoviviparous cockroach *Nauphoeta cinerea*. *Experientia* 41, 913–917.
- Leal, W.S., Hasegawa, M., Sawada, M., Ono, M., Tada, S., 1996. Scarab beetle *Anomala albopilosa* utilizes a more complex sex pheromone system than a similar species *A. cuprea*. *J. Chem. Ecol.* 22, 2001–2010.
- Lee, H.J., Wu, Y.L., 1994. Mating effects on the feeding and locomotion of the German cockroach, *Blattella germanica*. *Physiol. Entomol.* 19, 39–45.
- Lee, H.T.Y., 1948. A comparative morphological study of prothoracic glandular bands of some lepidopteran larvae with special reference to their innervation. *Ann. Entomol. Soc. America* 41, 200–205.
- Leitch, B., Laurent, G., Shepherd, D., 1992. Embryonic development of synapses on spiking local interneurons in locust. *J. Comp. Neurol.* 324, 213–236.
- Leppa, N.C., Koehler, P.G., Agee, H.R., 1989. Circadian rhythms of the German cockroach (Dictyoptera: Blattellidae): locomotion in response to different photoperiods and wavelengths of light. *J. Insect Physiol.* 35, 63–66.
- Lewy, A.J., Sack, R.L., Blood, M.L., Bauer, V.K., Cutler, N.L., et al., 1995. Melatonin marks circadian phase position and resets the endogenous circadian pacemaker in humans. In: Chadwick, D.J., Ackrill, K. (Eds.), *Circadian Clocks and their Adjustment*. Wiley, New York, pp. 303–321.
- Lezzi, M., Gatzka, F., Ineichen, H., Gruzdev, A.D., 1991. Transcriptional activation of puff site I-18C of *Chironomus tentans*: hormonal responsiveness changes in parallel with diurnal decondensation cycle. *Chromosoma* 100, 235–241.
- Liang, D., Schal, C., 1990. Circadian rhythmicity and development of the behavioral response to sex pheromone in male brown-banded cockroaches, *Supella longipalpa*. *Physiol. Entomol.* 15, 355–361.
- Lin, T.M., Lee, H.J., 1998. Parallel control mechanisms underlying locomotor activity and sexual receptivity of the female German cockroach, *Blattella germanica* (L.). *J. Insect Physiol.* 44, 1039–1051.
- Linn, C.E., 1997. Neuroendocrine factors in the photoperiodic control of male moth responsiveness to sex pheromone. In: Cardé, R.T., Minks, A.K. (Eds.), *Insect Pheromone Research: New Directions*. Chapman and Hall, London, pp. 194–209.
- Linn, C.E., Cambell, M.G., Poole, K.R., Wu, W.-Q., Roelofs, W.L., 1996. Effects of photoperiod on the circadian timing of pheromone response in male *Trichoplusia ni*: relationship to the modulatory action of octopamine. *J. Insect Physiol.* 42, 881–891.
- Linn, C.E., Cambell, M.G., Roelofs, W.L., 1992. Photoperiodic cues and the modulatory action of octopamine and 5-hydroxytryptamine on locomotor and pheromone response in male gypsy moths, *Lymantria dispar*. *Arch. Insect Biochem. Physiol.* 20, 265–284.
- Linn, C.E., Poole, K.R., Wu, W.Q., Roelofs, W.L., 1995. Circadian changes in melatonin in the nervous system and hemolymph of the cabbage looper moth, *Trichoplusia ni*. *J. Comp. Physiol. A* 176, 761–771.
- Linn, C.E., Roelofs, W.L., 1992. Role of photoperiod cues in regulating the modulatory action of octopamine on pheromone-response thresholds in the cabbage looper moth. *Arch. Insect Biochem. Physiol.* 20, 285–302.
- Liu, X., Lorenz, L., Yu, Q., Hall, J.C., Rosbash, M., 1988. Spatial and temporal expression of the *period* gene in *Drosophila melanogaster*. *Genes Devel.* 2, 228–238.
- Loeb, M.J., Brandt, E.P., Birnbaum, M.J., 1984. Ecdysteroid production by testes of the tobacco budworm, *Heliothis virescens*, from last larval instar to adult. *J. Insect Physiol.* 30, 375–381.
- Loeb, M.J., Brandt, E.P., Woods, C.W., Bell, R.A., 1988. Secretion of ecdysteroid by sheaths of testes of the gypsy moth, *Lymantria dispar*, and its regulation by testis ecdysiotropin. *J. Exp. Zool.* 248, 94–100.
- Löfstedt, L., Van Der Pers, J.N.C., Lofqvist, J., Lanne, B.S., Appelgren, M., et al., 1982. Sex pheromone components of the turnip moth, *Agrotis segetum*: chemical identification, electrophysiological evaluation and behavioral activity. *J. Chem. Ecol.* 8, 1305–1321.
- Loher, W., Zervas, G., 1979. The mating rhythm of the olive fruit fly, *Dacus oleae*. *Z. Angew. Entomol.* 88, 425–435.
- Lukat, R., Weber, F., Wiedenmann, G., 1989. Cyclic layer deposition in the cockroach (*Blaberus craniifer*) endocuticle: a decentral circadian clock? *J. Insect Physiol.* 35, 321–329.
- Ma, P.W.K., Garden, R.W., Niermann, J.T., O'Connor, M., Sweedler, J.V., et al., 2000. Characterizing the Hez-PBAN gene products in neuronal clusters with immunocytochemistry and MALDI MS. *J. Insect Physiol.* 46, 221–230.
- Ma, P.W.K., Knipple, D.C., Roelofs, W.L., 1998. Expression of a gene that encodes pheromone biosynthesis activating neuropeptide in the central nervous system of corn earworm, *Helicoverpa zea*. *Insect Biochem. Mol. Biol.* 28, 373–385.

- Ma, P.W.K., Roelofs, W.L., 1995. Sites of synthesis and release of PBAN-like factor in the female European corn borer, *Ostrinia nubilalis*. *J. Insect Physiol.* 41, 339–350.
- Martinez, T., Camps, F., 1988. Stimulation of sex pheromone production by head extract in *Spodoptera littoralis* at different times of the photoperiod. *Arch. Insect Biochem. Physiol.* 9, 211–220.
- Masler, E.P., Raina, A.K., Wagner, R.M., Kochansky, J.P., 1994. Isolation and identification of a pheromonotropic neuropeptide from the brain–suboesophageal ganglion complex of *Lymantria dispar*: a new member of the PBAN family. *Insect Biochem. Mol. Biol.* 24, 829–836.
- Matsumoto, S., Ozawa, R., Nagamin, T., Gil-Hah, K., Uchiumi, K., *et al.*, 1995. Intracellular transduction in the regulation of pheromone biosynthesis of the silkworm, *Bombyx mori*: suggested involvement of calmodulin and phosphoprotein phosphatase. *Biosci. Biotechnol. Biochem.* 59, 560–562.
- Maywood, E.S., Okamura, H., Hastings, M.H., 2002. Opposing actions of neuropeptide Y and light on the expression of circadian clock genes in the mouse supra-chiasmatic nuclei. *Eur. J. Neurosci.* 15, 216–220.
- Mbata, G.N., Ramaswamy, S.B., 1990. Rhythmicity of sex pheromone content in female *Heliothis virescens*: impact of mating. *Physiol. Entomol.* 15, 423–432.
- McNabb, S.L., Baker, J.D., Agapite, J., Steller, H., Riddiford, L.M., *et al.*, 1997. Disruption of a behavioral sequence by targeted death of peptidergic neurons in *Drosophila*. *Neuron* 19, 813–823.
- McNamara, P., Seo, S.B., Rubic, R.D., Seghali, A., Chakravarti, D., *et al.*, 2001. Regulation of CLOCK and MOP4 by nuclear hormone receptors in vasculature: a humoral mechanism to reset a peripheral clock. *Cell* 105, 877–889.
- Mesce, K.A., Fahrbach, S.E., 2002. Integration of endocrine signals that regulate insect ecdysis. *Frontiers Neuroendocrin.* 23, 179–199.
- Minis, D.H., Pittendrigh, C.S., 1968. Circadian oscillation controlling hatching: its ontogeny during embryogenesis of a moth. *Science* 159, 534–536.
- Mizoguchi, A., Dedos, S.G., Fugo, H., Kataoka, H., 2002. Basic pattern of fluctuation in hemolymph PTTH titers during larval–pupal and pupal–adult development of the silkworm, *Bombyx mori*. *Gen. Comp. Endocrinol.* 127, 181–189.
- Mizoguchi, A., Ishizaki, H., 1982. Prothoracic glands of the saturniid moth *Samia cynthia ricini* possess a circadian clock controlling gut purge timing. *Proc. Natl Acad. Sci. USA* 79, 2726–2730.
- Mizoguchi, A., Ishizaki, H., 1984a. Circadian clock controlling gut-purge rhythm of the saturniid *Samia cynthia ricini*: its characterization and entrainment mechanism. *J. Comp. Physiol. A* 155, 639–647.
- Mizoguchi, A., Ishizaki, H., 1984b. Further evidence for the presence of a circadian clock in the prothoracic glands of the saturniid moth *Samia cynthia ricini*: decapitated larvae can respond to light-dark changes. *Devel. Growth Differ.* 26, 607–611.
- Mizoguchi, A., Ohashi, Y., Hosoda, K., Ishibashi, J., Kataoka, H., 2001. Developmental profile of the changes in the prothoracicotropic hormone titer in hemolymph of the silkworm *Bombyx mori*: correlation with ecdysteroid secretion. *Insect Biochem. Mol. Biol.* 31, 349–358.
- Mizoguchi, A., Oka, T., Kataoka, H., Nagasawa, H., Suzuki, A., *et al.*, 1990. Immunohistochemical localization of prothoracicotropic-hormone producing neurosecretory cells in the brain of *Bombyx mori*. *Devel. Growth Differ.* 32, 501–598.
- Molis, T.M., Spriggs, L.L., Hill, S.M., 1994. Modulation of estrogen receptor mRNA expression by melatonin in MCF-7 human breast cancer cells. *Mol. Endocrinol.* 8, 1681–1690.
- Morton, D.B., Truman, J.W., 1988. The EGPs: the ecdision hormone and cyclic GMP-regulated phosphoproteins. 2. Regulation of appearance by the steroid hormone 20-hydroxyecdysone in *Manduca sexta*. *J. Neurosci.* 8, 1338–1345.
- Moshitzky, P., Fleischmann, I., Chaimov, N., Saudan, P., Klausner, S., *et al.*, 1996. Sex-peptide activates juvenile hormone biosynthesis in the *Drosophila melanogaster* corpus allatum. *Arch. Insect Biochem. Physiol.* 32, 363–374.
- Mullins, D.E., Keil, C.B., White, R.H., 1992. Maternal and paternal nitrogen investment in *Blattella germanica* (L.) (Dictyoptera: Blattellidae). *J. Exp. Biol.* 162, 55–72.
- Nässel, D.R., 2000. Functional roles of neuropeptides in the insect central nervous system. *Naturwissenschaften* 87, 439–449.
- Nässel, D.R., Shiga, S., Wikstrand, E.M., Rao, K.R., 1991. Pigment-dispersing hormone-immunoreactive neurons and their relation to serotonergic neurons in the blowfly and cockroach visual system. *Cell Tissue Res.* 265, 511–523.
- Nation, J.L., 1990. Biology of pheromone release by male Caribbean fruit flies, *Anastrepha suspensa* (Diptera: Tephritidae). *J. Chem. Ecol.* 16, 553–572.
- Neville, A.C., 1965. Circadian organization of chitin in some insect skeletons. *Q. J. Microsc. Sci.* 106, 315–325.
- Neville, A.C., 1967. A dermal light sense influencing skeletal structures in locusts. *J. Insect Physiol.* 13, 933–939.
- Neville, A.C., 1975. *Biology of Arthropod Cuticle*. Springer, Berlin.
- Neville, A.C., 1983. Daily cuticular growth layers and the teneral stage in adult insects: a review. *J. Insect Physiol.* 29, 211–219.
- Nickisch-Rosenegk, E. von, Krieger, J., Kubick, S., Laage, R., Strobel, J., *et al.*, 1996. Cloning of biogenic amine receptors from moths (*Bombyx mori* and *Heliothis virescens*). *Insect Biochem. Mol. Biol.* 26, 817–827.
- Nijhout, H.F., 1994. *Insect Hormones*. Princeton University Press, Princeton, NJ.
- Noldus, L.P.J.J., Potting, R.P.J., 1990. Calling behavior of *Mamestra brassicae* (Lepidoptera: Noctuidae): effects of age and photoperiod. *Entomol. Exp. Applic.* 56, 23–30.

- Nonaka, H., Emoto, N., Ikeda, K., Fukuya, H., Rohman, M.S., *et al.*, 2001. Angiotensin II induces circadian gene expression of clock genes in cultured vascular smooth muscle cells. *Circulation* 104, 1746–1748.
- Noronha, K.F., Lange, A.B., 1997. Proctolin's role in neurally evoked contractions of the locust oviducts. *J. Neurobiol.* 33, 139–150.
- O'Brien, M.A., Katahira, E.J., Flanagan, T.R., Arnold, L.W., Haughton, G., *et al.*, 1988. A monoclonal antibody to the insect prothoracicotropic hormone. *J. Neurosci.* 8, 3247–3257.
- Ono, T., Charlton, R.E., Cardé, R.T., 1990. Variability in pheromone composition and periodicity of pheromone titer in potato tuberworm moth, *Phthorimaea oerculella* (Lepidoptera: Gelechiidae). *J. Chem. Ecol.* 16, 531–542.
- Orchard, I., Donly, B.C., Fuse, M., Lange, A.B., Tobe, S.S., *et al.*, 1997. FMRFamide-related peptides in insects, with emphasis on the myosuppressins. *Neuropept. Dev. Aging* 814, 307–309.
- Orchard, I., Lange, A.B., 1988. The regulation of insect visceral muscle by octopamine. In: Boulton, A.A., Juorio, A.V., Downe, R.G. (Eds.), Trace Amines. Humana Press, Totowa, NJ, pp. 41–51.
- Ottiger, M., Soller, M., Stocker, R.F., Kubli, E., 2000. Binding sites of *Drosophila melanogaster* sex peptide pheromones. *J. Neurobiol.* 44, 57–71.
- Page, T.L., 1983. Regeneration of the optic lobe tracts and circadian pacemaker activity in the cockroach *Leucophaea maderae*. *J. Comp. Physiol. A* 152, 231–240.
- Page, T.L., 1985. Clocks and circadian rhythms. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 6. Pergamon, Oxford, pp. 577–652.
- Page, T.L., 1988. Circadian organization and the representation of circadian information in the nervous system of invertebrates. *Adv. Biosci.* 73, 67–79.
- Pang, S.F., Li, L., Ayre, E.A., Pang, C.S., Lee, P.P.N., *et al.*, 1998. Neuroendocrinology of melatonin in reproduction: recent developments. *J. Chem. Neuroanat.* 14, 157–166.
- Park, C., Jeon, S.K., Kim, M.Y., Han, S.S., Yu, C.H., *et al.*, 2001. Postembryonic localization of allatotropin- and allatostatin-producing cells in central nervous system of the silk moth, *Bombyx mori*. *Zool. Sci.* 18, 367–379.
- Park, Y., Filippov, V., Gill, S.S., Adams, M.E., 2002. Deletion of the ecdysis-triggering hormone gene leads to lethal ecdysis deficiency. *Development* 129, 493–503.
- Pelc, D., Steel, C.G.H., 1997. Rhythmic steroidogenesis by the prothoracic glands of the insect *Rhodnius prolixus* in the absence of rhythmic neuropeptide input: implications for the role of prothoracicotropic hormone. *Gen. Comp. Endocrinol.* 108, 358–365.
- Petri, B., Homberg, U., Loesel, R., Stengl, M., 2002. Evidence for the role of GABA and Mas-allatotropin in photic entrainment of the circadian clock of the cockroach *Leucophaea maderae*. *J. Exp. Biol.* 205, 1459–1469.
- Petri, B., Stengl, M., 1997. Pigment-dispersing hormone shifts the phase of the circadian pacemaker of the cockroach *Leucophaea maderae*. *J. Neurosci.* 17, 4087–4093.
- Petri, B., Stengl, M., Wuerden, S., Homberg, U., 1995. Immunocytochemical characterization of the accessory medulla in the cockroach *Leucophaea maderae*. *Cell Tissue Res.* 282, 3–19.
- Picimbon, J.-F., Bécard, J.-M., Sreng, L., Clément, J.-L., Gadenne, C., 1995. Juvenile hormone stimulates pheromonotropic brain factor release in the female black cutworm, *Agrotis ipsilon*. *J. Insect Physiol.* 41, 377–382.
- Picimbon, J.-F., Gadenne, C., Bécard, J.M., Clément, J.L., Sreng, L., 1997. Sex pheromone of the French black cutworm moth, *Agrotis ipsilon* (Lepidoptera: Noctuidae): identification and regulation of a multicomponent blend. *J. Chem. Ecol.* 23, 211–229.
- Pickard, G.E., Tang, W.-X., 1994. Pineal photoreceptors rhythmically secrete melatonin. *Neurosci. Lett.* 171, 109–112.
- Pittendrigh, C.S., 1993. Temporal organization: reflections of a Darwinian clock-watcher. *Annu. Rev. Physiol.* 55, 17–54.
- Pivnic, K.A., 1993. Response of males to female sex pheromone in the orange blossom midge, *Sitodiplosis mosellana* (Gehin) (Diptera: Cecidomyiidae). *J. Chem. Ecol.* 19, 1677–1689.
- Plautz, J.D., Kaneko, M., Hall, J.C., Kay, S.A., 1997. Independent photoreceptive circadian clocks throughout *Drosophila*. *Science* 278, 1632–1635.
- Pope, M.M., Gaston, L.K., Baker, T.C., 1982. Composition, quantification and periodicity of sex pheromone gland volatiles from individual *Heliothis virescens* females. *J. Chem. Ecol.* 8, 1043–1056.
- Pophof, B., 2000. Octopamine modulates the sensitivity of silkworm pheromone receptor neurons. *J. Comp. Physiol. A* 186, 307–313.
- Pophof, B., 2002. Octopamine enhances moth olfactory responses to pheromone, but not those to general odors. *J. Comp. Physiol. A* 188, 659–662.
- Predel, R., Eckert, M., 2000. Neurosecretion: peptidergic systems in insects. *Naturwissenschaften* 87, 343–350.
- Rafaeli, A., 1994. Pheromonotropic stimulation of moth pheromone gland cultures *in vitro*. *Arch. Insect Biochem. Physiol.* 25, 287–299.
- Rafaeli, A., Gileadi, C., 1997. Neuroendocrine control of pheromone production in moths. *Invert. Neurosci.* 3, 223–229.
- Rafaeli, A., Gileadi, C., Fan, Y., Cao, M., 1997. Physiological mechanisms of pheromonostatic responses: effects of adrenergic agonists and antagonists on moth (*Helicoverpa armigera*) pheromone biosynthesis. *J. Insect Physiol.* 43, 261–269.
- Rafaeli, A., Hirsch, J., Soroker, V., Kamensky, B., Raina, A.K., 1991. Spatial and temporal distribution of pheromone biosynthesis-activating neuropeptide in *Helicoverpa (Heliothis) armigera* using RIA and *in vitro* bioassay. *Arch. Insect Biochem. Physiol.* 18, 119–129.

- Rafaeli, A., Soroker, V., 1989. Influence of diel rhythm and brain hormone on pheromone production in two lepidopteran species. *J. Chem. Ecol.* 15, 447–456.
- Rafaeli, A., Soroker, V., Hirsch, J., Kamensky, B., Raina, A.K., 1993. The influence of photoperiod and age on the competence of pheromone glands and the distribution of immunoreactive PBAN in *Helicoverpa* spp. *Arch. Insect Biochem. Physiol.* 22, 169–180.
- Rafaeli, A., Zakharova, T., Lapsker, Z., Jurenka, R.A., 2003. The identification of an age- and female-specific putative PBAN membrane-receptor protein in pheromone glands of *Helicoverpa armigera*: possible up-regulation by juvenile hormone. *Insect Biochem. Mol. Biol.* 33, 371–380.
- Raina, A.K., Jaffe, H., Kempe, T.G., Keim, P., Blacher, R.W., et al., 1989. Identification of a neuropeptide hormone that regulates sex pheromone production in female moths. *Science* 244, 796–798.
- Raina, A.K., Klun, J.A., 1984. Brain factor control of sex pheromone production in the female corn earworm moth. *Science* 225, 531–533.
- Ramaswamy, S.B., Cardé, R.T., 1984. Rate of release of spruce budworm (*Choristoneura fumiferana*) pheromone from virgin females and synthetic lures. *J. Chem. Ecol.* 10, 1–8.
- Ramaswamy, S.B., Jurenka, R.A., Linn, C.E., Roelofs, W.L., 1995. Evidence for the presence of a pheromonotropic factor in hemolymph and regulation of sex pheromone production in *Helicoverpa zea*. *J. Insect Physiol.* 41, 501–508.
- Ramaswamy, S.B., Mbata, G.N., Cohen, N.E., Moore, A., Cox, N.M., 1994. Pheromonotropic and pheromonostatic activity in moths. *Arch. Insect Biochem. Physiol.* 25, 301–315.
- Rao, K.R., Riehm, J.P., 1993. Pigment-dispersing hormones. *Ann. New York Acad. Sci.* 680, 78–88.
- Reischig, T., Stengl, M., 1997. The accessory medulla is the presumptive circadian pacemaker in the cockroach *Leucophaea maderae*. *Verhandl. Deut. Zool. Gesells.* 89–3, 45.
- Rence, B.G., Lisy, M.T., Garves, B.R., Quinlan, B.J., 1988. The role of ocelli in circadian singing rhythms of crickets. *Physiol. Entomol.* 13, 201–212.
- Reppert, S.M., Weaver, D.R., 2001. Molecular analysis of mammalian circadian rhythms. *Ann. Rev. Physiol.* 63, 647–676.
- Reynolds, S.E., 1980. Integration of behavior and physiology in ecdysis. *Adv. Insect Physiol.* 15, 475–696.
- Reynolds, S.E., 1987. Endocrine timing signals that direct ecdysial physiology and behavior. In: Borkovec, A.B., Gelman, D. (Eds.), *Insect Neurochemistry and Neurophysiology*. Humana Press, Totowa, NJ, pp. 53–77.
- Richards, G., 1981. Insect hormones in development. *Biol. Rev.* 56, 501–549.
- Richter, K., 2001. Daily changes in neuroendocrine control of molting hormone secretion in the prothoracic gland of the cockroach, *Periplaneta americana* (L.). *J. Insect Physiol.* 47, 333–338.
- Richter, K., Pescjke, E., Peschke, D., 2000. A neuroendocrine releasing effect of melatonin in the brain of an insect, *Periplaneta americana* (L.). *J. Pineal Res.* 28, 129–135.
- Riddiford, L.M., 1985. Hormone action at the cellular level. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 8. Pergamon, Oxford, pp. 37–84.
- Riddiford, L.M., Cherbas, P., Truman, J.W., 2000. Ecdysone receptors and their biological actions. *Vitamins Hormones* 60, 1–73.
- Riddiford, L.M., Hewes, R.S., Truman, J.W., 1994. Dynamics and metamorphosis of an identifiable peptidergic neuron in an insect. *J. Neurobiol.* 25, 819–830.
- Riedl, H., Loher, W., 1980. Circadian control of oviposition in the codling moth, *Laspeyresia pomonella*, Lepidoptera: Olethreutidae. *Entomol. Exp. Applic.* 27, 38–49.
- Riemann, J.G., Giebultowicz, J.M., 1991. Secretion in the upper vas deferens of the gypsy moth correlated with the circadian rhythm of sperm release from the testes. *J. Insect Physiol.* 37, 53–62.
- Riemann, J.G., Thorson, B.J., 1971. Sperm maturation in the male and female genital tract of *Anagasta kühniella* (Lepidoptera: Pyralidae). *Int. J. Insect Morphol. Embryol.* 1, 11–19.
- Riemann, J.G., Thorson, B.J., 1976. Ultrastructure of the vasa deferentia of the Mediterranean flour moth. *J. Morphol.* 149, 483–506.
- Riemann, J.G., Thorson, B.J., Ruud, R.L., 1974. Daily cycle of release of sperm from the testes of the Mediterranean flour moth. *J. Insect Physiol.* 20, 195–207.
- Rosén, W.-Q., 2002. Endogenous control of circadian rhythms of pheromone production in the turnip moth, *Agrotis segetum*. *Arch. Insect Biochem. Physiol.* 50, 21–30.
- Roy, E.J., Wilson, M.A., 1981. Diurnal rhythm of cytoplasmic estrogen receptors in the rat brain in the absence of circulating estrogens. *Science* 213, 1525–1527.
- Saez, L., Young, M.W., 1988. *In situ* localization of the PER clock protein during development of *Drosophila melanogaster*. *Mol. Cell Biol.* 8, 5378–5385.
- Saifullah, A.S.M., Tomioka, K., 2002. Serotonin sets the day state in the neurons that control coupling between the optic lobe circadian pacemakers in the cricket, *Gryllus bimaculatus*. *J. Exp. Biol.* 205, 1305–1314.
- Saifullah, A.S.M., Tomioka, K., 2003. Pigment-dispersing factor sets the night state of the medulla bilateral neurons in the optic lobe of the cricket, *Gryllus bimaculatus*. *J. Insect Physiol.* 49, 231–239.
- Sakai, T., Ishida, N., 2001. Circadian rhythms of female mating activity governed by clock genes in *Drosophila*. *Proc. Natl Acad. Sci.* 98, 9221–9225.
- Sakamoto, K., Shimizu, I., 1994. Photosensitivity in the circadian hatching rhythm of the carotenoid-depleted silkworm, *Bombyx mori*. *J. Biol. Rhythms* 9, 61–70.

- Sakurai, S., 1983. Temporal organization of endocrine events underlying larval-larval ecdysis in the silkworm, *Bombyx mori*. *J. Insect Physiol.* 29, 919-932.
- Sakurai, S., 1984. Temporal organization of endocrine events underlying larval-pupal metamorphosis in the silkworm, *Bombyx mori*. *J. Insect Physiol.* 30, 657-664.
- Sakurai, S., Kaya, M., Satake, S., 1998. Hemolymph ecdysteroid titer and ecdysteroid-dependent developmental events in the larval-pupal stadium of the silkworm, *Bombyx mori*: role of low ecdysteroid titer in larval-pupal metamorphosis and a reappraisal of the head critical period. *J. Insect Physiol.* 44, 867-881.
- Sall, C., Tsoupras, G., Kappler, C., Lagueux, M., Zachary, D., et al., 1983. Maternal conjugated ecdysteroids during embryonic development in *Locusta migratoria*. *J. Insect Physiol.* 29, 491-507.
- Sasaki, M., Jibiki, F., Hirobe, T., 1984. Comparison of the daily rhythmic behavior between wild and domestic silkmoths, *Bombyx mandarina* and *Bombyx mori*. *Bull. Fac. Agric. Tamagawa Univ.* 24, 26-42.
- Sasaki, M., Yamazaki, S., Chiba, K., 1987. Brain photoreception in the calling rhythm of a noctuid moth, *Anadevidia peponis*: method of making eyeless moth and micro-irradiation of brain with fiber optics. *Bull. Fac. Agric. Tamagawa Univ.* 27, 81-92.
- Sato, Y., Oguchi, M., Menjo, N., Imai, K., Saito, H., et al., 1993. Precursor polypeptide for multiple neuropeptides secreted from the suboesophageal ganglion of the silkworm *Bombyx mori*: characterization of the cDNA encoding the diapause hormone precursor and identification of additional peptides. *Proc. Natl Acad. Sci. USA* 90, 3251-3255.
- Sauman, I., Reppert, S.M., 1996a. Circadian clock neurons in the silkworm *Antheraea pernyi*: novel mechanisms of period protein regulation. *Neuron* 17, 889-900.
- Sauman, I., Reppert, S.M., 1996b. Molecular characterization of prothoracicotropic hormone (PTTH) from the giant silkworm *Antheraea pernyi*: developmental appearance of PTTH-expressing cells and relationship to circadian clock cells in central brain. *Devel. Biol.* 178, 418-429.
- Sauman, I., Reppert, S.M., 1998. Brain control of embryonic circadian rhythms in the silkworm *Antheraea pernyi*. *Neuron* 20, 741-748.
- Sauman, I., Tsai, T., Roca, A.L., Reppert, S.M., 1996. Period protein is necessary for circadian control of egg hatching behavior in the silkworm *Antheraea pernyi*. *Neuron* 17, 901-909.
- Saunders, D.S., 2002. (with Steel, C.G.H., Vafopoulou, X., Lewis, R.D.). *Insect Clocks*. Elsevier, Amsterdam, p. 560.
- Schal, C., Cardé, R.T., 1986. Effects of temperature and light on calling in the tiger moth *Holomelina lamae* (Freeman) (Lepidoptera: Arctiidae). *Physiol. Entomol.* 11, 75-87.
- Schwartz, L.M., Truman, J.W., 1983. Hormonal control of rates of metamorphic development in the tobacco hornworm *Manduca sexta*. *Devel. Biol.* 99, 103-114.
- Sefiani, M., 1987. Regulation of egg laying and *in vitro* contractions in *Gryllus bimaculatus*. *J. Insect Physiol.* 33, 215-222.
- Sehadová, H., Sauman, I., Sehna, F., 2003. Immunocytochemical distribution of pigment-dispersing hormone in the cephalic ganglia of polyneopteran insects. *Cell Tissue Res.* 312, 113-125.
- Seth, R.K., Rao, D.K., Reynolds, S.E., 2002. Movement of spermatozoa in the reproductive tract of adult male *Spodoptera litura*: daily rhythm of sperm descent and the effect of light regime on male reproduction. *J. Insect Physiol.* 48, 119-131.
- Shearer, P.W., Jones, V.P., Riedl, H., 1995. Diel periodicity and circadian control of oviposition by *Cryptophlebia illepidia* (Lepidoptera: Tortricidae). *Environ. Entomol.* 24, 1229-1233.
- Shimizu, I., Kawai, Y., Taniguchi, M., Aoki, S., 2001. Circadian rhythm and cDNA cloning of the clock gene *period* in the honeybee, *Apis cerana japonica*. *Zool. Sci.* 18, 779-789.
- Shirai, Y., Aizono, Y., Iwasaki, T., Yanagida, A., Mori, H., et al., 1993. Prothoracicotropic hormone is released five times in the 5th-larval instar of the silkworm, *Bombyx mori*. *J. Insect Physiol.* 39, 83-88.
- Shorey, H.H., 1966. The biology of *Trichoplusia ni*. 4. Environmental control of mating. *Ann. Entomol. Soc. America* 59, 502-506.
- Shorey, H.H., Gaston, L.K., 1965. Sex pheromones of noctuid moths. 5. Circadian rhythm of pheromone-responsiveness in males of *Autographa californica*, *Heliothis virescens*, *Spodoptera exigua* and *Trichoplusia ni*. *Ann. Entomol. Soc. America* 58, 597-611.
- Shu, S., Koepnick, W.L., Mbata, G.N., Cork, A., Ramaswamy, S.B., 1996. Sex pheromone production in *Callosobruchus maculatus* (Coleoptera: Bruchidae): electroantennographic and behavioral responses. *J. Stored Prod. Res.* 32, 21-30.
- Shu, S., Park, Y.I., Ramaswamy, S.B., Srinivasan, A., 1998. Temporal profiles of juvenile hormone titers and egg production in virgin and mated females of *Heliothis virescens* (Noctuidae). *J. Insect Physiol.* 44, 1111-1117.
- Siegmund, T., Korge, G., 2001. Innervation of the ring gland of *Drosophila*. *J. Comp. Neurol.* 431, 481-491.
- Simmons, L.W., Gwynne, D.T., 1993. Reproductive investment in bushcrickets: the allocation of male and female nutrients to offspring. *Proc. R. Soc. London B* 252, 1-5.
- Slama, K., 1980. Homeostatic function of ecdysteroids in ecdysis and oviposition. *Acta Entomol. Bohemoslavaca* 77, 145-168.
- Slinger, A.J., Isaac, R.E., 1988. Ecdysteroid titers during embryogenesis of the cockroach *Periplaneta americana*. *J. Insect Physiol.* 34, 1119-1126.
- Smith, A.F., Schal, C., 1991. Circadian calling behavior of the adult female brown-banded cockroach, *Supella longipalpa* (F.) (Dictyoptera: Blattellidae). *J. Insect Behav.* 4, 1-14.

- Smith, W.A., Rountree, D.B., Bollenbacher, W.E., Gilbert, L.I., 1986. Dissociation of the prothoracic glands of *Manduca sexta* into hormone-responsive cells. In: Borkovec, A.B., Gelman, D.B. (Eds.), *Insect Neurochemistry and Neurophysiology*. Humana Press, Totowa, NJ, pp. 319–322.
- So, W.V., Sarov-Blat, L., Kotarski, C.K., McDonald, M.J., Allada, R., et al., 2000. *Takeout*, a novel *Drosophila* gene under circadian clock transcriptional regulation. *Mol. Cell Biol.* 20, 6935–6944.
- Sokolove, P.G., 1975. Localization of the cockroach optic lobe circadian pacemaker with microlesions. *Brain Res.* 87, 13–21.
- Sokolove, P.G., Loher, W., 1975. Role of eyes, optic lobes and pars intercerebralis in locomotor and stridulatory circadian rhythms of *Teleogryllus commodus*. *J. Insect Physiol.* 21, 785–799.
- Soller, M., Bownes, M., Kubli, E., 1997. Mating and sex-peptide stimulate the accumulation of yolk in oocytes of *Drosophila melanogaster*. *Eur. J. Biochem.* 243, 732–738.
- Soller, M., Bownes, M., Kubli, E., 1999. Control of oocyte maturation in sexually mature *Drosophila* females. *Devel. Biol.* 208, 337–351.
- Sower, L.L., Shorey, H.H., Gaston, L.K., 1970. Sex pheromones of noctuid moths. 21. Light–dark cycle regulation and light inhibition of the sex pheromone release by females of *Trichoplusia ni*. *Ann. Entomol. Soc. America* 63, 1090–1092.
- Stanewsky, R., 2002. Clock mechanisms in *Drosophila*. *Cell Tissue Res.* 309, 11–26.
- Stanewsky, R., 2003. Genetic analysis of the circadian system in *Drosophila melanogaster* and mammals. *J. Neurobiol.* 54, 111–147.
- Stanley-Samuelson, D.W., 1994. Prostaglandins and related eicosanoids in insects. *Adv. Insect Physiol.* 24, 115–212.
- Stay, B., Tobe, S.S., Bendena, W.G., 1994. Allatostatins: identification, primary structures, functions and distribution. *Adv. Insect Physiol.* 25, 267–337.
- Steel, C.G.H., Ampleford, E.J., 1984. Circadian control of hemolymph ecdysteroid titres and the ecdysis rhythm in *Rhodnius prolixus*. In: Porter, R., Collins, G.M. (Eds.), *Photoperiodic Regulation of Insect and Molluscan Hormones*. Pitman Press, London, pp. 150–169.
- Steel, C.G.H., Davey, K.G., 1985. Integration in the insect endocrine system. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 8. Pergamon, Oxford, pp. 1–35.
- Steel, C.G.H., Vafopoulou, X., 1989. Ecdysteroid titer profiles during growth and development of arthropods. In: Koolman, J. (Ed.), *Ecdysone*. Georg Thieme Verlag, Stuttgart, pp. 221–231.
- Steel, C.G.H., Vafopoulou, X., 1999. A tango of hormone rhythms directs insect development: circadian regulation of prothoracicotropic hormone and ecdysteroids. In: Roubos, E.W., Wenderlaar-Bonga, S.E., Vaudry, H., DeLoof, A. (Eds.), *Recent Developments in Comparative Endocrinology and Neurobiology*. Shaker, Maastricht, pp. 94–97.
- Steel, C.G.H., Vafopoulou, X., 2001. Regulation of rhythmic steroidogenesis by light and neuropeptide inputs during development in the insect *Rhodnius prolixus*. In: Goos, H.J.T., Rastogi, R.K., Vaudry, H., Peirantoni, R. (Eds.), *Perspectives in Comparative Endocrinology: Unity and Diversity*. Monduzzi Editore, Bologna, pp. 301–307.
- Steel, C.G.H., Vafopoulou, X., 2002. Physiology of circadian systems. In: Saunders, D.S., with Steel, C.G.H., Vafopoulou, X., Lewis, R., *Insect Clocks*. Elsevier, Amsterdam, pp. 115–188.
- Stengl, M., Homberg, U., 1994. Pigment-dispersing hormone-immunoreactive neurons in the cockroach *Leucophaea maderae* share properties with circadian pacemaker neurons. *J. Comp. Physiol. A* 175, 203–213.
- Stout, J.F., Atkins, G., Zacharias, D., 1991. Regulation of cricket phonotaxis through hormonal control of the threshold of an identified auditory neuron. *J. Comp. Physiol. A* 169, 765–772.
- Stout, J.F., Gerard, G., Hasso, S., 1976. Sexual responsiveness mediated by the corpora allata and its relationship to phonotaxis in the female cricket, *Acheta domesticus*. *J. Comp. Physiol. A* 108, 1–10.
- Stout, J.F., Hao, J., Kim, P., Mbungu, D., Bronsert, M., et al., 1998. Regulation of the phonotactic threshold of the female cricket, *Acheta domesticus*: juvenile hormone III, allatectomy, L1 auditory neuron thresholds and environmental factors. *J. Comp. Physiol. A* 182, 635–645.
- Sybchev, M.A., Stamenova, M.B., Borisova, Y.A., 1986. Effect of the time of the day, age of insects and pheromone dose on the sexual reaction of *Musca domestica* (Diptera; Muscidae). *Ekologiya (Sofia)* 1, 62–67.
- Takeda, M., 1983. Ontogeny of the circadian system governing ecdysial rhythms in a holometabolous insect, *Diatraea grandiosella* (Pyralidae). *Physiol. Entomol.* 8, 321–331.
- Tang, J.D., Charlton, R.E., Cardé, R.T., Yin, C.M., 1992. Diel periodicity and influence of age and mating on sex pheromone titer in gypsy moth *Limantria dispar* (L.). *J. Chem. Ecol.* 18, 749–760.
- Tawata, M., Ichikawa, T., 2001. Circadian firing of neurosecretory cells releasing pheromonotropic neuropeptides in the silkworm, *Bombyx mori*. *Zool. Sci.* 18, 645–649.
- Teal, P.E.A., Davis, N.T., Meredith, J.A., Christensen, T.A., Hildebrand, J.G., 1999a. Role of the ventral nerve cord and terminal abdominal ganglion in the regulation of sex pheromone production in the tobacco budworm (Lepidoptera: Noctuidae). *Ann. Entomol. Soc. America* 92, 891–901.
- Teal, P.E.A., Meredith, J.A., Gomez, S.Y., 1999b. Isolation and identification of terpenoid sex pheromone components from extracts of hemolymph of males of the Caribbean fruit fly. *Arch. Insect Biochem. Physiol.* 42, 225–232.

- Teal, P.E.A., Tumlinson, J.H., Oberlander, H., 1989. Neural regulation of sex pheromone biosynthesis in *Heliothis* moths. *Proc. Natl Acad. Sci. USA* 86, 2488–2492.
- Temin, G., Zander, M., Roussel, J.P., 1986. Physico-chemical (GC-MS) measurements of juvenile hormone III titers during embryogenesis of *Locusta migratoria*. *Int. J. Invert. Reprod.* 9, 105–112.
- Terry, K.L., Steel, C.G.H., 2004. Confocal analysis of PERIOD- and TIMELESS-immunoreactivity in the brain and prothoracic glands of *Rhodnius prolixus*: localization of potential circadian oscillators in the neuroendocrine system. *J. Comp. Neurol.* (in press).
- Thompson, K.G., Siegler, M.V.S., 1993. Development of segment specificity in identified lineages of the grasshopper CNS. *J. Neurosci.* 13, 3309–3318.
- Thompson, K.J., Sivanesan, S.P., Campell, H.R., Sanders, K.J., 1999. Efferent neurons and specialization of abdominal segments in grasshoppers. *J. Comp. Neurol.* 415, 65–79.
- Thompson, M.J., Svodoba, J.A., Lozano, R., Wilzer, K.R., Jr., 1988. Profile of free and conjugated ecdysteroids and ecdysteroid acids during embryonic development of *Manduca sexta* (L.) following maternal incorporation of carbon-14 cholesterol. *Arch. Insect Biochem. Physiol.* 7, 157–172.
- Thorson, B.J., Riemann, J.G., 1977. Abdominally entrained periodicities of testis and vas deferens activity in the Mediterranean flour moth. *J. Insect Physiol.* 23, 1189–1197.
- Thorson, B.J., Riemann, J.G., 1982. Effects of 20-hydroxyecdysone on sperm release from the testes of the Mediterranean flour moth, *Anagasta kuehniella* (Zeller). *J. Insect Physiol.* 28, 1013–1019.
- Thummel, C.S., 2002. Ecdysone-regulated puff genes 2000. *Insect Biochem. Mol. Biol.* 32, 113–120.
- Tillman, J.A., Seybold, S.J., Jurenka, R.A., Blomquist, G.J., 1999. Insect pheromones: an overview of biosynthesis and endocrine regulation. *Insect Biochem. Mol. Biol.* 29, 481–514.
- Tischkau, S.A., Mitchell, J.W., Tyan, S.H., Buchanan, G.F., Gillette, M.U., 2003. Ca^{2+} /cAMP response element protein (CREB)-dependent activation of *Per1* is required for light-induced signaling in the suprachiasmatic nucleus clock. *J. Biol. Chem.* 278, 718–723.
- Toma, D.P., Bloch, G., Moore, D., Robinson, G.E., 2000. Changes in *period* mRNA levels in the brain and division of labour in honey bee colonies. *Proc. Natl Acad. Sci. USA* 97, 6914–6919.
- Tomioka, K., Wakatsuki, T., Shimono, K., Chiba, Y., 1991. Circadian control of hatching in the cricket, *Gryllus bimaculatus*. *J. Insect Physiol.* 37, 365–371.
- Touhara, K., Lerro, K.A., Bonning, B.C., Hammock, B.D., Prestwich, G.D., 1993. Ligand binding by a recombinant insect juvenile hormone binding protein. *Biochemistry* 32, 2068–2075.
- Touhara, K., Prestwich, G.D., 1992. Binding site mapping of a photoaffinity-labelled juvenile hormone binding protein. *Biochem. Biophys. Res. Commun.* 182, 466–473.
- Truman, J.W., 1972a. Physiology of insect rhythms. 1. Circadian organization of the endocrine events underlying the molting cycle of larval tobacco hornworm. *J. Exp. Biol.* 57, 805–820.
- Truman, J.W., 1972b. Physiology of insect rhythms. 2. The silkworm brain as the location of the biological clock controlling eclosion. *J. Comp. Physiol.* 81, 99–114.
- Truman, J.W., 1974. Physiology of insect rhythms. 4. Role of the brain in the regulation of the flight rhythm of the giant silkmoths. *J. Comp. Physiol.* A 95, 281–296.
- Truman, J.W., 1992. The eclosion hormone system of insects. *Progr. Brain Res.* 92, 361–374.
- Truman, J.W., Copenhaver, P.F., 1989. The larval eclosion hormone neurones in *Manduca sexta*: identification of the brain–proctodeal neurosecretory system. *J. Exp. Biol.* 147, 457–470.
- Truman, J.W., Horodyski, F.M., Hewes, R.S., Riddiford, L.M., 1991. Eclosion hormone: from genes to behavior. In: Menn, J.J., Kelly, T.J., Masler, E.P. (Eds.), *Insect Neuropeptides: Chemistry, Biology and Action*. American Chemical Society, Washington, pp. 95–99.
- Truman, J.W., Morton, D.B., 1990. The eclosion hormone system: an example of coordination of endocrine activity during the molting cycle of insects. In: Epple, A., Scane, C.G., Stetson, M.H. (Eds.), *Progress in Comparative Endocrinology*. Wiley-Liss, New York, pp. 300–308.
- Truman, J.W., Riddiford, L.M., 1970. Neuroendocrine control of ecdysis in silkmoths. *Science* 167, 1624–1626.
- Truman, J.W., Riddiford, L.M., 1974. Physiology of insect rhythms. 3. The temporal organization of the endocrine events underlying pupation of the tobacco hornworm. *J. Exp. Biol.* 60, 371–382.
- Truman, W.J., Riddiford, L.M., 2002. Endocrine insights into the evolution of metamorphosis in insects. *Annu. Rev. Entomol.* 47, 467–500.
- Truman, J.W., Rountree, D.B., Reiss, S.E., Schwartz, L.M., 1983. Ecdysteroids regulate the release and action of eclosion hormone in the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol.* 29, 895–900.
- Truman, J.W., Taghert, P.H., Copenhaver, P.F., Tublitz, N.J., 1981. Hormonal control of ecdysis through embryonic and post-embryonic development. In: Sehna, F., Zabza, A., Menn, J.J., Cymborowski, B. (Eds.), *Regulation of Insect Development and Behavior*. Wrocław Technical University Press, Wrocław, pp. 1011–1020.
- Turek, F.W., 1994. Circadian rhythms. *Recent Progr. Hormone Res.* 49, 43–90.
- Vafopoulou, X., Sim, C.-H., Steel, C.G.H., 1996. Prothoracicotropic hormone in *Rhodnius prolixus*: *in vitro* analysis and changes in amounts in the brain and retrocerebral complex during larval–adult development. *J. Insect Physiol.* 42, 407–415.

- Vafopoulou, X., Steel, C.G.H., 1989. Developmental and diurnal changes in ecdysteroid biosynthesis by prothoracic glands of *Rhodnius prolixus* (Hemiptera) *in vitro* during the last larval instar. *Gen. Comp. Endocrinol.* 74, 484–493.
- Vafopoulou, X., Steel, C.G.H., 1991. Circadian regulation of synthesis of ecdysteroids by prothoracic glands of the insect *Rhodnius prolixus*: evidence of a dual oscillator system. *Gen. Comp. Endocrinol.* 83, 27–34.
- Vafopoulou, X., Steel, C.G.H., 1992. *In vitro* photosensitivity of ecdysteroid synthesis by prothoracic glands of *Rhodnius prolixus* (Hemiptera). *Gen. Comp. Endocrinol.* 86, 1–9.
- Vafopoulou, X., Steel, C.G.H., 1996a. The insect neuro-peptide prothoracicotrophic hormone is released with a dialy rhythm: re-evaluation of its role in development. *Proc. Natl Acad. Sci. USA* 93, 3368–3372.
- Vafopoulou, X., Steel, C.G.H., 1996b. Circadian regulation of a daily rhythm of release of prothoracicotrophic hormone from the brain–retrocerebral complex of *Rhodnius prolixus* (Hemiptera) during larval–adult development. *Gen. Comp. Endocrinol.* 102, 123–129.
- Vafopoulou, X., Steel, C.G.H., 1998. A photosensitive circadian oscillator in an insect endocrine gland: photic induction of rhythmic steroidogenesis *in vitro*. *J. Comp. Physiol. A* 182, 343–349.
- Vafopoulou, X., Steel, C.G.H., 1999. Daily rhythm of responsiveness to prothoracicotrophic hormone in prothoracic glands of *Rhodnius prolixus*. *Arch. Insect Biochem. Physiol.* 41, 117–123.
- Vafopoulou, X., Steel, C.G.H., 2001. Induction of rhythmicity in prothoracicotrophic hormone and ecdysteroids in *Rhodnius prolixus*: roles of photic and neuroendocrine Zeitgebers. *J. Insect Physiol.* 47, 935–941.
- Vafopoulou, X., Steel, C.G.H., 2002. Prothoracicotrophic hormone of *Rhodnius prolixus*: Partial characterization and rhythmic release of neuropeptides related to *Bombyx* PTTH and bombyxin. *Invert. Reprod. Devel.* 42, 111–120.
- Vafopoulou, X., Steel, C.G.H., Terry, K.L., 2001. Ecdysteroid receptor (EcR) cycles with tissue-specific phases in the insect *Rhodnius prolixus*: relationships with circadian regulation of blood ecdysteroid levels. In: Goos, H.J.T., Rastogi, R.K., Vaudry, H., Pierantoni, R. (Eds.), *Perspective in Comparative Endocrinology: Unity and Diversity*. Monduzzi Editore, Bologna, pp. 837–844.
- Vafopoulou, X., Steel, C.G.H., Terry, K.L., 2004a. Circadian cycling of the insect ecdysteroid hormone nuclear receptor (EcR) occurs in some tissues, but not others, during development in *Rhodnius prolixus*. *Cell Tissue Res.* (in press).
- Vafopoulou, X., Steel, C.G.H., Terry, K.L., 2004b. Immunohistochemical localization and developmental studies of neurons expressing prothoracicotrophic hormone- and bombyxin-like peptides in the brain of the insect *Rhodnius prolixus*. *J. Comp. Neurol.* (in press).
- van Esseveldt, L.K.E., Lehman, M.N., Boer, G.J., 2000. The suprachiasmatic nucleus and the circadian time-keeping system revisited. *Brain Res. Revs.* 33, 34–77.
- Veelaert, D., Schoofs, L., Tobe, S.S., Yu, C.G., Vullings, H.G.B., et al., 1995. Immunological evidence for an allatostatin-like neuropeptide in the central nervous system of *Schistocerca gregaria*, *Locusta migratoria* and *Neobelliera bullata*. *Cell Tissue Res.* 279, 601–611.
- Vivien-Roels, B., Pevet, P., 1993. Melatonin: presence and formation in invertebrates. *Experientia* 49, 642–647.
- Walker, W.F., 1979. Mating behavior in *Oncopeltus fasciatus*: circadian rhythms of coupling, copulation duration and “rocking” behavior. *Physiol. Entomol.* 4, 275–283.
- Warren, J.T., Petryk, A., Marques, G., Jarcho, M.P., Purvy, J.P., et al., 2002. Molecular and biochemical characterization of two P450 enzymes in the ecdysteroidogenic pathway of *Drosophila Melanogaster*. *Proc. Natl Acad. Sci. USA* 99, 11043–11048.
- Weber, F., 1995. Cyclic layer deposition in the cockroach (*Blaberus cranifer*) endocuticle: a circadian rhythm in leg pieces cultured *in vitro*. *J. Insect Physiol.* 41, 153–161.
- Webster, R.P., Cardé, R.T., 1982. Relationships among pheromone titer, calling and age in the omnivorous leaf-roller moth (*Platynota stultana*). *J. Insect Physiol.* 28, 925–934.
- Webster, R.P., Yin, C.M., 1997. Effects of photoperiod and temperature on calling behavior of the gypsy moth, *Lymantria dispar* L. (Lepidoptera: Lymantriidae). *Can. Entomol.* 129, 843–854.
- Westbrook, A.L., Bollenbacher, W.E., 1990. The development of identified neurosecretory cells in the tobacco hornworm, *Manduca sexta*. *Devel. Biol.* 140, 291–299.
- Wiedenmann, G., 1983. Splitting in a circadian activity rhythm: the expression of bilaterally paired oscillators. *J. Comp. Physiol.* 150, 51–60.
- Wiedenmann, G., Krüger-Alef, K., Martin, W., 1988. The circadian control of calling song and walking activity patterns in male crickets (*Teleogryllus commodus*). *J. Exp. Biol.* 47, 127–137.
- Wiedenmann, G., Loher, W., 1984. Circadian control of singing in crickets: two different pacemakers for early-evening and before-dawn activity. *J. Insect Physiol.* 30, 145–151.
- Wiedenmann, G., Lukat, R., Weber, F., 1986. Cyclic layer deposition in the cockroach endocuticle: a circadian rhythm? *J. Insect Physiol.* 32, 1019–1027.
- Wigglesworth, V.B., 1934. The physiology of ecdysis in *Rhodnius prolixus* (Hemiptera). 2. Factors controlling molting and “metamorphosis” *Q. J. Microsc. Sci.* 77, 191–222.
- Wigglesworth, V.B., 1940. The determination of characters at metamorphosis in *Rhodnius prolixus* (Hemiptera). *J. Exp. Biol.* 17, 201–222.
- Wigglesworth, V.B., 1952. The thoracic gland in *Rhodnius prolixus* (Hemiptera) and its role in molting. *J. Exp. Biol.* 29, 561–570.

- Wise, S., Davis, N.T., Tyndale, E., Noveral, J., Folwell, M.G., *et al.*, 2002. Neuroanatomical studies of *period* gene expression in the hawkmoth, *Manduca sexta*. *J. Comp. Neurol.* 447, 366–380.
- Wojtasek, H., Prestwich, G.D., 1995. Key disulfide bonds in an insect hormone binding protein: cDNA cloning of a juvenile hormone binding protein of *Heliothis virescens* and ligand binding by native and mutant forms. *Biochemistry* 34, 5234–5241.
- Wyatt, G.R., Davey, K.G., 1996. Cellular and molecular actions of juvenile hormone. 2. Roles of juvenile hormone in adult insects. *Adv. Insect Physiol.* 26, 1–155.
- Yagi, Y., Ishibashi, J., Nagata, K., Kataoka, H., Suzuki, A., *et al.*, 1995. The brain neurosecretory cells of the moth *Samia cynthia ricini*: immunohistochemical localization and developmental changes of the *Samia* homologues of the *Bombyx* prothoracicotropic hormone and bombyxin. *Devel. Growth Differ.* 37, 505–516.
- Yagita, K., Tamanini, F., van der Horst, G.T.J., Okamura, H., 2001. Molecular mechanisms of the biological clock in cultured fibroblasts. *Science* 292, 278–281.
- Yamaoka, K., Hirao, T., 1981. Mechanisms of ovipositional behavior in *Bombyx mori*: time-gating and accumulation of the internal factor. *Int. J. Invert. Reprod.* 4, 169–180.
- Yannielli, P.C., Harrington, M.E., 2000. Neuropeptide Y applied *in vitro* can block the phase shifts induced by light *in vivo*. *Neuroreport* 11, 1587–1591.
- Závodská, R., Šauman, I., Sehnal, F., 2003. Distribution of PER protein, pigment-dispersing hormone, prothoracicotropic hormone, and eclosion hormone in the cephalic nervous system of insects. *J. Biol. Rhythms* 18, 106–122.
- Zhao, C.-H., Li, Q., Gao, W., 2002. Stimulation of sex pheromone production by PBAN-like substance in the pine caterpillar moth, *Dendrolimus punctatus* (Lepidoptera: Lasiocampidae). *Arch. Insect Biochem. Physiol.* 49, 137–148.
- Zhao, Z., Zera, A.J., 2004. The hemolymph JH titer exhibits a large-amplitude, morph-dependent, diurnal cycle in the wing-polymorphic cricket, *Gryllus firmus*. *J. Insect Physiol.* 50, 93–102.
- Zhu, J.W., Millar, J., Löfstedt, C., 1995. Hormonal regulation of sex pheromone biosynthesis in the turnip moth, *Agrotis segetum*. *Arch. Insect Biochem. Physiol.* 30, 41–59.
- Žitňan, D., Adams, M.E., 2000. Excitatory and inhibitory roles of central ganglia in initiation of the insect ecdysis behavioral sequence. *J. Exp. Biol.* 203, 1329–1340.
- Žitňan, D., Kingan, T.G., Beckage, N.E., 1995a. Parasitism-induced accumulation of FMRFamide-like peptides in the gut innervation and endocrine cells of *Manduca sexta*. *Insect Biochem. Mol. Biol.* 25, 669–678.
- Žitňan, D., Kingan, T.G., Kramer, S.J., Beckage, N.E., 1995b. Accumulation of neuropeptides in the cerebral neurosecretory system of *Manduca sexta* larvae parasitized by the braconid wasp, *Cotesia congregata*. *J. Comp. Neurol.* 356, 83–100.
- Žitňan, D., Ross, L.S., Zitnanova, I., Hermesman, J.L., Gill, S.S., *et al.*, 1999. Steroid induction of a peptide hormone gene leads to orchestration of a defined behavioral sequence. *Neuron* 23, 523–535.
- Žitňan, D., Sehnal, F., Bryant, P.J., 1992. Neurons producing specific neuropeptides in the central nervous system and pupariation-delayed *Drosophila*. *Devel. Biol.* 156, 117–135.

10 Transposable Elements for Insect Transformation

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10.1. Introduction

The ability to create genetically transformed organisms has played a central role in the history of modern genetics, in particular, to our understanding of gene expression and development. Indeed, the pioneering transformation experiments of *Pneumococcus* by Griffith (1928) and subsequent systematic analyses by Avery *et al.* (1944), that showed transformation from a “rough” to a “smooth” bacterial cell wall phenotype, were instrumental in defining DNA as the inherited genetic material. The importance of these initial transformation experiments to prokaryotic genetic analysis was widely appreciated, and continued studies by many other laboratories laid the foundation for modern molecular biology.

The importance of “transformation” technology to eukaryotic genetic studies was apparent, and several of the initial attempts to create transgenic

animals were performed in insects, though the means of achieving and assessing insect transformation were not straightforward. The primary reasons why these early attempts to create transgenic insects were largely unsuccessful were the inability to isolate and reproduce individual genetic elements that could be used as transformation vectors and markers, and the lack of efficient means of introducing DNA into germ cells. Most of the initial studies of insect transformation relied on soaking embryos or larvae, with visible mutant phenotypes, in solutions of total wild-type genomic DNA in hopes of reverting the mutant phenotype. The first experiments performed on *Bombyx* and *Ephestia* met with some success where mutant wing color pattern phenotypes were reverted in some organisms, though inheritance was inconsistent and transformation events could not be confirmed unequivocally

(Caspari and Nawa, 1965; Nawa and Yamada, 1968; Nawa *et al.*, 1971). Similar results were obtained in *Drosophila* studies (Fox and Yoon, 1966, 1970; Fox *et al.*, 1970), and for all of these initial experiments, it is most likely that the observed phenotypic changes resulted from extra-chromosomal maintenance of introduced DNA in the somatic tissue by an unknown mechanism. A different approach involving the microinjection of wild-type genomic DNA into embryos homozygous for a recessive eye color mutation (*vermilion*), resulted in transformants with a reversion to the normal red eye color phenotype. While the reversion event was genetically mapped away from the mutant locus, a thorough molecular analysis to verify a transformation event, before the lines were lost, was not achieved and so the nature of the phenomenon observed in this experiment remains unexplained (Germeraad, 1976).

In the mid-1970s a turning point in insect science occurred with the extension of molecular genetic analysis to *Drosophila melanogaster*. These early studies and subsequent studies not only provided many of the tools and reagents necessary for developing and critically assessing genetic transformation in insects, but they also emphasized the need for a technology that would facilitate a more complete understanding of the genes being isolated using recombinant DNA methods. One technology that was clearly needed was a means to integrate DNA molecules into the chromosomes of germ cells where it remained stable, resulting in heritable germline transformation. The simple introduction of raw linearized DNA into preblastoderm embryos in the hope of fortuitous recombination into host chromosomes was clearly not reliable. Interest was growing, however, in the use of mobile genetic elements as vectors for DNA integration, including retrotransposons and transposons that were being isolated in *Drosophila* for the first time. Foremost among these was the *P* transposable element, isolated from certain mutant alleles of the *white* gene. The subsequent testing and success of transformation mediated by the *P* element in the *Drosophila* germline proved to be a dramatic turning point in the genetic analysis of an insect species. The eventual impact of this technology on understanding genetic mechanisms in all eukaryotic systems cannot be understated. The success with *P* in *Drosophila* gave hope that this system could be straightforwardly extended to genetic manipulation of other insect species, and especially those highly important to agriculture and human health. While there was reason for optimism, we now realize that this was a naive expectation given what we now understand

about the natural history of *P* elements relative to other Class II transposable elements, in particular its extremely limited distribution and its dependence on species-specific host factors. The inability of *P* to function in non-drosophilids, however, was a motivating force to more completely understand transposon regulation and the identification and testing of new vector systems. These included other transposable elements, as well as viral and bacterial vectors.

The development of routine methods for insect gene-transfer was probably delayed by a decade due to attention being focused exclusively on the *P* element. Yet, this delay has resulted in a more varied toolbox of vectors and markers that now allow nearly routine transformation for many important species, and the potential for transformation of most insects (see Handler, 2001). Indeed, some of the tools developed for testing the *P* element, in particular embryonic mobility assays, are now routinely used for initial tests for function of other vectors in an insect species before more laborious and time-consuming transformation experiments are attempted.

The creation of this varied toolbox was first related to the potential need for different vector and marker systems for different insect species. We now realize that the future of genetic analysis will depend on multiple vector and marker systems for each of these species, since genomics and functional genomics studies will require multiple systems for DNA integration and reporters for gene expression. Indeed, germline transformation is essential for the insertional mutagenesis and functional genomics studies that are critical underpinnings for both assessing genomic architecture and relating sequences to gene expression. Notably, the continuing functional analysis of the *Drosophila* genome now relies on the vectors and markers, described in this chapter, that were first developed for nondrosophilid insect species.

10.2. *P* Element Transformation

10.2.1. *P* Element

The use of transposable element-based vectors for *Drosophila* transformation followed the discovery of short inverted terminal repeat-type elements similar to the *Activator* (*Ac*) element discovered in maize by McClintock (see Federoff, 1989). The first such element to be discovered in insects was the *P* element, the factor responsible for hybrid dysgenesis that occurred in crosses of males from a *P* strain (containing *P* factor) with females from an

M strain (devoid of *P* factor) females (Kidwell *et al.*, 1977). The identification of *P* sequences resulted from the molecular analysis of *P*-induced *white* mutations that occurred in dysgenic hybrids (Rubin *et al.*, 1982). While the initial *P* elements isolated as insertion sequences were incomplete, nonautonomous elements, complete functional elements were later isolated and characterized by O'Hare and Rubin (1983).

P is 2907 bp in length with 31 bp inverted terminal repeats (ITRs) and 11 bp subterminal inverted repeats that occur approximately 125 bp from each terminus (Figure 1). Other repeat sequences exist within *P*, but their functional significance, if any, remains unknown. A defining signature for *P*, as with other transposable elements, is the nature of its insertion site which consists of an 8 bp direct repeat duplication. The extensive use of *P* for transformation and transposon mutagenesis has shown the element to have a distinctly nonrandom pattern of integration. It is now clear that *P* elements are blind to a significant fraction of the genome and new gene vectors are being employed in *Drosophila* to complement these limitations. *P* elements and all transposable elements currently used as insect gene vectors belong to a general group of transposable elements known as Class II short inverted terminal repeat transposons (see Finnegan, 1989). These elements transpose via a DNA intermediate and generally utilize a cut-and-paste mechanism that creates a duplication of the insertion site. These are distinguished from Class I elements, or retrotransposons, that have long direct terminal repeats (LTRs) and transpose via reverse transcription an RNA intermediate.

The original use of *P* for germline transformation was accomplished by inserting a marker gene within the element so that it did not disrupt activity of the terminal sequences or the transposase gene. The *rosy*⁺ gene was inserted at the 3' end of the transposase-coding region, but upstream of the 3' subterminal inverted repeat sequence. Plasmids containing this vector were injected into preblastoderm (syncytial) embryos homozygous for *ry*⁻ so the *P* vector could transpose into germ cell nuclei. Germline transformation events were identified in the following generation (G₁) by virtue of reversion of the mutant *ry*⁻ eye color phenotype to wild-type. These experiments not only proved the feasibility of transposon-mediated transformation, but also permitted structure–function relationships within the *P* element to be defined (Karess and Rubin, 1984). The *P* transcriptional unit was found to be composed of four exons separated by three introns. Further analysis determined that the *P* transposase function is cell-type specific owing to differential transcript splicing of the third intron that is limited to the germline. The lack of splicing in the soma results in production of nonfunctional truncated polypeptides in this tissue (Rio *et al.*, 1986).

While the original *P* vector allowed efficient transformation, the presence of a functional transposase gene within the vector made the system self-mobilizable (autonomous) and inherently unstable, allowing potential excision or transposition of the original insertion event. Subsequent vector development resulted in a binary system in which the vector transposase was deleted or made defective by insertion of a marker gene. The ability of the transposase

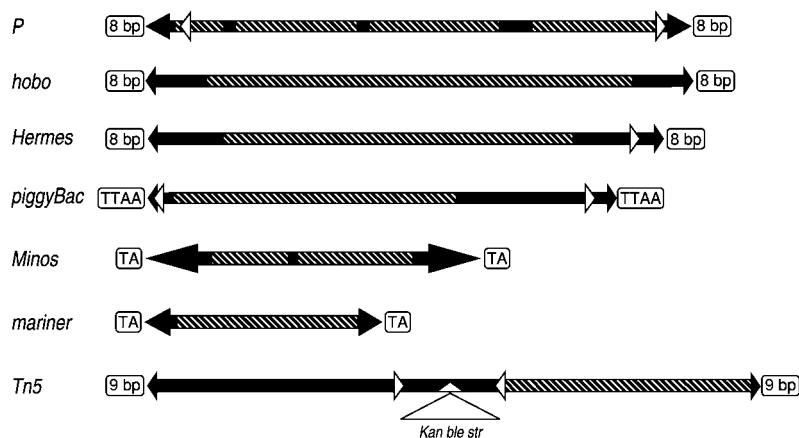


Figure 1 Diagram of transposable elements currently in use for the germline transformation of insect species. The left arms represent the 5' termini and right arms represent the 3' termini. Transposon sizes and specific internal elements are shown in relative positions but are not at precise scale. Major structural elements include duplicated insertion sites (open boxes); inverted terminal repeat sequences (black arrowheads); internal subterminal repeat sequences (white arrowheads); transposase coding region (boxed diagonals); and intron sequences (black boxes). The *Tn5* element is a composite transposable element consisting of two functional elements flanking three antibiotic resistance genes. Refer to text for specific details on nucleotide lengths and relative positions.

to act *in trans* allowed transposase to be provided by a separate plasmid (helper), that could facilitate vector integrations when cointroduced with the vector-containing plasmid into the same nucleus (Rubin and Spradling, 1982). Integrations would remain stable if the helper did not integrate, but the original helpers, such as $\pi 25.1$, were autonomous *P* elements themselves that could integrate along with the vector. While helper integration was diminished by injecting much higher concentrations of vector plasmid, this possibility was only eliminated with the creation of defective helpers having one or both of their terminal sequences deleted (known as “wings-clipped” helpers). The first of these was $\pi 25.7wc$, which was immobilized by deletion of 3' terminal sequences (Karess and Rubin, 1984). This prototype vector system served as a model for binary systems of nonautonomous vector: helper elements used for all the transposon-based transformation systems currently in use (Table 1).

A notable characteristic of *P* elements was not only their discontinuous distribution within the species (*P* and *M* strains), but their discontinuous interspecific distribution. Based on its distribution patterns it has become apparent that *P* was recently introduced into *D. melanogaster* from *D. willistoni* by an unknown mechanism (Daniels and Strausbaugh, 1986). Regardless of the mechanism since the 1950s, *P* elements have thoroughly invaded wild populations of *D. melanogaster* (Anxolabéhère *et al.*, 1988), and without the existence of *M* strain laboratory stocks that were removed from nature before this time, the development of *P* vectors might never have been realized. This is due to the repression of *P* mobility in *P*-containing strains that was first observed in hybrid dysgenesis studies, which also showed that movement was not repressed in *M* strains devoid of *P*. The basis for *P* strain repression appears to be due to a number of factors including repressor protein synthesis, transposase titration by resident defective elements, and regulation of transposase gene transcription (Handler *et al.*, 1993b; Simmons *et al.*, 2002). As will be discussed further on, other vector systems in use have thus far been shown to be widely functional in several orders of insects, and the presence of the same or related transposon in a host insect does not necessarily repress vector transposition. In this and several other aspects, the *P* vector system appears to be the exception rather than the rule for transposon-mediated gene transfer in insects.

10.2.2. *P* Vectors and Markers

Regardless of regulatory differences between *P* and other transposon vector systems currently in use,

methods developed for *P* transformation of *Drosophila* serve as a paradigm for all other insect vector systems. Those familiar with *Drosophila* transformation will be in the best position to attempt these methods in other insects. Current techniques developed for other insect species are variations on a theme, although as we describe, considerable modifications have been made. Several comprehensive reviews are available for more specific details on the structure, function, and use of *P* for transformation in *Drosophila*, which are highly relevant to the understanding and use of other vector systems (see Karess, 1985; Spradling, 1986; Engels, 1989; Handler and O'Brochta, 1991). Particularly useful are the books and method manuals by Ashburner (1989a, 1989b) that review the various vectors, markers, and methodologies used for *Drosophila* transformation, as well as early techniques used to manipulate *Drosophila* embryos. This information is especially applicable to other insect systems.

The first consideration for transformation is the design of vector and helper plasmids, and the marker system used for transformant selection. The first *P* vectors and helpers were actually autonomous vectors, which was probably a useful starting point since the actual sequence requirements for vector mobility and transposase function were unknown. As noted, the first nonautonomous helper had a 3' terminal deletion that prevented its transposition, providing greater control over vector stability. However, this source of transposase was inefficient, until it was placed under *hsp70* regulation which allowed transposase induction by heat shock (Steller and Pirrotta, 1986). All other vector system helper constructs have similarly taken advantage of heat shock promoters, mostly from the *D. melanogaster hsp70* gene, but other *hsp* promoters have been tested including those from the host species being transformed. Other constitutive promoters such as those from the genes for *actin* and $\alpha 1$ -*tubulin* have proven successful for helper transposase regulation, and will be discussed further on.

While sufficient transposase production is critical for transposition, the structure of the vector is equally important, and for some, very subtle changes from the autonomous vector can dramatically decrease or eliminate mobility. These variations include critical sequences (typically in the termini and subtermini), and placement and amount of exogenous DNA inserted within the termini. For some vectors the amount of plasmid DNA external to the vector can affect transposition rates. Subsequent to the initial test of several *P* vectors, the terminal sequence requirements for *P* mobility were determined to

Table 1 Transposon-mediated germline transformation

Transposon	Host species	Marker	Reference
Hermes	<i>Aedes aegypti</i>	<i>Dm-cinnabar</i> ⁺	Jasinskiene <i>et al.</i> (1998)
	<i>Culex quinquefasciatus</i>	actin5C-EGFP	Pinkerton <i>et al.</i> (2000)
		actin5C-EGFP	Allen <i>et al.</i> (2001)
	<i>Ceratitis capitata</i>	<i>Cc-white</i> ⁺	Michel <i>et al.</i> (2001)
	<i>Drosophila melanogaster</i>	<i>Dm-white</i> ⁺	O'Brochta <i>et al.</i> (1996)
		actin5C-EGFP	Pinkerton <i>et al.</i> (2000)
		3xP3-EGFP	Horn <i>et al.</i> (2000)
	<i>Stomoxys calcitrans</i>	actin5C-EGFP	O'Brochta <i>et al.</i> (2000)
	<i>Tribolium castaneum</i>	3xP3-EGFP	Berghammer <i>et al.</i> (1999)
	hobo	<i>Drosophila melanogaster</i>	<i>Dm-mini-white</i> ⁺
<i>Drosophila virilis</i>		<i>Dm-mini-white</i> ⁺	Lozovskaya <i>et al.</i> (1996); Gomez and Handler (1997)
mariner (Mos1)	<i>Aedes aegypti</i>	<i>Dm-cinnabar</i> ⁺	Coates <i>et al.</i> (1998)
	<i>Drosophila melanogaster</i>	<i>Dm-white</i> ⁺	Garza <i>et al.</i> (1991); Lidholm <i>et al.</i> (1993)
Minos	<i>Drosophila virilis</i>	3xP3-EGFP	Horn <i>et al.</i> (2000)
		<i>Dm-white</i> ⁺	Lohe and Hartl (1996a)
	<i>Anopheles stephensi</i>	actin5C-EGFP	Catteruccia <i>et al.</i> (2000b)
	<i>Ceratitis capitata</i>	<i>Cc-white</i> ⁺	Loukeris <i>et al.</i> (1995b)
P	<i>Drosophila melanogaster</i>	<i>Dm-white</i> ⁺	Loukeris <i>et al.</i> (1995a)
	<i>Drosophila melanogaster</i>	<i>Dm-rosy</i> ⁺	Rubin and Spradling (1982)
piggyBac	<i>Drosophila melanogaster</i>	<i>Dm-white</i> ⁺	Hazelrigg <i>et al.</i> (1984); Pirrota <i>et al.</i> (1985)
		<i>Dm-hsp70-mini-white</i> ⁺	Klemenz <i>et al.</i> (1987)
	<i>Drosophila simulans</i>	pUChsneo	Steller and Pirrota (1985)
		<i>Dm-rosy</i> ⁺	Scavarda and Hartl (1984)
	<i>Aedes aegypti</i>	<i>Dm-cinnabar</i> ⁺	Lobo <i>et al.</i> (2002)
	<i>Anastrepha suspensa</i>	3xP3-EGFP	Kokoza <i>et al.</i> (2001)
		PUB-nls-EGFP	Handler and Harrell (2000)
		PUB-nls-EGFP	Perera <i>et al.</i> (2002)
		hr5-ie1:EGFP	Grossman <i>et al.</i> (2001)
		actin5C-DsRed	Nolan <i>et al.</i> (2002)
	<i>Anopheles gambiae</i>	<i>BmA3-EGFP, hsp70-GFP</i>	Sumitani <i>et al.</i> (2003)
	<i>Anopheles stephensi</i>	<i>Cc-white</i> ⁺	Handler and McCombs (2000)
	<i>Athalia rosae</i>	PUB-nls-EGFP	Handler and McCombs (unpublished data)
	<i>Bombyx mori</i>	<i>BmA3-EGFP</i>	Tamura <i>et al.</i> (2000)
		3xP3-EGFP	Thomas <i>et al.</i> (2002); Uhlirva <i>et al.</i> (2002)
<i>Ceratitis capitata</i>	<i>Cc-white</i> ⁺	Handler <i>et al.</i> (1998)	
	PUB-nls-EGFP	Handler and Krasteva (unpublished data)	
<i>Drosophila melanogaster</i>	PUB-DsRed1	Handler and Krasteva (unpublished data)	
	PUB-nls-EGFP	Allen <i>et al.</i> (2004)	
	<i>Dm-white</i> ⁺ , PUB-nls-EGFP	Handler and Harrell (1999)	
	PUB-DsRed1	Handler and Harrell (2001)	
	3xP3-EGFP	Horn <i>et al.</i> (2000)	
	3xP3-EYFP	Horn and Wimmer (2000)	
	3xP3-ECFP	Horn and Wimmer (2000)	
	3xP3-DsRed	Horn <i>et al.</i> (2002)	
	PUB-nls-EGFP	Heinrich <i>et al.</i> (2002)	
	3xP3-EGFP	Hediger <i>et al.</i> (2000)	
<i>Lucilia cuprina</i>	<i>BmA3-EGFP</i>	Peloquin <i>et al.</i> (2000)	
<i>Musca domestica</i>	3xP3-EGFP	Berghammer <i>et al.</i> (1999)	
<i>Pectinophora gossypiella</i>	3xP3-EGFP	Lorenzen <i>et al.</i> (2003)	
<i>Tribolium castaneum</i>	3xP3-EGFP	O'Brochta (unpublished data)	
Tn5	<i>Aedes aegypti</i>	3xP3-DsRed	O'Brochta (unpublished data)

EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; EYFP, enhanced yellow fluorescent protein; ECFP, enhanced cyan fluorescent protein.

include 138 bp of the 5' end and 216 bp of the 3' end. While the inverted repeat sequences within these terminal regions are identical, the adjacent sequences were found not to be interchangeable in terms of vector mobility (Mullins *et al.*, 1989). Of interest was the discovery that the strongest binding affinity for the *P* transposase was at sequences approximately 50 bp internal to the terminal repeats (Rio and Rubin, 1988; Kaufman *et al.*, 1989). While the minimal sequences required for mobility may be used in vectors, typically the rate of mobility decreases with the decreased length of terminal sequence. Specific sequences may be required for binding of transposase or other nuclear factors, and conformational changes needed for recombination may be dependent upon sequence length and position.

P vector mobility was also found to be influenced by the amount of exogenous DNA inserted between the termini, with transformation frequency diminishing with increasing size. Initial tests with 8 kb vectors marked with *rosy* yielded transformation frequencies of approximately 50% per fertile G₀, while use of 15 kb vectors resulted in 20% frequencies (see Spradling, 1986). Larger vectors could transpose but frequencies approached 1% or less.

Of equal importance to creating an efficient vector system is having marker genes and appropriate host strains that will allow efficient and unambiguous identification or selection of transgenic individuals. Indeed, the genetic resources available for *Drosophila* also provided cloned wild-type DNA and appropriate mutant hosts for use in visible mutant-rescue marker systems that made testing *P* transformation possible. As noted, the first of these used the *ry*⁺ eye color gene, but this required a relatively large genomic fragment of nearly 8 kb. The *white* (*w*) eye color gene was then tested, but this required a genomic sequence that was longer than *ry*, and resultant transformation frequencies were relatively low (Hazelrigg *et al.*, 1984; Pirrotta *et al.*, 1985). New *w* markers, known as mini-*white*, that had the large first intron deleted decreased the marker insert to 4 kb resulting in much more efficient transformation, and placing the mini-*white* marker under *hsp70* regulation increased efficiencies further (Klemenz *et al.*, 1987). Use of *w* markers, especially in CaSpeR vectors (Pirrotta, 1988), has been a mainstay of *Drosophila* transformation, yet expression of the *w* gene in particular is subject to position effect variegation/suppression (PEV) that typically diminishes eye pigmentation. PEV, indeed, was originally discovered as a result of translocating *w*⁺ proximal to heterochromatin (Green, 1996), and it routinely manifests itself in *w*⁻ flies

transformed with *w*⁺. This effect has been observed with the use of eye pigmentation markers in several other insect species as well.

Other markers based upon chemical selections or enzymatic activity were also developed for *Drosophila*, though none has found routine use. These included alcohol dehydrogenase (*Adh*) (Goldberg *et al.*, 1983) and dopa decarboxylase (*Ddc*) (Scholnick *et al.*, 1983) that complemented existing mutations, and neomycin phosphotransferase (NPT or *neo*) (Steller and Pirrotta, 1985), β-galactosidase (Lis *et al.*, 1983), organophosphorus dehydrogenase (*opd*) (Benedict *et al.*, 1995), and dieldrin resistance (*Rdl*) (French-Constant *et al.*, 1991) which are dominant selections not requiring preexisting mutations (see French-Constant and Benedict, 2000).

10.2.3. *P* Transformation of Non-Drosophilids

Given the straightforward procedures for transforming *Drosophila* with *P* elements, there were high expectations that the system would function in other insects. The ability to test this was facilitated by the development of the neomycin (G418)-resistance marker system (Steller and Pirrotta, 1985), and neomycin resistance-containing *P* vectors were widely tested in tephritid flies and mosquitoes (see Walker, 1990; Handler and O'Brochta, 1991). Unfortunately, the neomycin resistance system was generally unreliable, and recovery of resistant individuals that were not transgenic was common. In three mosquito species, however, neomycin resistant transgenic insects were recovered but they arose from rare transposition-independent recombination events (Miller *et al.*, 1987; McGrane *et al.*, 1988; Morris *et al.*, 1989). Other dominant chemical resistance markers, including *opd* and *Rdl*, which had had some success in *Drosophila*, were also tested, but no transformation events could be verified in other insects. A major limitation of these experiments was that, given the numerous variables involved, it was impossible to determine which components in the system were failing. This limitation led to efforts to determine systematically whether the transposon vector system was, indeed, functional in host embryos, which resulted in the development of rapid transposon mobility assays as described below. The first of these assays tested *P* excision in drosophilid and non-drosophilid embryos, revealing that *P* function decreased in drosophilids as a function of relatedness to *D. melanogaster*, with no function evident in non-drosophilids (O'Brochta and Handler, 1988; Handler *et al.*, 1993a). These results were the first indication that for transposon-mediated germline transformation to succeed in non-drosophilids, new

vector systems would have to be created from existing and newly discovered transposon systems.

10.3. Excision and Transposition Assays for Vector Mobility

Assessing the ability of an insect gene vector to function in a particular species can be challenging. The procedures required to create a transgenic insect using transposable element-based gene vectors require a great deal of technical skill and the ability to perform basic genetic manipulations. Depending on the insect, its generation time, and its amenability to being reared in the laboratory, the process of genetic transformation can be quite lengthy. At the early stages of developing non-drosophilid transformation technology there was little experience in manipulating and injecting the embryos of the various non-drosophilid species of insects. In addition, the genetic markers available to select for, or recognize, transgenic insects were limited and none could be confidently expected to function optimally in the species being tested at that time. Consequently, early efforts to test the functionality of potential gene vectors, by attempting to create transgenic insects, required simultaneous success in dealing with a

number of daunting challenges. The failure of these efforts to yield a transgenic insect could not, unfortunately, be ascribed to the failure of any one particular step in the process (see Handler and O'Brochta, 1991). These efforts, therefore, did not represent an isolated test of the gene vector since failure to obtain a transgenic insect might have been due to a failure in DNA delivery, expression of the genetic marker, or the failure of the transposable element vector system. Technology development under these conditions was very difficult. What was needed was an experimental system that permitted the activity of the transposable element system to be assessed in the species of interest independent of any prospective genetic marker system and DNA delivery system. Such a system was developed for investigating the mobility properties of the *D. melanogaster* P element, and was very adaptable to other transposable element and insect systems (Figure 2).

The system developed for P elements involved transfecting *Drosophila* cells with a mixture of two plasmids – one containing a P element inserted into the coding region of the LacZ α peptide of a common cloning vector, and a second containing the P element transposase gene under the regulatory control

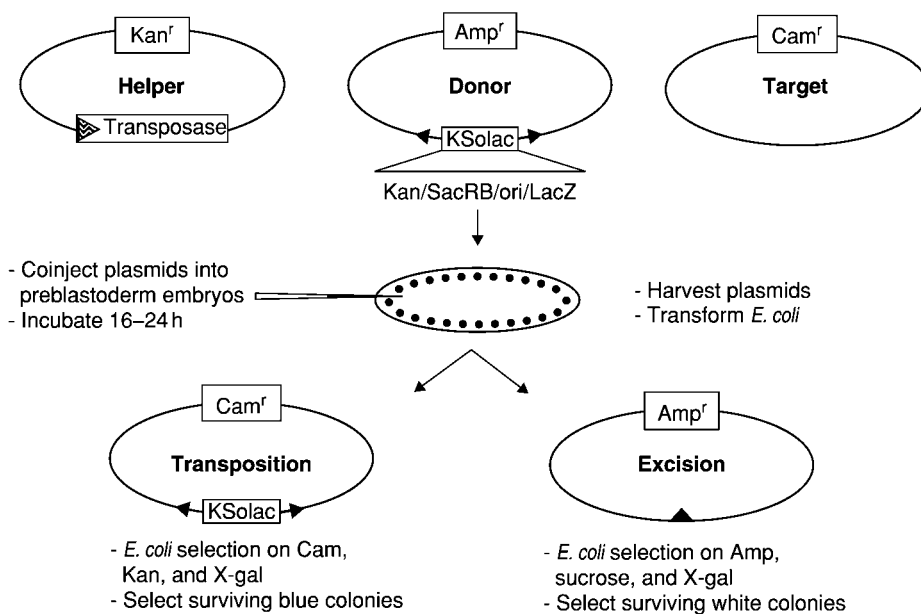


Figure 2 Plasmid-based transposable element mobility assays. A mixture of three plasmids is coinjected into preblastoderm embryos to insure incorporation into nuclei. After approximately 24 h the plasmids are extracted from the embryos and introduced into *E. coli*. Transient expression of the transposase gene on the helper plasmid in the developing embryos results in the production of functional transposase. If the transposase catalyzes excision and transposition of the element, excision will result in the loss of element-specific markers on the donor plasmid. In the example shown sucrose sensitivity, β -galactosidase activity, and kanamycin resistance are lost, and others could be used. Transposition results in the target plasmid acquiring all of the element-specific markers. In this example the target plasmid is from a Gram-positive bacteria and incapable of replicating in *E. coli* unless it acquires the origin of replication present on the element. Assays can be completed in 3 days and rates of movement of 0.001% or greater are routinely detectable.

of a strong promoter (Rio *et al.*, 1986). Transient expression of the transposase gene resulted in the production of transposase, catalyzing the excision of *P* elements from the “excision indicator plasmids.” Subsequent recovery of the injected plasmids from the cells, followed by their introduction into an appropriate strain of *Escherichia coli*, permitted plasmids that had lost the *P* element through excision to be recognized by virtue of their restored LacZ α peptide coding capacity. This transient *P* element excision assay was readily adaptable to use in *Drosophila* embryos through the process of direct microinjection of preblastoderm embryos, and it played a critical role in assessing the functionality of the *P* element system in a variety of drosophilid and non-drosophilid insect systems (O’Brochta and Handler, 1988).

As originally configured the excision assay only permitted the identification and recovery of excision events that resulted in the restoration of the open reading frame of the LacZ α peptide reporter gene. Various modifications in this basic assay were adopted that permitted precise and imprecise excisions to be identified and recovered (O’Brochta *et al.*, 1991). For example, marker genes such as the *E. coli* LacZ α peptide coding region, *E. coli supF*, sucrase (*SacRB*) from *Bacillus subtilis*, and streptomycin sensitivity, were incorporated into the transposable element (O’Brochta *et al.*, 1991; Coates *et al.*, 1997; Sundararajan *et al.*, 1999). Plasmids recovered that lacked marker gene expression were usually excision events. Further refinements of the excision assay involved the use of transposable element-specific restriction endonuclease sites as a means for selecting for excision events. Digesting plasmids recovered from embryos with restriction endonucleases with sites only in the transposable element was a very powerful method of physically removing plasmids that had not undergone excision from the pool of plasmids recovered from embryos and used to transform *E. coli*. Each restriction site was essentially a single dominant genetic marker and therefore transposable elements with multiple restriction sites provided a very powerful system for selecting against plasmids that have not undergone excision (O’Brochta, unpublished data).

Continued development of element mobility assays lead to assays in which interplasmid transposition could be measured. These assays involved the coinjection of a transposase-encoding helper plasmid, an element “donor” plasmid and a “target” plasmid. Typically the target plasmid contains a gene whose inactivation results in a selectable phenotype. For example, the *SacRB* gene has been used since its inactivation eliminates sucrose sensitivity. If the donor element also contains unique genetic

markers then transposition events would lead to a recombinant plasmid with a new combination of a variety of markers (Saville *et al.*, 1999). Perhaps the most powerful transposition assay developed for assessing transposable elements in insect embryos involved the use of a genetic marker cassette containing a plasmid origin of replication, an antibiotic resistance marker, and the LacZ α peptide coding region, in combination with a target consisting of a Gram-positive plasmid (pGDV1) (Sarkar *et al.*, 1997a). pGDV1 contains an origin of replication that cannot function in *E. coli*, although it does have a chloramphenicol resistance gene that is functional in this species. Transposition of the marked transposable element into pGDV1 converts it into a functional replicon in *E. coli*. Because of the absolute *cis*-dependence of origins of replication, and the complete inability of pGDV1 to replicate in *E. coli*, transposition events can be readily detected even at low frequencies.

Transient mobility assays are now a standard for defining vector competence in insect embryos, and in particular, when assessing a vector in a species for the first time. For this application, transposition assays provide the most information relevant to the potential for successful germline transformation, and can be used as a system to test helper construct function. As noted below, however, there may be differing constraints on plasmid and chromosomal transpositions for particular transposons. The use of these assays for analyzing transposon function is discussed in more detail in the relevant sections below.

Embryonic assays also provide an essential test system for assessing potential transgene instability by mobilizing or cross-mobilizing systems within a host genome, which is a critical information for the risk analysis of transgenic insects being considered for release. The importance of excision assays for this purpose became evident by the *hobo* excision assays in *Musca domestica* that revealed the existence of the *Hermes* element (Atkinson *et al.*, 1993), and the subsequent assays that defined the interaction between the two transposons (Sundararajan *et al.*, 1999). Since cross-mobilizing systems do not always promote precise excisions, assays that reveal imprecise as well as precise excisions are most sensitive for this purpose. Since successful transposition may depend on precise excision, transposition assays may only reveal the existence of mobilizing systems that have a high level of functional relatedness.

10.4. Transformation Marker Systems

The availability and development of selectable marker systems has played a large part in recent

advancements in insect transformation, that have been equal to the importance of vector development. The rapid implementation and expansion of *P* transformation in *Drosophila* was possible, in large part, due to the availability of several eye color mutant-rescue systems. These systems depend on the transgenic expression of the dominant-acting wild-type gene for an eye color mutation present in the host strain (see Sarkar and Collins, 2000). Successful transformation of non-drosophilid species was similarly dependent upon the development of analogous systems, with the first transformations in the medfly, *Ceratitis capitata*, and a mosquito, *Aedes aegypti*, relying on *white* and *cinnabar* mutant-rescue systems. While chemical resistance markers were used initially for non-drosophilid transformation, and can be highly useful for specific applications, their inefficiency and inconsistency when used alone provided ambiguous results for several species (see French-Constant and Benedict, 2000). Eye color marker systems are generally efficient and reliable, and cloned wild-type genes from *Drosophila* often complement orthologous mutant alleles in other insects. However, only a handful of species have stable mutant strains that can serve as suitable hosts for mutant-rescue strategies. The most significant advancement in marker gene development for the wide use of insect transformation has been the development of fluorescent protein markers (Higgs and Sinkins, 2000; Horn *et al.*, 2002). As dominant-acting neomorphs, that do not depend on preexisting mutations, they are directly useful in almost all host strains. When compared to the *white* eye color marker in *Drosophila*, the enhanced green fluorescent protein (EGFP) gene seemed to be less affected by position effect suppression, and thus has the additional advantage of more reliable detection (Handler and Harrell, 1999). Certainly for the foreseeable future, fluorescent protein markers will continue to be the markers of choice for most insect transformation strategies.

10.4.1. Eye Color Markers

The first insect transformations used mutant-rescue systems to identify transformant individuals, but in these experiments total genomic DNA was used, rather unreliably, to complement mutations in the respective host strains. The most reliable of these, however, was reversion of the *vermilion* eye color mutation in *D. melanogaster* (Germeraad, 1976). The success of the initial *P* element transformations in *Drosophila* also depended on reversion of eye color mutant strains, but the use of cloned *rosy* and *white* genomic DNA within the vector plasmid

allowed for much greater efficiency and reliability. The first non-drosophilid transformations in medfly (Loukeris *et al.*, 1995b; Handler *et al.*, 1998; Michel *et al.*, 2001) similarly relied on use of the wild-type medfly *white* gene cDNA that was placed under *Drosophila hsp70* regulation (Zwiebel *et al.*, 1995). This gene complemented a mutant allele in a *white eye* medfly host strain that was isolated more than 20 years earlier. The medfly *white* gene also complemented the orthologous gene mutation in the oriental fruit fly, yielding in one line a nearly complete reversion (Handler and McCombs, 2000). The first transformations in *A. aegypti* used a kynurenine hydroxylase-*white* mutant host strain, but for these tests the complementing marker was the *D. melanogaster cinnabar* gene (Cornel *et al.*, 1997). The *D. melanogaster vermilion* gene, that encodes tryptophan oxygenase (*to*) has also been used to complement the orthologous *green* eye color mutation in *M. domestica* (White *et al.*, 1996), and the *Anopheles gambiae* tryptophan oxygenase gene complements *vermilion* in *Drosophila* (Besansky *et al.*, 1997). The *vermilion* and *cinnabar* orthologs have also been cloned from *Tribolium*, and while the *white* mutation in this species is complemented by tryptophan oxygenase, no preexisting eye color mutation is complemented by kynurenine hydroxylase (Lorenzen *et al.*, 2002). The use of eye color mutant-rescue systems has certainly been critical to initial advances in insect transformation, and these markers should have continued utility for those species that have been successfully tested. The use of these markers, however, for the development of insect transformation in other species will be limited by the availability of suitable mutant host strains.

10.4.2. Chemical Selections

Previous to the development of mutant-rescue marker systems, transformant selections in non-drosophilid insects focused on genes that could confer resistance to particular chemicals or drugs. Importantly, these types of selections could be used for screening transformants en masse by providing the selectable chemical or drug in culture media. Ideally, only transformant individuals would survive the selection, allowing the rapid screening of large numbers of G₁ insects. For vectors that are inefficient and insects that are difficult to rear, the efficient screening of populations can be essential to identifying transformant individuals. The first drug resistance selection tested used the bacterial neomycin phosphotransferase gene (NPT II or *neo-mycin^r*) that conferred resistance by inactivation of the neomycin analog G418 (or Geneticin) (Steller

and Pirrotta, 1985). This seemed straightforward since the selection and *hsneo* marker system (putting NPT II under heat shock regulation) was already developed and tested in *Drosophila* for mass transformant screens, and the bacterial resistance gene was thought to be functional in most eukaryotes. The initial *P* transformations in *Drosophila* using the pUCHsneo vector were generally reliable; however, the marker was not easily transferable to other species. G418 resistance was highly variable, most likely due to species differences in diet, physiology, and symbiotic bacteria, and indeed, variations in resistance in transformed *Drosophila* have been attributed to strains of yeast used in culture media (Ashburner, 1989a). Other chemical resistance markers, including *opd* conferring resistance to paraoxan (Phillips *et al.*, 1990; Benedict *et al.*, 1995), and the gene for dieldrin resistance (*Rdl*) (ffrench-Constant *et al.*, 1991), that were initially tested in *Drosophila* were also problematic when tested in other species. These failures were due in large part to ineffective vector systems, but a common attribute in these studies was the selection of individuals having nonvector related or natural resistance to the respective chemical. While naturally resistant insects could be selected out by molecular tests in primary transformant screens, the recurrence of resistant insects in subsequent generations would make use of the transgenic strains highly impractical.

While the problems cited made chemical resistance selections frustrating for several species, and they have not been used for any recent transformation experiments, some successes were reported and the need for mass screening still exists. The initial tests for *P* transformation in several mosquito species used the pUCHsneo vector with G418 resistant transformants being selected, though transformation frequencies were low and all of them resulted from fortuitous recombination events and not *P*-mediated transposition (Miller *et al.*, 1987; McGrane *et al.*, 1988; Morris *et al.*, 1989). Nonetheless, chemical selections can be very powerful, and if reliable, they would dramatically improve the efficiency of transformation screens for most insects. It is quite possible that many species will not be amenable to current transformation techniques without markers that allow selection en masse. A potential means of increasing the reliability of chemical resistance screens would be to link a resistance marker to a visible marker within the vector. Initial G_1 transformants could be screened en masse by chemical resistance, with surviving individuals verified as transformants and maintained in

culture by use of the visible marker. This type of marking is tested by linking the *hsneo* construct with a red fluorescent protein marker in the *piggyBac* vector. Thus far initial results in *Drosophila* are highly encouraging (Handler and Harrell, unpublished data).

Of the enzyme systems tested for chemical selection in *Drosophila* that might be extended to other insects, the *Adh* system might have the most promise (Goldberg *et al.*, 1983). An *Adh* marker gene can complement the *adh* mutation in *Drosophila*, eliminating lethal sensitivity to ethanol treatment in mutant hosts. An *adh* gene has been cloned from the medfly, and a strategy has been developed to use it for genetic sexing by male-specific overexpression (Christophides *et al.*, 2001). Conceivably a similar strategy could be extended to transformant selections, though its use would be limited to medfly and possibly other tephritid species.

10.4.3. Fluorescent Protein Markers

The dramatic advancement of insect transformation in recent years has been due, primarily, to the development of fluorescent protein markers which are dominant-acting neomorphs that do not depend on preexisting mutations. The first of these to be tested was the green fluorescent protein (GFP) gene that was isolated from the jellyfish *Aequorea victoria* (Prasher *et al.*, 1992) and which initially exhibited heterologous function in the nematode *Caenorhabditis elegans* (Chalfie *et al.*, 1994). GFP expression was then tested in transformant *Drosophila* where it was used as a reporter for gene expression (Plautz *et al.*, 1996; Hazelrigg *et al.*, 1998), and several other species for both *in vivo* and *in vitro* studies. GFP was first tested in non-drosophilid insects as marker for Sindbis viral infection in *Aedes aegypti* (Higgs *et al.*, 1996), and the dramatic somatic expression of GFP in adults was highly encouraging for the further use of GFP for germline transformants.

This possibility was first tested in *Drosophila* using a construct that linked EGFP to a *polyubiquitin* promoter and nuclear localizing sequence (Lee *et al.*, 1988; Davis *et al.*, 1995; Handler and Harrell, 1999). A control for transformation, that also allowed a direct comparison of EGFP expression to that from the visible mini-*white* marker, was the initial use of a *piggyBac* vector, pB[Dmw, PUBnlsEGFP], that linked the two markers in a mutant *white* strain. The results from this experiment indicated that not only was the PUBnlsEGFP marker efficient and easily detectable under epifluorescence optics, but that many of the G_1 transformants that expressed GFP did not express a detectable level

of *white*⁺. Presumably the chromosomal position effects that suppressed *white* had a negligible effect on GFP expression. This result was highly encouraging for the use of GFP as a marker in non-drosophilids and several subsequent transformation experiments used EGFP regulated by a variety of promoters in *piggyBac*, *Hermes*, and *Minos* vectors. Notably, this allowed germline transformation to be tested in several species that otherwise have no visible marker systems, such as the Caribbean fruit fly, *Anastrepha suspensa*, which was transformed with pB[PUBnlsEGFP] (Handler and Harrell, 2000). This vector was subsequently tested in the Australian sheep blowfly, *Lucilia cuprina* (Heinrich *et al.*, 2002) and the mosquito *Anopheles albimanus* (Perera *et al.*, 2002). Similarly, a *Hermes* vector marked with EGFP regulated by the *Drosophila actin5C* promoter was first tested in *Drosophila* (Pinkerton *et al.*, 2000), and was used then to efficiently select transformants in *Aedes aegypti* (Pinkerton *et al.*, 2000), *Stomoxys calcitrans* (O'Brochta *et al.*, 2000), and *Culex quinquefasciatus* (Allen *et al.*, 2001). A *Minos* vector marked with *actin5C*-EGFP was used to select *Anopheles stephensi* transformants (Catteruccia *et al.*, 2000b), and a *piggyBac* vector marked with EGFP under *Bombyx actin 3A* promoter regulation was used to transform the lepidopteran species *Bombyx mori* (Tamura *et al.*, 2000) and *Pectinophora gossypiella* (Peloquin *et al.*, 2000).

Both the *polyubiquitin* and *actin* promoters have activity in all tissues throughout development, making insects marked in this fashion particularly useful for some applications such as the marking of insects used in biocontrol release programs (see Handler, 2002b). However, the detection of these markers can be limited due to quenching or obstruction by melanized cuticle or scales, and fluorescent protein expression regulated by a strong tissue-specific promoter can be valuable for difficult insects and particular applications. Foremost among these markers have been a series of fluorescent protein constructs regulated by the artificial 3xP3 promoter derived from the *Drosophila eyeless* gene (Sheng *et al.*, 1997; Horn *et al.*, 2000). The markers express strongly from the larval nervous system and adult eyes and ocelli. A 3xP3-EGFP marker within *piggyBac* was first used to transform *D. melanogaster* and *Tribolium castaneum* (Berghammer *et al.*, 1999) and has since been used in *M. domestica* (Hediger *et al.*, 2000), *Aedes aegypti* (Kokoza *et al.*, 2001), and the sawfly *Athalia rosae* (Sumitani *et al.*, 2003). The particular strengths and weaknesses for a marker construct such as 3xP3-EGFP is evident

from experiments where it allowed transformant selection in *Bombyx* embryos prior to larval hatching (Thomas *et al.*, 2002), while it was undetectable in *Aedes aegypti* adults having normal eye pigmentation (Kokoza *et al.*, 2001). It must, therefore, be recognized that the utility of fluorescent protein markers must be considered in the context of the host insect's structure and physiology during development.

GFP expression is less sensitive to position effect suppression than eye color markers, yet there is much evidence for quantitative and qualitative variabilities in fluorescent protein expression from transgenes. It is likely that tissue-specific variations are primarily due to position effects, while expression of new tissue phenotypes are due to proximal enhancer effects. Polyubiquitin-regulated EGFP expression is most intense in the thoracic flight muscles in *Drosophila* and tephritid fruit fly adults. In Caribbean fruit fly transgenic adult lines, EGFP was only observed in the thorax, but spectrofluorometric assays revealed as much as fivefold differences in fluorescence between lines having the same number of vector integrations (Handler and Harrell, 1999), and differences in expression that are stable within lines are often observed by inspection. In contrast to typical thoracic expression in tephritid flies, adult PUB-EGFP expression in *Lucilia* was limited to female ovaries (Heinrich *et al.*, 2002), and a PUB-DsRed transgenic medfly line exhibits most intense expression in the tarsi while another line expresses in abdominal tracheal apertures at the dorsal-ventral midline (Handler and Krasteva, unpublished data). In *Tribolium*, 3xP3-EGFP expresses typically from the eyes and brain, though several lines are atypical with one having muscle-specific expression throughout development (Lorenzen *et al.*, 2003). In *Anopheles stephensi*, the 3xP3-EGFP marker showed atypical expression in the pylorus, epidermal cells and in a subset of cells in the rectum (O'Brochta, Kim, and Koo, unpublished data).

The use of GFP will certainly be continued for transformant identification in many, if not most, other species where transformation is tested. Yet continuing studies in species already transformed will require multiple marking systems that are distinguishable from one another, especially when coexpressed. This will allow the detection of multiple independent transgenes when used in concert for conditional gene expression systems and gene discovery methods such as enhancer traps (Bellen *et al.*, 1989; Wilson *et al.*, 1989; Brand *et al.*, 1994). After testing 3xP3-EGFP, the 3xP3 promoter was linked to the GFP redshifted variants that emit blue (BFP), cyan (CFP), and yellow (YFP) fluorescence; these were

tested in *Drosophila*, and have proven useful individually as reporters and for identifying transformants (Horn and Wimmer, 2000). BFP and GFP have distinct enough emission spectra to be used together, though BFP photobleaches quickly and is not useful for many applications. While use of EGFP with ECFP is also problematic, ECFP and EYFP can be distinguished when using appropriate filter sets. For details on appropriate filter sets for particular applications see Horn *et al.* (2002) and the website for Chroma Technology Corp. (Chroma, 2004) which manufactures filters for most of the stereozoom fluorescence microscopes used for insect studies.

The most spectrally distinct fluorescent protein distinct from GFP and its variants is a red fluorescent protein, known as DsRed, isolated from the Indo-Pacific sea coral *Discosoma striata* (Matz *et al.*, 1999). It was first tested in insects by linking it to the *polyubiquitin* promoter in a *piggyBac* vector (pB[PUB-DsRed1]) and tested in *Drosophila*, where it exhibited highly intense expression (Handler and Harrell, 2001). Importantly, DsRed expression was completely distinguishable from EGFP when the two transgenic lines were interbred, and when coexpressed as an hsp70-Gal4/UAS-DsRed reporter in lines having vectors marked with EGFP. DsRed has since been incorporated into several mosquito and fruit fly species (Nolan *et al.*, 2002; Handler, unpublished data).

Both EGFP and DsRed are highly stable and generally resistant to photobleaching, and could be detected in tephritid flies several weeks after death, though DsRed1 was relatively the more stable of the two. This is highly advantageous for the use of these genes as markers for released insects that might only be retrieved several weeks after death in traps. A drawback for fluorescent proteins, and DsRed in particular, is that they require oligomerization and slow maturation that can take up to 48 h, resulting in low intensity in early development. However, variants of DsRed with shorter maturation times (Campbell *et al.*, 2002), and new fluorescent proteins with enhanced properties for specific applications are becoming available on a consistent basis (see Matz *et al.*, 2002).

10.4.3.1. Detection methods for fluorescent proteins Once heterologous expression of GFP in nematodes was discovered it was realized that use of the marker for whole-body analysis of gene expression would require an optical system allowing a large depth of field and a stage with working space for culture plates. Up to this time, most epifluorescence systems were linked to compound or inverted microscopes that had limited field depth

and capability to manipulate organisms under observation. This led to the development of an epifluorescence module using a mercury lamp that could be attached to a Leica stereozoom microscope system. Most major microscope manufacturers now market integrated epifluorescent stereozoom microscopes with capabilities for several filter systems.

A lower cost alternative for GFP screening is use of a lamp module using ultra bright blue light emitting diodes (LEDs) with barrier filters that attaches to the objective lens of most stereozoom microscopes (BLS Ltd., Budapest, Hungary). It costs considerably less than a mercury lamp system, but at present, it only has capabilities for detecting GFP and YFP.

The use of fluorescent protein markers, and especially multiple markers will be greatly aided by the use of fluorescence activated embryo sorters. A device first developed to sort *Drosophila* embryos expressing GFP (Furlong *et al.*, 2001) has been modified and commercially marketed for *Drosophila* and other organisms by Union Biometrica (Somerville, MA, USA). The latest sorting machines are highly sensitive having the ability not only to distinguish different fluorescent proteins, but also to discriminate between levels of fluorescence from the same protein. Thus, these systems may have enormous importance to the straightforward screening for transgenics, and more sophisticated assays such as those for enhancer traps. Practical applications could include the screening of released transgenic insects caught in traps (in systems adapted for adults), or for genetic-sexing of embryos having a Y-linked or male-specific fluorescent marker.

10.5. Transposon Vectors

10.5.1. *Hermes*

10.5.1.1. Discovery, description, and characteristics *Hermes* is a member of the *hAT* family of transposable elements and is related to the *hobo* element of *D. melanogaster*, the *Ac* element from maize, *Zea mays*, and the *Tam3* element from *Antirrhinum majus* (Warren *et al.*, 1994). The initial interest in this family of elements by those interested in creating new insect gene vectors stemmed from two observations. First, during the middle and late 1980s the mobility characteristics of the *Ac/Ds* element system were being extensively studied because the element was recognized as having great potential to serve as a gene-analysis and gene-finding tool in maize and other plants. In addition, the mobility properties of *Ac/Ds* were being extensively tested in species of plants other than maize and in almost

every case evidence for *Ac/Ds* mobility was obtained (Fedoroff, 1989). *Ac/Ds* appeared to be a transposable element with a very broad host range, unlike, for example, the *P* element from *D. melanogaster*, which only functions in closely related species (O'Brochta and Handler, 1988). Because transposable elements with broad host ranges were of interest to those attempting to develop insect transformation technology, *Ac*-like elements warranted attention. The second significant observation at this time was that the *hobo* element from *D. melanogaster* had notable DNA sequence similarity to *Ac/Ds*, suggesting that it was a distant relative of this broadly active element (Calvi *et al.*, 1991). Investigation into the host range of *hobo* using plasmid-based mobility assays (as described above) ensued (O'Brochta *et al.*, 1994). It was during the investigation of *hobo* that *Hermes* was discovered (Atkinson *et al.*, 1993). Atkinson *et al.* (1993) performed plasmid-based *hobo* excision assays in embryos of *M. domestica* as part of an initial attempt to assess the host range of *hobo*. Assays were performed in the presence of *hobo*-encoded transposase and *hobo* excision events were recovered suggesting that *hobo*, like *Ac/Ds*, would have a broad host range. However, when the assays were performed without providing *hobo*-encoded transposase *hobo*, excision events were still recovered in *M. domestica*. The movement of *hobo* in the absence of *hobo*-transposase was completely dependent upon the inverted terminal repeats of *hobo* and the resulting excision events had all of the characteristics of a transposase-mediated process. It was proposed that *M. domestica* embryos contained a *hobo* transposase activity and that this activity arose from the transposase gene of an endogenous *hobo*-like transposable element (Atkinson *et al.*, 1993). These investigators were eventually able to confirm their hypothesis and the element they discovered was called *Hermes* (Warren *et al.*, 1994).

Hermes is 2749 bp in length and is organized like other Class II transposable elements in that it contains ITRs and a transposase-coding region (Figure 1). It contains 17 bp ITRs with 10 of the distal 12 nucleotides being identical to the 12 bp ITRs of *hobo*. *Hermes* encodes for a transposase with a predicted size of 72 kDa and based on the amino acid sequence is 55% identical and 71% similar to *hobo* transposase (Warren *et al.*, 1994). The cross-mobilization of *hobo* by *Hermes* transposase that was proposed by Atkinson *et al.* (1993) was tested directly by Sundararajan *et al.* (1999). These investigators used plasmid-based excision assays in *D. melanogaster* embryos to show that *hobo* transposase could mobilize *Hermes* elements

and that *Hermes* transposase could mobilize *hobo* elements (Sundararajan *et al.*, 1999). The phenomenon of cross-mobilization has important implications for the future use of transposable element-based gene vectors in non-drosophilid insects and will be discussed below. As is typical of transposable elements, *Hermes* is present as a middle repetitive sequence within the genomes of multiple strains of *M. domestica* and in all populations examined there appeared to be full-length copies of the element. The natural history of this element within *M. domestica* has not been investigated and its mobility properties within this species and the existence of any regulatory system remain unknown.

10.5.1.2. Patterns of integration The integration behavior of *Hermes* has been examined in a variety of contexts. Sarkar *et al.* (1997a, 1997b) tested the ability of *Hermes* to transpose, using a plasmid-based assay, in five species of Diptera. They recovered transpositions of *Hermes* in the target plasmid at a frequency of approximately 10^{-3} in all species tested. In addition they examined the distribution of 127 independent transposition events into the 2.8 kb plasmid used as a target in their assay and observed a distinctly nonrandom pattern of integrations. Most notable was the existence of three sites that were targets for *Hermes* integration 10 or more times each. In an experiment in which any site used two or more times was considered a hot spot for integration, the three sites used 10 or more times constitute sites with unusual characteristics. The precise nature of those characteristics however could not be defined. The sites shared four of eight nucleotides of the target site in common (GTNNNNAC); however, other sites with this nucleotide composition were not equally attractive as integration sites indicating that other factors must be influencing target choice. Saville *et al.* (1999) demonstrated that sequences flanking *hobo* integration hot spots were critical for determining the targeting characteristics of a site. These investigators were able to move an 8 bp *hobo* target site from plasmid to plasmid without losing its target characteristics as long as they included 20 bp of flanking sequence on each side of the target. It was suggested that proximity to a preferred integration site increased the likelihood of a site being used as a target (Sarkar *et al.*, 1997a). They found that sites 80 and 160 bp flanking the integration hot spot were also preferred integration sites. The authors suggested that nucleosomal organization of the target contributes significantly to the target site selection process and contributed to the local juxtaposition of hot spots and flanking DNA.

10.5.1.3. Structure–function relationships Many Class II transposable elements contain a distinct amino acid motif within their catalytic domains consisting of two aspartate residues and a glutamate. This DD35E motif can be found in many but not all Class II transposable elements. The presence of this motif in *Hermes* transposase has been unclear. Bigot *et al.* (1996) proposed the existence of a DDE motif among members of the *hAT* family. However, they proposed that the second aspartate was replaced by a serine in *Ac*, *hobo*, and *Hermes*. Capy *et al.* (1996) concluded that *hAT* elements, like *P* elements from *Drosophila*, do not contain the DDE motif based on sequence alignments, and Lerat *et al.* (1999) supported this conclusion based on the lack of similarity in predicted secondary structure of the transposase of members of the *mariner/Tc* superfamily and *hobo* transposase. Michel *et al.* (2002) examined experimentally the importance of D402, S535, and E572 to the proper functioning of *Hermes* transposase. They found that mutations D402N and E572Q abolished transposase activity while the mutations S535A and S535D had no effect on transposase activity. The work of Michel *et al.* (2002) provided the first experimental data to support the hypothesis that the positive charge of residues D402 and E572 are required for transposition. The authors concluded, based on these data, that D402, S535, and E572 do not constitute the catalytic center of *Hermes* transposase because one of the residues was not essential for activity. Therefore, *Hermes* (and *hAT* elements in general) do not appear to be of the DD35E type of transposable elements, making them distinct from *mariner/Tc* elements.

Because *Hermes* transposase acts within the nucleus it is expected to contain a nuclear localization signal to direct the mature transposase from the ribosome to the nucleus. Deletion and site-directed mutagenesis analysis were performed that demonstrated that the *Hermes* nuclear localization signal is located at the amino acid end of the protein and divided among three domains (Michel and Atkinson, 2003).

The ITRs of transposable elements play an essential role in their mobility. Altering the sequence of ITRs can, depending on the element, lead to loss of function, hyperactivity of the element, or switching the mode of transposition from a cut-and-paste mechanism to a replicative mechanism. *Hermes* contains imperfect ITRs with a 2 bp mismatch within the ITR (Warren *et al.*, 1994). In addition, a naturally occurring polymorphism in the terminal nucleotide of the right 3' ITR exists. Elements with a cytosine in the terminal position of the right ITR

have no activity within *D. melanogaster* but are capable of undergoing an aberrant form of transposition in mosquitoes. Small pentanucleotide motifs in the subterminal regions of both *Hermes* and *hobo* have been found to be important for the mobilization of *Hermes* and *hobo*. The sequences GTGGC and GTGAC are interspersed throughout the subterminal region of the element, and similar repeats are present in the subterminal regions of *Ac* and are known to be transposase binding sites. In *Hermes*, altering a single repeat can eliminate transpositional activity (Atkinson *et al.*, 2001).

Hermes transposase is capable of dimerizing and one region of the protein critical for dimerization is located in the C-terminus of the protein including amino acids 551–569. This region is not only essential for dimerization but is also required for transposition activity. A second region that affects dimerization is located in the N-terminus of the protein within the first 252 amino acids of the transposase. However, this region apparently plays a nonspecific role in dimerization (Michel *et al.*, 2003).

10.5.1.4. Host range of *Hermes* *Hermes* has a wide insect host range and has been found to function (as measured by either plasmid-based mobility assays or by germline transformation) in at least 13 species of insects including 11 flies, one beetle, and one moth (Atkinson *et al.*, 2001). *Hermes* functions rather efficiently in *D. melanogaster* and transforms this species at rates of 20–40% (O'Brochta *et al.*, 1996). In all other species tested the efficiency of transformation was considerably lower and tended to be less than 10%. For example, *T. castaneum* was transformed at a rate of 1%, *A. aegypti* at 5%, *C. quinquefasciatus* at 11%, *C. capitata* at 3%, and *S. calcitrans* at 4% (Atkinson *et al.*, 2001). In all insects except mosquitoes, *Hermes* appeared to use a standard cut-and-paste type mechanism as is typical of most Class II transposable elements. Such integrations are characterized by the movement of only those sequences delimited by the ITRs, and the integrated elements are flanked by direct duplications of 8 bp. Integration of *Hermes* into the germline of *A. aegypti* and *C. quinquefasciatus* appears to occur by a noncanonical mechanism resulting in the integration of DNA sequences originally flanking the element on the donor plasmid. The amount of flanking DNA that accompanies the integration of *Hermes* in these mosquito species varies. In some cases two tandem copies of the *Hermes* element were transferred to the chromosome and each copy was separated by plasmid DNA sequences (Jasinskiene *et al.*, 2000). Although

these transposition reactions are unusual they are dependent upon *Hermes* transposase since the introduction of *Hermes*-containing plasmid DNA in the absence of *Hermes* transposase failed to yield transformation events. The germline integration behavior of *Hermes* in mosquitoes is not unique; however, other elements being used as gene vectors such as *mariner* and *piggyBac* have occasionally shown similar behavior in *A. aegypti* (O'Brochta, unpublished data). Transposition assays performed with plasmids in developing mosquito embryos and in mosquito cell lines showed that *Hermes* could transpose via a canonical cut-and-paste type mechanism under these conditions (Sarkar *et al.*, 1997b). The basis for the difference in types of integration events between plasmid-based transposition assays and chromosomal integrations is unknown but may reflect differences in somatic and germ cells. In *Aedes*, canonical cut-and-paste transposition has been readily detected in the somatic tissues of insects containing an autonomous element. Germline transposition in these same insects has not been detected. It has been suggested that mosquitoes might contain endogenous *hAT* elements that affect the ability of *Hermes* elements to be integrated precisely. An alternative suggestion is that *Hermes* may have a second mode of transposition as do the transposable elements *Tn7*, *IS903*, and *Mu*, which utilize a replicative mechanism of integration. Such a mechanism would result in integration products that resemble those observed in the germline of *A. aegypti* and *C. quinquefasciatus*. Replicative transposition of *Hermes* has not been demonstrated experimentally and direct tests of the "alternate mechanism" hypothesis have not been reported.

10.5.1.5. Postintegration behavior Once integrated into the genome of *D. melanogaster*, *Hermes* maintains its ability to be remobilized and has shown mobility characteristics that are similar to other transposable elements. Following the introduction of an autonomous *Hermes* element in which the transposase gene was under *hsp70* promoter regulation, and also contained an EGFP marker gene under constitutive regulatory control of the *actin5C* promoter, Guimond *et al.* (2003) found that the element continued to transpose in the germline at a rate of 0.03 jumps per element per generation. The element used in this study was also active in the somatic tissue and they used this as a means of collecting approximately 250 independent transposition events. Analysis of somatic integration events revealed a number of interesting patterns. First, they found that transpositions were clustered around the original integration event. On average

39% of the *Hermes* transpositions recovered were intrachromosomal and 17% were within the same numbered polytene chromosome division. Ten percent of the new insertions were at sites within 2 kb of the donor element, indicating that *Hermes*, like other transposable elements, shows the characteristic of local hopping. Local hopping refers to the tendency of some elements to preferentially integrate into closely linked sites. Local hopping has been described for a number of elements and is likely to be a general characteristic of Class II transposable elements although the mechanistic basis for this behavior is unknown. Certain regions of the *D. melanogaster* genome, as defined by numbered divisions of the polytene chromosomes, are preferred as integration sites, with these regions being repeatedly targeted by *Hermes*. The observed clustering of independent transposition events in regions of the chromosome seems to reflect undefined aspects of the transposition process that might be influenced by the chromatin landscape. With one exception, the clustering observed by Guimond *et al.* (2003) was not correlated with any common feature of the chromosomes or the genes within a region. This type of nonrandom pattern of integration with regional differences has also been reported for other elements. Interestingly, there does not seem to be any strong correlation between the preferred insertion-site regions of the elements *P*, *hobo*, and *Hermes*, at least with respect to chromosome 3 of *D. melanogaster* (see figure 7 from Guimond *et al.*, 2003). Guimond *et al.* (2003) also observed a notable clustering of integrations in polytene chromosome division 5. Eight of the 11 integration events recovered from division 5 (3.2% of all the transposition events examined) were within the 2.7 kb segment of DNA upstream of the cytoplasmic actin gene, *actin5C*. This same 2.7 kb segment of the 5' regulatory region of *actin5C* was also present within the autonomous *Hermes* element, as a promoter for the EGFP marker, which the investigators tracked as it jumped within the genome.

The strong clustering of transpositions in a target sequence that is homologous to a sequence contained within the vector has been referred to as "homing." This type of target site selection bias was first described for *P* elements and has been reported on a number of occasions. It was initially reported as a strong bias in the integration site distribution of a number of primary germline integration events in which a *P* element containing the *engrailed* gene preferentially integrated into the *engrailed* region of the host genome (Hama *et al.*, 1990; Kassis *et al.*, 1992). A similar biasing of integration site selection was also observed with *P* elements containing *Bithorax*

and *Antennapedia* regulatory sequences (Engstrom *et al.*, 1992; Bender and Hudson, 2000). Taillebourg and Dura (1999) reported a remarkable example of homing of a remobilized *P* element in *D. melanogaster*. This element contained either an 11 kb or 1.6 kb fragment of the 5' region of the *linotte* gene, and it was found that 20% of the remobilized elements integrated into the 5' region of the *linotte* gene. Insertions in this case were highly localized and most occurred within a 36 bp fragment of the *linotte* regulatory region. *Hermes* homing indicates that the phenomenon is not element specific, but may be a general characteristic of Class II elements. Guimond *et al.* (2003) suggested that homing was a special case of local hopping, and the physical proximity between donor elements and target sites seems to underlie the phenomenon of local hopping. The presence of transgene regulatory sequences (e.g., *actin5C* 5' region) may promote tethering of the donor elements to similar regulatory regions via proteins with common DNA binding sites. Deliberate tethering or transposable elements to selected sequences may be a means to regulate target site selection and to minimize the detrimental mutagenic effects of transposable element integration (Bushman, 1994; Kaminski *et al.*, 2002).

The postintegration behavior of the same autonomous *Hermes* element described above in *A. aegypti* had quite different characteristics. In this case germline transposition of the autonomous *Hermes* element was never detected, and it should be noted that the primary integration events in the germline involved the integration of DNA sequences flanking the element (Jasinskiene *et al.*, 1998, 2000). Despite the fact that the element was intact and that functional transposase was expressed, the element was immobile in the germline. This was not the case, however, in the soma of *A. aegypti* where *Hermes* excision and cut-and-paste transpositions were readily detected. Transposition events in the soma had all of the hallmarks of Class II cut-and-paste integration. Only those sequences precisely delimited by the ITRs moved and integration resulted in the creation of 8 bp direct duplications at the target site. Excision of *Hermes* was imprecise and led, in some cases, to the creation of small deletions. The basis for the difference in behavior of the *Hermes* element in the germline versus the somatic tissue of *A. aegypti* is unknown. Clearly the postintegration behavior of *Hermes* in this species will influence how this element will be employed, and in situations where germline stability is essential, *Hermes* will be particularly useful. It will not be useful in its present form for constructing gene-finding tools such as

enhancer and promoter traps that rely heavily on transposable element vector remobilization to be effective.

10.5.1.6. Extrachromosomal forms of *Hermes*
Excision of *Hermes* in *M. domestica*, and autonomous *Hermes* integrations in *D. melanogaster* and *A. aegypti*, lead to the formation of circularized *Hermes* elements in which the terminal inverted repeats are jointed end-to-end in various ways following the excision reaction (Atkinson and O'Brochta, unpublished data). The most common configuration results in the ends being joined end-to-end with a short spacer sequence between them. The spacer sequence was most often 1, 3, or 4 bp but could also be as much as 200 bp. The extrachromosomal *Hermes* elements found in *M. domestica* are particularly interesting because they have been found in all populations tested and in great abundance in somatic tissue. These data provide evidence for the somatic activity of *Hermes* in the insect from which it was originally isolated. Circularized forms of excised transposable elements of a number of types have been reported in the past (Sundaresan and Freeling, 1987). For example, circularized forms of *Ac/Ds* have been described as well as *Minos* (Arca *et al.*, 1997; Gorbunova and Levy, 1997), yet the significance of extrachromosomal forms of transposable elements has remained unclear. In some cases the circularized elements do not contain intact ITRs and consequently the elements are not expected to be integration competent. Based on rather limited data it has generally been concluded that such forms represent byproducts of aborted or interrupted transposition reactions. A study of the extrachromosomal forms of *Hermes* suggests that these elements may have some biological significance. Circularized *Hermes* elements with intact ITRs are integration-competent, potentially allowing them to contribute to forward transposition (Atkinson and O'Brochta, unpublished data). The ability of circularized forms of excised *Hermes* elements to reintegrate may have an impact on the transmission potential of this element. The existence of a large pool of functional extrachromosomal transposable elements may have implications for the ability of the element to be transferred horizontally and may provide an additional means of vertical transmission (e.g., maternally inherited), both of which will potentially enhance the element's ability to increase in frequency within a population. The biology of extrachromosomal *Hermes* elements needs to be investigated further.

10.5.1.7. *hAT* elements have been found in other insects The Queensland fruit fly, *Bactrocera tryoni*, contains members of at least two distinct *hAT*-like transposable elements (Pinkerton *et al.*, 1999). *Homer* is a 3789 kb element whose sequence is 53% identical to *Hermes* and 54% identical to *hobo*. The transposase coding region is approximately 53% identical and 71% similar to the transposases of *Hermes* and *hobo*. Similarly, the ITRs of *Homer*, which are 12 bp in length, are identical to those of the *hobo* and *Hermes* elements at 10 of 12 positions. There are also *Homer*-like elements within *B. tryoni*. There are fewer than ten copies per genome, and while these elements have not been fully characterized, a conceptual translation of the transposase of this *Homer*-like element reveals 48% identity and 66% similarity to the transposase of *Homer*. These *Homer*-like elements are as similar to *hobo* as they are to *Homer*. Although *Homer* appears to be weakly functional in *D. melanogaster* based on plasmid-based excision assays, all *Homer*-like elements contain inactivating frameshift mutations.

The blowfly *L. cuprina* contains a nonfunctional *hAT* element called *hermit*. *Hermit* was initially found by low stringency hybridization screening of an *L. cuprina* genomic library using a DNA probe homologous to *hobo* (Coates *et al.*, 1996). *Hermit* is 2716 bp and contains perfect 15 bp ITRs, the distal 12 of which are identical to the *hobo* ITRs at 10 of 12 positions. Although inactive because of frameshift mutations within the transposase coding region, its amino acid sequence is 42% identical and 64% similar to *hobo* transposase. *Hermit* is unusual in that it is present as a unique sequence within *L. cuprina*, in contrast to multiple copies that exist for most transposons. Although present only once within this species it does appear to have arisen within the genome as a result of transposition since the existing copy of the element is flanked by an 8 bp direct duplication of a sequence that is similar to the consensus target site duplication derived from other *hAT* elements. *Hermit* appears to have become inactivated soon after integrating into the *L. cuprina* genome.

Several *hAT* elements have been discovered in tephritid fruit flies using a polymerase chain reaction (PCR) approach similar to that used to discover *Hermes* (Handler and Gomez, 1996). Of these elements, a complete *hAT* transposon (*hopper*) was isolated from a genomic library of the wild Kahuku strain of the Oriental fruit fly, *B. dorsalis*, using the *B. dorsalis hobo*-related element (Bd-HRE) PCR product as a hybridization probe (Handler and Gomez, 1997). A complete 3120 kb element was isolated having 19 bp ITRs. However, the putative

transposase-coding region was frameshifted and it did not have a duplicated 8 bp insertion site, suggesting that it had accumulated mutations and was nonfunctional. The Kahuku sequence was used to isolate additional *hopper* elements using an inverse and direct PCR approach, and a new 3131 bp *hopper* was isolated from the *B. dorsalis white eye* strain (Handler, 2003). This element has an uninterrupted coding region and an 8 bp duplicated insertion site. Notably, *hopper* is highly diverged from all other known insect *hAT* elements and its transposase is distantly yet equally related to the coding regions of *hobo* and *Ac*. Of the terminal 12 nucleotides only five are identical to those of *hobo*, while six are identical to the ITRs of *Homer* (*B. tryoni*). *hopper* also exists in the melonfly, *B. cucurbitae*, and another *hAT* element originally discovered in the melonfly, that is closely related to *hobo* and *Hermes*, also exists in *B. dorsalis* (Handler and Gomez, 1996).

hAT elements have been also reported in the human malaria vector *A. gambiae*. Approximately 25 copies of sequences that resemble *hAT* transposases were discovered although none appeared to be part of an intact transposable element. More recently, however, search criteria were used based on unique aspects of *hAT* transposable elements such as length and spacing of ITRs and the characteristics of *hAT* element target sites. This search revealed a *hAT* element in *A. gambiae* that contained perfect 12 bp ITRs flanked by 8 bp direct duplications and a 603 amino acid transposase open reading frame that appeared to contain no internal stop codons. This element (*Herves*) is most closely related to *hopper* and the ability of this element to excise and transpose in *A. gambiae* or other species has not been determined (Atkinson and Arenburger, personal communication).

10.5.2. *piggyBac*

10.5.2.1. Discovery of *piggyBac* and other TTAA-specific elements Similar to several other insect transposable element systems, the *piggyBac* element was discovered fortuitously in association with a mutant phenotype. However, unlike all the other transposons used for insect transformation, the mutant phenotype was the result of a functional element that had transposed into an infectious organism. Fraser and colleagues (see Fraser, 2000) discovered several Few Polyhedra (FP) mutations in the baculoviruses, *Autographa californica* nucleopolyhedrovirus (AcNPV), and *Galleria mellonella* nucleopolyhedrovirus (GmNPV), after passage through the *Trichoplusia ni* cell line TN-368 (Fraser *et al.*, 1983, 1985). Among these elements that inserted specifically into tetranucleotide TTAA sites

was *piggyBac* (then named IFP2), which transposed into AcNPV. Although it might be assumed that IFP2 was an autonomous functional element based on its mobility, another TTAA insertion-site element, *tagalong* (then called TFP3), discovered in AcNPV and GmNPV, was later found not to have a transposase coding region and thus had to be mobilized by another TFP3 or related element. Autonomous functional elements have not yet been found for *tagalong*, though the original IFP2 *piggyBac* element was indeed functional (Wang *et al.*, 1989; Wang and Fraser, 1993). All the *piggyBac* elements discovered in TN-368 were found to be identical, having a length of 2472 kb with 13 bp perfect ITRs and 19 bp subterminal repeats located 31 bp from the 5' ITR and 3 bp from the 3' ITR (Cary *et al.*, 1989) (Figure 1). The transposase coding region exists as a single reading frame of 2.1 kb that encodes a protein with a predicted molecular mass of 64 kDa. The functionality of *piggyBac* and the precise nature of its transposition was further verified by a series of viral and plasmid transposition and excision assays. A *piggyBac* indicator plasmid was marked with *polh/lacZ*, and assays in the fall armyworm, *Spodoptera frugiperda* cell line SF21AE showed that the original *piggyBac* element, within the p3E1.2 plasmid, could mobilize the marked element. These assays proved that the 3E1 *piggyBac* element encoded a functional transposase, and defined the element's TTAA insertion-site specificity and the precise nature of its transposition. Importantly, these assays also showed directly that *piggyBac* could be mobilized in other lepidopteran species (Fraser *et al.*, 1995), indicating that it might function similarly as a vector for germline transformation. This was a critical realization given the failure of *P* to be mobilized in non-drosophilids, which was consistent with its failure as a vector in these species.

10.5.2.2. *piggyBac* transformation The failure of *P* vectors to transform non-drosophilid species made the testing of other available transposon systems a high priority. The other systems found to be functional in non-drosophilids, however, were first tested successfully for gene transfer vector function in *Drosophila*. For *piggyBac*, germline transformation was first attempted in the Mediterranean fruit fly, *Ceratitis capitata*. This was possible due to the availability of a marker system that had been tested previously by medfly transformation with the *Minos* transposon vector. The medfly *white* gene cDNA was linked to the *Drosophila hsp70* promoter, and was used as a mutant-rescue system in a white eye host strain (Loukeris *et al.*, 1995b; Zwiebel *et al.*, 1995). In the absence of data for the minimal

sequence requirements for *piggyBac* mobility, the first *piggyBac* vector was constructed by insertion of the 3.6 kb *hsp-white* cDNA marker into the unique *HpaI* site within *piggyBac* in the p3E1.2 plasmid. None of the *piggyBac* sequence was deleted though the insertion interrupted the coding region eliminating transposase function. Construction of the first helper was a simple deletion of the 5' ITR sequence resulting from a *SacI* digestion and religation of p3E1.2. There is some uncertainty as to whether the upstream *SacI* site cuts within the *piggyBac* promoter (Cary *et al.*, 1989), yet transposase expression was indeed sufficient to support germline transpositions from the vector plasmid. The first experiment with this helper in the medfly resulted in one transgenic line at a transformation frequency of 5% per fertile G₀. However, sibling sublines exhibited two and three independent integrations (Handler *et al.*, 1998). This experiment with a *piggyBac*-regulated helper was repeated with five additional G₁ lines isolated, but at approximately the same frequency. These attempts at *piggyBac* transformation yielded relatively low transformation frequencies, but it was notable that a lepidopteran transposon vector system had autonomous function in a dipteran species.

Subsequent to the medfly transformation, *piggyBac* transformation was tested in *Drosophila* using the mini-*white* marker from that species (Handler and Harrell, 1999). Using the self-regulated helper, transformants were isolated at a similar frequency of 1–3%, but tests with a *hsp70*-regulated transposase increased the frequency to above 25%, consistent with *P* and *hobo* transformations using heat shock promoted transposase.

Given that *piggyBac* was first isolated from a lepidopteran species, there was some optimism that it would be functional as a vector in other moth species. Function was first tested by transposition assays in the pink bollworm, *Pectinophora gossypiella* (Thibault *et al.*, 1999), which then led to successful germline transformation of this species using the *phspBac* helper and a vector marked with EGFP regulated by the *Bombyx actinA3* promoter (Peloquin *et al.*, 2000). Concurrent experiments were also performed in the silkworm *B. mori* using a similar *actinA3*-regulated EGFP marker, but for this species transformation was achieved with an *actinA3*-regulated transposase helper (Tamura *et al.*, 2000). While these are the only moth species reported to be transformed with *piggyBac*, several other dipteran species have been transformed, as well as species in the orders Coleoptera and Hymenoptera. The dipteran species transformed include several of medical and agricultural importance, such

as the mosquitoes *Aedes aegypti* (Kokoza *et al.*, 2001; Lobo *et al.*, 2002), *A. gambiae* (Grossman *et al.*, 2001), *A. albimanus* (Perera *et al.*, 2002), and *A. stephensi* (Nolan *et al.*, 2002), and the tephritid fruit flies *Anastrepha suspensa* (Handler and Harrell, 2000) and *Bactrocera dorsalis* (Handler and McCombs, 2000). Other transformed dipterans include *M. domestica* (Hediger *et al.*, 2000) and *L. cuprina* (Heinrich *et al.*, 2002). Of particular importance at this time, has been the use of *piggyBac* to transform a coleopteran, the red flour beetle, *Tribolium castaneum* (Berghammer *et al.*, 1999; Lorenzen *et al.*, 2003), and a hymenopteran, the sawfly *Athalia rosae* (Sumitani *et al.*, 2003). Notably, all of these species were primarily transformed using a helper regulated by the *Drosophila hsp70* promoter, and with vectors marked with EGFP, though other fluorescent proteins have since been used for some as well.

Although most of these transformations occurred at frequencies between 3% and 5% per fertile G₀, dramatic differences between species have been observed as well, and in some of the same species performed by different laboratories. A single transformant line was reported for *Anopheles gambiae*, at a frequency of approximately 1% (Grossman *et al.*, 2001), while transformation in *A. albimanus* occurred at frequencies ranging from 20% to 40% (Perera *et al.*, 2002). The first transformations of *Tribolium* occurred at an unusually high frequency of 60% (Berghammer *et al.*, 1999).

Many of the transformations were preceded by testing *piggyBac* function by embryonic transposition assays that were first developed for *piggyBac* mobility in the pink bollworm (Thibault *et al.*, 1999). As discussed previously, these assays can rapidly assess the relative mobility of *piggyBac* in a specific host species in a few days. Positive results from these assays provided some assurance that more tedious and time-consuming transformation experiments had some likelihood of success. For some studies the assays also were used to test promoter function in helper plasmids, or provided insights into insertion site specificity, or determined the likelihood of a particular vector construct retaining function in the absence of specific sequences (Lobo *et al.*, 2001). For example, *piggyBac* helper promoters were tested by transposition assays and germline transformation in *D. melanogaster* and *L. cuprina* (Li *et al.*, 2001a; Heinrich *et al.*, 2002). It was found that in *Drosophila*, an *hsp70*-regulated helper yielded the highest transposition frequency, while a constitutive $\alpha 1$ -*tubulin*-regulated helper was more effective for germline transformation. By comparison, in *Lucilia* the *hsp70*

helper was most effective for both plasmid and germline transpositions, while the *Drosophila* $\alpha 1$ -*tub* helper failed to support transformation. Transposition assays have also shown target site preferences among the TTAA sites within the pGDV1 target plasmid, and assays in *Drosophila* indicated a bias for sites having A or T nucleotides at positions -3, -1, +1, and +3 relative to TTAA (Li *et al.*, 2001a). However, a sequence analysis of 45 genomic integrations sites in *Tribolium*, after *piggyBac* vector remobilization, failed to show this bias (Lorenzen *et al.*, 2003), which may be an indication of species specificity for insertion site preference.

Mobility assays also provide a rapid means of testing sequence requirements for vector mobility, which allow modifications for more efficient vector function. Since vector mobility is known to be affected negatively with increasing size, this information should allow minimal vectors to be created that retain optimal function. However, minimal sequence requirements for plasmid transpositions may differ from those for chromosomal transposition. For example, excision and transposition assays performed in *Trichoplusia ni* embryos showed that the *piggyBac* inverted terminal repeat and subterminal repeat sequences were sufficient for transposition (35 bp from the 5' terminus and 63 bp from the 3' terminus), but that an outside spacer region between the ITRs of greater than 40 bp is necessary for optimal transposition from a plasmid (Li *et al.*, 2001b). Use of similar vectors in *Drosophila*, however, did not result in germline transformants (Handler, unpublished data). The minimal sequence requirements for *piggyBac* transformation verified thus far for *Drosophila* are 300 bp from the 5' terminus and 250 bp from the 3' terminus (Li, Fraser, and Handler, unpublished data).

10.5.2.3. Phylogenetic distribution of *piggyBac* and implications for transgene stability Unlike most other transposons used for transformation, *piggyBac* is not an apparent member of a larger family, or superfamily of related elements such as the *mariner/Tc* or *hAT* families. Until recently, the only *piggyBac* elements known were the functional elements originally discovered in *T. ni* (Fraser *et al.*, 1983). Thus, an unexpected finding from the Southern analysis of *B. dorsalis* transformants was that 8 to 10 *piggyBac*-related elements exist in the host strain genome (Handler and McCombs, 2000). PCR analysis of internal coding sequence indicated that these were nearly identical elements, though none has been found to be identical to *piggyBac*, nor are their coding regions consistent with transposase functionality. The isolation of complete *B. dorsalis*

piggyBac elements as genomic clones and by inverse PCR indicate that complete elements exist with conserved terminal and subterminal sequences that are integrated into duplicated TTAA insertion sites. *Bactrocera dorsalis* is part of a larger species complex and PCR analysis indicated that, indeed, *piggyBac* exists throughout the complex having nucleotide sequence identities of 92% among all the elements sequenced, with individual identities of 96–99% to one another and the *T. ni* 3E1 *piggyBac* (Handler, unpublished data).

10.5.2.4. *piggyBac* in other insects The evolutionary distance between *T. ni* and *Bactrocera* strongly suggests that the transposon moved between these species by recent horizontal transmission, and the separation of their geographical habitats raises the possibility that this movement may have been mediated by intermediary species. A Southern blot survey for *piggyBac* in more than 50 species showed the most clear evidence for multiple *piggyBac* elements in *S. frugiperda*, but hybridization patterns suggested that most of the elements are defective and nonfunctional (Handler, unpublished data; see Handler, 2002a). Evidence for *piggyBac* in other insects and other organisms, including mosquitoes and humans, comes from recent sequence data from genome projects. Although discrete sequence similarities suggests that *piggyBac* has an ancient history, there is little evidence at present to indicate that *piggyBacs* have coevolved as functional elements, and related complete elements have yet to be discovered.

The first functional *piggyBac* elements were discovered in a *T. ni* cell line, but little analysis has been done to characterize *piggyBac* in the organismal genome. Recent hybridization analysis of *piggyBac* from larval *T. ni* genomes indicates that *piggyBac* exists, and that its general structure is consistent with full-length functional elements. However, a PCR survey of these genomic sequences has only identified nonfunctional elements, having a level of identity no greater than many of the elements found in *Bactrocera* (>96%) (Zimowska and Handler, unpublished data). If functional *piggyBacs* do not exist *in vivo*, this could be explained by genomic instability that may arise from a highly active transposon. Functional elements may create a genetic load resulting in organismal lethality, which is more easily withstood in cell lines. If this is the case, it is therefore intriguing to consider how the functional element arose in the cell line.

It is also intriguing to consider how horizontal transmission of *piggyBac* may have occurred, considering that the element was originally discovered by virtue of its transposition into an infectious

baculovirus. This could potentially explain a distribution among lepidopterans, but not the apparent recent movement between moths and flies. Understanding the interspecies movement of *piggyBac*, as well as all other vectors used for practical application, will be critical to understanding and eliminating risk associated with the release of transgenic insects.

10.5.3. *mariner*

10.5.3.1. Discovery, description, and characteristics The *mariner* element was first discovered as an insertion element responsible for the *white-peach* (w^{pch}) mutant allele of *D. mauritiana* (Haymer and Marsh, 1986; Jacobson *et al.*, 1986). This particular allele was interesting when discovered because it was highly unstable with reversions to wild type occurring at a frequency of approximately 10^{-3} per gene per generation. *white-peach* individuals also had a high frequency of mosaic eyes, at an approximate frequency of 10^{-3} , suggesting somatic instability. Molecular analysis of the w^{pch} allele indicated that it was the result of a 1286 bp transposable element insertion into the 5' untranslated leader region of the *white* gene (Jacobson *et al.*, 1986) (Figure 1). The *mariner* element is a Class II type transposable element with 28 bp imperfect inverted repeats with four mismatches. The element recovered from w^{pch} contained a single open reading frame capable of encoding a 346 amino acid polypeptide (Jacobson *et al.*, 1986). While the original w^{pch} was highly unstable, another strain of *D. mauritiana* was discovered in which mosaicism of the eyes occurred in every fly (Bryan *et al.*, 1987). This mosaicism factor was found to be heritable and was referred to as *Mos1* (Mosaic eyes). *Mos1* was a dominant autosomal factor on chromosome 3 and was subsequently found to be identical to *mariner* except for six amino acid differences in the putative transposase coding region (Medhora *et al.*, 1988). *Mos1* encodes for a functional transposase while the 346 amino acid polypeptide of the w^{pch} *mariner* element was not a functional transposase.

One of the most notable characteristics of *mariner* and *mariner*-like elements (MLEs) is their widespread distribution. MLEs are found not only in insects and invertebrates but also in vertebrates and plants (Robertson, 2000; Robertson and Zumpano, 1997). Not long after the *D. mauritiana* *mariner* elements were described, a related element was discovered in the *cecropin* gene of the moth *Hyalophora cecropia* (Lidholm *et al.*, 1991). Based on the sequence comparison between the *mariner* elements from *D. mauritiana* and *H. cecropia*, Robertson (1993) designed degenerate PCR primers

and surveyed 404 species of insects for the presence of related sequences. He found that 64 of the genomes examined contained *MLEs*, and within this group are five subgroups referred to as the *mauritiana*, *cecropia*, *mellifera*, *irritans*, and *capitata* subgroups (Robertson and MacLeod, 1993). Since that original analysis insect *MLEs* have continued to be discovered and currently there are two additional subgroups recognized, known as *mori* and *briggsae* (Lampe *et al.*, 2000). Additional subgroups are likely to be recognized in the future as additional representatives of this family of elements are found. Elements from different subgroups are typically about 50% identical at the nucleotide sequence level while the transposases encoded by elements from different subgroups are usually between 25% and 45% identical at the amino acid level. A notable feature of the phylogenetic relationships of the *MLEs* is their incongruence with the phylogenetic relationships of the insects from which they were isolated. The implication is that many of these elements were introduced into their host genome via a horizontal gene transfer event (Robertson and Lampe, 1995a). The abundant examples of horizontal transfer of *mariner* elements have led to the conclusion that such transfers occur relatively frequently. Hartl *et al.* (1997) estimated that the rate of horizontal transmission of *MLEs* is about the same as the rate of speciation, at least within the *D. melanogaster* species subgroup. The widespread occurrence of horizontal transmission of *MLEs* has been proposed to be critical for the long-term survival of these elements. Horizontal transmission provides a means for invading naive genomes where element proliferation can occur before inactivating influences of mutation and host regulation can occur (Hartl *et al.*, 1997).

Although hundreds of *MLEs* have been reported, only two (*Mos1* from *D. mauritiana* and *Himar1* from *Haematobia irritans*) have been demonstrated to be functional or active. *Haematobia irritans* contains approximately 17 000 copies of *Himar1*, although all of the copies examined were highly defective. Functional elements could be reconstructed based on the consensus sequence of *Himar1* and then constructed by modifying the closely related *Cpmar1* element from the green lacewing, *Chrysoperla plorabunda*, to match the *Himar* consensus sequence (Robertson and Lampe, 1995b; Lampe *et al.*, 1998). Purification of the transposase from a bacterial expression system and its use in an *in vitro* mobility assay demonstrated the functionality of the *Himar1* protein and the ITRs of the element (Lampe *et al.*, 1996).

10.5.3.2. Structure–function relationships The transposases of *MLEs* belong to a large group of integrases and transposases that share a significant feature of their catalytic domains. Specifically, *MLEs* contain the highly conserved DD35E motif within the active site of the protein (Robertson, 2000). This part of the active site interacts with a divalent cation that is essential for catalysis. Transposase binds to the ITRs of the element, and gel retardation assays were used to assess the binding activity of eight mutant transposases with deletions at the N- or C-termini (Auge-Gouillou *et al.*, 2001a). It was possible to show that amino acids 1–141 were sufficient for binding to the ITRs. The ITR binding domain of *Mos1* transposase differs somewhat from that of *Tc1* elements in that it is composed of two different structural motifs, a helix–turn–helix motif and an α -helical region (Auge-Gouillou *et al.*, 2001a).

The ITRs of *Mos1* are not identical and differ in sequence at four positions, which have effects on the activity of the element *in vitro*. Auge-Gouillou *et al.* (2001b) reported a 10-fold higher affinity of *Mos1* transposase for the 3' ITR compared to the 5' ITR. In addition, modified 5' ITRs that were made to resemble 3' ITRs at one of the four variable positions resulted in an increase in transposase binding. These investigators also showed that a *Mos1* element with two 3' ITRs had 104 times the transposition activity of the native ITRs (Auge-Gouillou *et al.*, 2001b). This hyperactive double-ended configuration has not been tested *in vivo*.

Hyperactive transposase mutants of the *Himar1* transposase have been reported (Lampe *et al.*, 1999) and one of the mutants contains two amino acid changes (at positions 131 and 137) in the ITR binding domain of the protein. Although not tested directly, it is possible that these hyperactive mutants result in increased binding of the transposase and consequently higher rates of movement. Paradoxically, neither *Himar1* nor any of the hyperactive mutants shows any transpositional activity in insects (Lampe *et al.*, 2000).

10.5.3.3. Host range of *mariner* The widespread distribution of *MLEs* in nature and the frequent examples of their horizontal transfer between species suggest that these elements have a broad host range. Empirical studies in which *Mos1* has been employed as a gene vector in a wide variety of organisms supports this conclusion. *Mos1* has been used successfully to create transgenic *D. melanogaster* (Lidholm *et al.*, 1993), *D. virilis* (Lohe and Hartl, 1996a), and *Aedes aegypti* (Coates *et al.*,

1998). In each of these species the frequency of transformation was approximately 5%. This element has also been used to create transgenic *B. mori* cells in culture (Wang *et al.*, 2000). In addition to transgenic insects, *Mos1* has been used to create transgenic *Leishmania* (Gueiros-Filho and Beverley, 1997), *Plasmodium* (Mamoun *et al.*, 2000), zebrafish (Fadool *et al.*, 1998), and chickens (Sherman *et al.*, 1998). Similarly the *Himar1* element has been shown to function in *E. coli* (Rubin *et al.*, 1999), Archaeobacteria (Zhang *et al.*, 2000), and human cells (Zhang *et al.*, 1998). However, this element has not been shown to be active in *D. melanogaster* or any other insect species, for reasons that are not clear (Lampe *et al.*, 2000).

10.5.3.4. Postintegration behavior The postintegration behavior of *Mos1* has been investigated in *D. melanogaster* and *A. aegypti*. *mariner* gene vectors used to create transgenic *D. melanogaster* have been found to be uncommonly stable even in the presence of functional transposase. Lidholm *et al.* (1993) created two lines of transgenic *D. melanogaster* with a *mariner* vector derived from *Mos1* and containing the mini-*white* gene as a genetic marker. When these lines were crossed to *Mos1* transposase-expressing lines, eye mosaicism was found in only 1% of the progeny, while these same *Mos1* expressing lines resulted in 100% mosaicism of the *w^{pch}* element. Similarly, germline transposition occurred at rates of less than 1% (Lidholm *et al.*, 1993), and Lohe *et al.* (1995) reported similar evidence for postintegration stability of *mariner* vectors. Lozovsky *et al.* (2002) suggested, after investigating the postintegration mobility of a number of *mariner* vectors containing different genetic markers in different locations within the element, that *mariner* mobility is highly dependent upon critical spacing of subterminal sequences and ITRs. They found that vectors with simple insertions of exogenous DNA of varying lengths and in varying positions showed levels of somatic and germline excision that were at least 100-fold lower than that observed with uninterrupted *mariner* elements. Only vectors consisting of two, almost complete, elements flanking the marker gene showed detectable levels of both somatic and germline mobility. Approximately 10% of the insects with these composite vectors had mosaic eyes when transposase was present. Germline excision rates of approximately 0.04% were observed in these same insects. Again, these values are considerably less than those reported for uninterrupted elements. In addition to the potential importance of subterminal sequence spacing (Lozovsky *et al.*, 2002), Lohe and

Hartl (2002) suggested that efficient mobilization of *mariner in vivo* also depends on the presence of critical sequences located quite distant from the ITRs. Based on the mobility characteristics of about 20 *mariner* elements with a wide range of internal deletions, they concluded that there are three regions within the element that play an important role in *cis*. Region I is approximately 350 bp in length and is located 200 bp from the left 5' ITR. Region II is approximately 50 bp in length and located approximately 500 bp from the right 3' ITR. Region III is about 125 nucleotides in length and located approximately 200 bp from the right ITR (Lohe and Hartl, 2002). While the presence of subterminal sequences that play a critical role in the movement of many Class II transposable elements is not unusual, what is uncommon in the case of *mariner* is the location of these *cis*-critical sequences. Their dispersed distribution within the element is unique, and consequently, manipulating the element for the purposes of creating gene vectors and associated tools without disrupting these important relationships may be difficult.

The postintegration mobility of *Mos1* can also be regulated by nonstructural aspects of the system including "overproduction inhibition" and "dominant-negative complementation." Increasing the copy number of *Mos1* in the genome resulted in a 25% decrease in the rate of germline excision. Copy number increases in *Mos1* presumably lead to increased transposase levels and, by an unknown mechanism, to the inhibition of excision (Lohe and Hartl, 1996b). High concentrations of transposase may lead to nonspecific associations of the protein resulting in inactive oligomers of transposase. In addition, the presence of mutated forms of *Mos1* transposase can repress the activity of functional transposase. Because the transposases of other transposable elements act as dimers or multimers it is thought that mutated *Mos1* transposases may become incorporated into multimers with functional transposases, thereby inactivating the entire complex (Lohe and Hartl, 1996b).

The possibility that transposase overproduction may negatively affect its own activity is a highly important concept in terms of vector system development. Most systems have the helper transposase under strong promoter regulation to optimize transpositional activity, though this may, indeed, be counterproductive. For *mariner* vectors, and potentially other systems, optimal transformation may require testing various helper promoters and a range of plasmid concentrations.

The postintegration mobility properties of *mariner* were also examined in *A. aegypti* (Wilson *et al.*,

2003). As part of an effort to create an enhancer trapping and gene discovery technology for *A. aegypti*, they created nonautonomous *mariner*-containing lines and lines expressing *Mos1* transposase. By creating heterozygotes between these two lines, they attempted to detect and recover germline transposition events, but only a single germline transposition event was recovered after screening 14 000 progeny. Somatic transpositions were detected, and while precise estimates of rates of somatic transposition were not possible because of the detection method, the authors observed fewer than one event per individual which they estimated to be an indication of a very low rate of movement. The vectors used by Wilson *et al.* (2003) resembled the simple vectors reported by Lozovsky *et al.* (2002) which had apparently disrupted spacing of the ITRs, and partial deletions of *cis*-critical sequences described by Lohe and Hartl (2002).

While the postintegration stability of *mariner* has been described in two species and appears to be a general mobility characteristic of this element, and not a reflection of a species-specific host effect, paradoxical observations remain to be explained. First, the use of *mariner* as a primary germline transformation vector in non-drosophilid insects and in non-insect systems is an effective means for creating transgenic organisms. Indeed, the host range of *mariner* as a gene transformation vector is unrivaled by any of the other gene vectors currently employed for insect transformation. *mariner* has been used as a gene vector in microbes, protozoans, insects, and vertebrates. The rates of germline transformation using *mariner*-based vectors in insects is approximately 10% or less, and is comparable to the efficiency of *Hermes*, *Minos*, and *piggyBac* gene vectors. This raises the question of whether *mariner* vectors present on plasmids behave the same as *mariner* vectors integrated into insect chromosomes. Given the rates of germline integration from plasmids it appears that the *mariner* vectors being used are not suffering from “critical spacing/critical sequence” defects. In addition, the *in vitro* behavior of *mariner* also differs from the behavior of chromosomally integrated elements. Tosi and Beverly (2000) demonstrated that only 64 nucleotides from the left end, and 33 nucleotides from the right end, of *mariner* were essential for transposition of a 1.1 kb vector *in vitro*. The rate of transposition of a minimal *mariner* vector *in vitro* was only two-fold less than that of a vector containing essentially a complete *mariner* element. These results suggest that *mariner* mobility has relatively simple sequence requirements and that the role of subterminal sequences is minimal *in vitro*. These apparently

conflicting data suggest that host factors may play an important role in the transposition process *in vivo*, and may influence the relative importance of *cis* sequences in the *mariner* transposition process. The broad distribution of MLEs and host range of *mariner/Mos1* suggest, however, that host factors play little role in the movement of these elements.

The postintegration behavior of *mariner/Mos1* seems to indicate that this element will not be a good candidate for developing gene-finding tools such as promoter/enhancer trapping and transposon tagging systems in *A. aegypti* or perhaps other insects. On the other hand, if a high level of post-integration stability is desired, then *mariner* is an appropriate element to consider in insects. The potential of this element to be lost through excision or transposition is low, even in the presence of functional *mariner* transposase. As currently configured and used, *mariner* vectors may be considered as suicide vectors in insects since they essentially become dysfunctional upon integration.

10.5.3.5. MLEs have been found in other insects While hundreds of MLEs have been described, few have been shown to be functional. The original *mariner* element from the *white-peach* allele was transpositionally competent although it did not produce a functional transposase. *Mos1* is a functional autonomous element and has been the basis for constructing all *mariner* gene vectors that function in insects. *Himar1* is a functional element from the *irritans* subgroup that was reconstructed based on multiple sequence comparisons of elements within this group. It has not been shown to be functional in insects despite significant efforts to do so. Lampe *et al.* (2000) report that at least eight other elements from the other subgroups are likely to be active or made active by minor modifications.

10.5.4. Minos

The first germline transformation of a non-drosophilid insect mediated by a transposon-based vector system was achieved with the *Minos* element. *Minos* was originally isolated as a fortuitous discovery in *D. hydei* during the sequencing of the non-coding region of a ribosomal gene (Franz and Savakis, 1991). *Minos* was found to be a 1.4 kb element having, unlike the other Class II transposons used as vectors, relatively long ITRs of 255 bp, with its transcriptional unit consisting of two exons (Figure 1). Additional *Minos* elements were isolated from *D. hydei* having small variations of one or two nucleotides, though the new elements had a transition change that restored the normal reading frame allowing translation of a functional transposase.

The sequence homology, general structure, and TA insertion-site specificity placed *Minos* within the *Tc* transposon family (Franz *et al.*, 1994). *Minos* was first used to transform *D. melanogaster* with *Minos*-mediated events demonstrated by sequencing insertion sites and remobilization of integrations (Loukeris *et al.*, 1995a). The first non-drosophilid transformation with *Minos* was achieved in a medfly *white eye* host strain using a cDNA clone for the medfly *white* gene as a marker (Zwiebel *et al.*, 1995), at an approximate frequency of 1–3% per fertile G_0 (Loukeris *et al.*, 1995b). *Minos* transposition was subsequently demonstrated in dipteran and lepidopteran cell lines (Klinakis *et al.*, 2000; Catteruccia *et al.*, 2000a), with germline transformation reported for *Anopheles stephensi* (Catteruccia *et al.*, 2000b) and *D. virilis* (Megna and Cline, personal communication). Recently transformation frequencies have been substantially increased in *Drosophila* and medfly by the use of *in vitro* synthesized transposase mRNA as helper (Kapetanaki *et al.*, 2002).

Although *Minos* has not been widely used for insect transformation, embryonic and cell line mobility assays in several insect species in the Diptera, Lepidoptera, and Orthoptera have indicated a broad range of function. Notably, *Minos* transposition in the cricket *Gryllus bimaculatus* was driven by transposase regulated by a *Gryllus* actin gene promoter, and not by the *Drosophila hsp70* promoter that has been widely used in dipterans (Zhang *et al.*, 2002). The broad function of the *Minos* vector is further supported by its ability to transpose in a mouse germline (Drabek *et al.*, 2003).

Minos structure places it within the *mariner/Tc* transposon superfamily, though knowledge of the distribution of *Minos* is thus far limited to the genus *Drosophila* (Arca and Savakis, 2000). In *Drosophila*, *Minos* is clearly widely distributed in the *Drosophila* and *Sophophora* subgenera, though discontinuously in the *Sophophora*. As noted for the *hAT*, *mariner*, and *piggyBac* elements, *Minos* may have also undergone horizontal transfer between *Drosophila* species.

10.5.5. *Tn5*

Tn5 is one of a number of very well-characterized transposable elements from prokaryotes. Recently, hyperactive forms of this element have been created in the laboratory that have proven to be the basis for the development of a number of commercially useful genomics tools (Goryshin and Reznikoff, 1998; Epicentre, 2004). *Tn5*-based genomics tools can be used in a wide variety of bacterial species and given the system's independence from host-encoded

factors, might be applicable to eukaryotic systems as well (Goryshin *et al.*, 2000). Efforts to use *Tn5* as an insect gene vector have been successful.

Tn5 is a prokaryotic transposon 5.8 kb in length, and it is often referred to as a composite transposon because it consists of five independently functional units (review: Reznikoff, 2000) (Figure 1). It contains three antibiotic resistance genes that are flanked by 1.5 kb inverted repeat sequences. Each inverted repeat is actually a copy of an *IS50* insertion sequence that are themselves functional transposons. Each *IS50* element contains 19 bp terminal sequences known as outside end (OE) and inside end (IE), and while OE and IE are very similar, they are not identical. *IS50* also encodes for two proteins: transposase (Tnp) is 476 amino acids long and catalyzes transposition while the second protein is an inhibitor of transposition (Inh). The *IS50* elements present at each end of *Tn5* are not identical and only *IS50R* is fully functional. *IS50L* contains an ochre codon that prematurely terminates the Tnp and Inh proteins resulting in a loss of function of both proteins.

The transposition reaction and all of the components involved in the reaction have been studied in great detail (Reznikoff *et al.*, 1999). Transposition proceeds by a cut-and-paste process involving binding of Tnp to the end sequences followed by dimerization of the bound Tnp to form a synaptic complex. Cleavage at the ends of the element results in an excised transposon with bound transposase that interacts with a target DNA molecule. Strand transfer results in the integration of the element into the target, and *in vitro*, this reaction requires only a donor element, a target DNA molecule, transposase, and Mg^{2+} (Goryshin and Reznikoff, 1998). Modifications of both the transposase and the terminal 19 bp sequences have led to the creation of *Tn5* elements consisting of little more than two copies of end sequences that can be mobilized a 1000-fold more efficiently than an unmodified *Tn5* element. This hyperactive *Tn5* system has been developed into a powerful tool for genetic analysis of a variety of organisms. *Tn5* has been attractive as a broad host range genomics tool because its pattern of integration is random and its biochemical requirements very simple. *Tn5* has been shown to function in a variety of bacterial and nonbacterial systems.

Current insect transformation protocols consist of microinjecting a mixture of two plasmids into preblastoderm embryos (see Section 10.6.3). One plasmid contains a nonautonomous transposable element with the transgenes and genetic markers of interest while the second plasmid contains a copy of the transposase gene. Transient expression of the

transposase gene is required postinjection and is followed by element excision and integration. Previous experiments examining the frequency of element excision of elements, such as *Hermes*, *mariner*, *Minos*, and *piggyBac*, from plasmids injected into insect embryos along with helper plasmids indicated that only one plasmid per thousand injected underwent an excision event. Therefore, 99.9% of the donor plasmids introduced into insect embryos will contribute nothing to the transformation efforts. The introduction of preexcised elements configured as active intermediates, such as synaptic complexes, was considered a means to permit higher integration rates and overall efficiency of transformation.

Transgenic *Aedes aegypti* were created using a *Tn5* vector containing DsRed under the regulatory control of the 3xP3 promoter (Rowan *et al.*, 2004). Preexcised vectors in the form of synaptic complexes were injected into preblastoderm embryos. Nine hundred adults were obtained from the injected embryos and families consisting of approximately 10 G₀ individuals were established. Two families of G₀ individuals produced transgenic progeny for an estimated transformation frequency of 0.22% (2/900). Analysis of the transgenic progeny showed that multiple integrations of *Tn5* occurred in each line. The patterns of integrations were complex with evidence of the *Tn5* vector integrating into *Tn5* vector sequences. The integration of the vector into copies of itself followed by the integration of the resulting concatamers was very unusual, and in no case was a simple cut-and-paste integration of the *Tn5* vector found with characteristic 9 bp direct duplications flanking the element. The complex pattern of *Tn5* integration was thought to be a direct consequence of injecting preassembled intermediates, that were inactive in the absence of Mg²⁺. Therefore, as soon as the synaptic complexes were injected they became activated and the first target sequences the elements were likely to encounter were other *Tn5* synaptic complexes. At the time of injection, *A. aegypti* embryos only contain approximately four to eight nuclei making genomic target DNA relatively rare. Furthermore, the synaptic complexes injected were expected to have a very short half-life. Therefore, although active intermediates were being introduced, a number of factors contributed to the inefficiency observed with this system including a short half-life of the active intermediate and low numbers of genomic target sequences. Injecting binary plasmid systems (as is done with *Hermes*, *mariner*, *Minos*, and *piggyBac*), while relatively inefficient in producing active transposition intermediates, achieves persistence over an extended period of time. Consequently,

more target genomes are exposed to active vectors over a longer period of time, resulting in higher transformation rates. The limitations of injecting synaptic complexes is unlikely to be specific to the *Tn5* system and similar approaches with other insect gene vectors are likely to encounter similar problems. It should be noted, however, that the results of Rowan *et al.* (2004) demonstrate that *Tn5* is functional in insects and, while injecting active intermediates is not recommended, using *Tn5* in a more conventional binary plasmid system consisting of a donor and helper plasmids is likely to be a viable option for creating transgenic insects.

10.6. Transformation Methodology

The technical methodology for insect transformation has largely remained the same or only slightly modified from the techniques originally used to transform *Drosophila*. The references cited for *P* transformation are relevant to this, as well as several recent articles that focus on methods for non-drosophilid transformation (Handler and O'Brochta, 1991; Morris, 1997; Ashburner *et al.*, 1998; Handler, 2000; Handler and James, 2000). The most variable aspect of this method is the preparation of embryos for DNA microinjection, though arguably, the lack of new techniques for DNA introduction has been the primary limitation in the more widespread use of the technology. While all successful insect transformations have utilized microinjection, variations on this method have been necessary for different types of embryos, and most of the procedures must be tested empirically and modified for particular insect species. This may be extended to different strains and for a variety of local ambient conditions including temperature and humidity. The apparatus for microinjection is usually the same for all species, though a wide variety of variations and modifications are possible and sometimes required. The basic equipment includes an inverted microscope or a stereozoom microscope with a mechanical stage having a magnification up to 60 to 80×; a micromanipulator that is adjustable in three axes with an appropriate needle holder; and a means to transmit the DNA into the egg. For dechorionated eggs, transmitted light allows precise positioning of the needle within the egg posterior, while direct illumination is needed for nondechorionated eggs that typically include mosquitoes and moths.

The standard for gene transfer methodology in general, and embryo microinjection in particular, was originally developed for *Drosophila*. The standard method involves collecting preblastoderm embryos

within 30 min of oviposition, and dechorionating them either manually or chemically. The timing of egg collection and DNA injection is related to the need to inject into preblastoderm embryos during a phase of nuclear divisions previous to cellularization. This allows the injected DNA to be taken up into the nuclei, and specifically into the primordial germ cell nuclei that are the gamete progenitors. For *Drosophila*, cellularization of the pole cells begins at approximately 90 min after fertilization at 25 °C, with blastoderm formation occurring about 30 min afterwards. The timing of these events and location of the pole cells varies among insects, and thus some knowledge of early embryogenesis in the desired host insect is highly advantageous. In the absence of this information for a particular species, the most prudent time of injection would be the earliest time after oviposition that does not compromise viability.

10.6.1. Embryo Preparation

Manual dechorionation of *Drosophila* eggs is achieved by gently rolling the eggs on double-stick tape with a forcep until the chorions peel off. While gentle on the eggs and requiring little desiccation time, manual dechorionation is tedious and has not been applicable to any other insect. Chemical dechorionation is typically achieved by soaking eggs in a 50% bleach solution (2.5% hypochlorite) for 2–4 min and washing at least three times in 0.02% Triton X-100. Tephritid fruit fly eggs usually have thinner chorions that can be dechorionated in 30% bleach (1.25% hypochlorite) in 2–3 min, but this must be determined empirically since they are easily overbleached resulting in death, either directly or after injection. Some species, such as *M. domestica*, can be only partially dechorionated, but bleached eggs can be released from the chorion by agitation. We have found the simplest and most precise method for bleach dechorionation with rapid washes is by using a 42 mm Buchner funnel with a filter flask attached to a water vacuum. Eggs can be washed into the funnel on filter paper and swirled within the funnel with the solution gently sucked out by regulating the water flow or the seal between the funnel and flask. The last wash is done on black filter paper that allows the eggs to be easily detected, which facilitates their mounting for injection (see below).

Many insects eggs cannot be dechorionated without a high level of lethality, and must be injected without dechorionation. These include most moth and mosquito species. *Drosophila* and tephritid flies can, similarly, be injected without dechorionation, and while embryo viability after injection is often lower than for dechorionated eggs, the frequency of

transformation in surviving embryos is often higher. It is more difficult to determine a precise site for injection in nondechorionated eggs, though this can be aided by adding food coloring to the DNA injection mix.

After dechorionation, fruit fly embryos are typically placed on a thin strip (~1 mm) of double stick tape placed on a microscope slide or 22 × 30 mm cover slip, though use of a cover slip is more adaptable for subsequent operations. A thin strip of tape is suggested due to anecdotal reports of toxic solvents from the tape affecting survival, though some particular tapes are considered to be nontoxic (3M Double Coated Tape 415; 3M, St. Paul, MN, USA) and some are useful for particular applications such as aqueous conditions needed for mosquito eggs. Adhesives resistant to moisture include Toupee tape (TopStick™, Vapon Inc.) and Tegaderm (3M). When eggs are injected under oil, the tape strip is placed within a thick rectangle created with a wax pencil that can retain the oil. It is important that the wax fence not be breached by oil when overlaying the eggs, since the loss of oil will result in embryo death.

Where possible, eggs are placed on the tape in an orientation having their posterior ends facing outwards towards the needle, but at a slight angle. All fruit fly eggs must be desiccated to some extent before injection. The interior of the egg is normally under positive pressure, and yolk and injected DNA will invariably flow out after injection without desiccation. This will result in lethality, sterility (from loss of pole plasm), or the lack of transformation if the plasmid DNA is lost. The time and type of desiccation, however, must be evaluated empirically, and sometimes varied during the course of an injection period. A major factor for dechorionated eggs is the length of time they are kept on moist filter paper before being placed on the tape. Typically we desiccate embryos on one strip of tape (15–20 embryos) for 8 to 10 min. Depending on the ease of injection the time can be varied by 1–2 min. In ambient conditions that are humid, it may be necessary to desiccate in a closed chamber with a drying agent (e.g., drierite), with or without a gentle vacuum. An important consideration is that a very short variation in the time for desiccation can be the difference between perfect desiccation and overdesiccation resulting in death, and that the optimal desiccation time will vary for different eggs on the tape. Thus, it is unlikely that all the eggs will respond well to the set conditions, which must be modified so that the majority of eggs can be injected with DNA at a high level of survival and fertility. After the determined time for desiccation, the eggs

must be placed immediately under Halocarbon 700 oil, or oil of similar density, to stop the desiccation process. Desiccation of most nondechorionated eggs is more challenging and one approach is to soak eggs in 1 M NaCl for several minutes. In contrast, for nondechorionated mosquito eggs, desiccation can occur within 1–2 min after removal from water, which is evidenced by slight dimpling of the egg surface, and this must be observed to avoid overdesiccation. Due to the rapidity of desiccation, mosquito eggs are typically arranged on moist filter paper and blotted together onto a taped cover slip from above, and after desiccation, the eggs are submerged in Halocarbon oil. Nondechorionated eggs from many species do not require oil, and it may be lethal for some insects such as moths, yet oil submersion was helpful for the survival of *Drosophila* and tephritid flies.

10.6.2. Needles

The type of needle and its preparation is possibly the most important component of successful embryo injections. Most dechorionated fruit fly eggs can be injected easily with borosilicate needles, which are drawn out to a fine tip and broken off to a 1 to 2 μm opening. Opening the tip is typically achieved by scraping the needle against the edge of the slide carrying the eggs to be injected. Opening the needle by beveling, however, creates consistently sharp tips that are much more important for nondechorionated eggs, and stronger alumina-silicate and quartz needles also provide an improvement to easily pierce chorions or tough vitelline membranes. Beveled needles are also critical when a large tip opening is required for large plasmids that are susceptible to shearing. Preparation of borosilicate needles, pulled from 25 μl capillary stock that has been silanized, can be achieved with several types of vertical or horizontal needle pullers, and we find the Sutter Model P-30 (Sutter Instruments, Novato, CA, USA) vertical micropipette puller to be highly effective. Alumina-silicate needles, and certainly quartz needles, require more sophisticated pullers that allow for fine programmable adjustment of high filament temperatures and pulling force, and the Sutter Models P-97 and P-2000 fulfill this need. Several needle bevelers are available, with the Sutter BV-10 used by many laboratories.

10.6.3. DNA Preparation and Injection

A mixture of highly purified vector and helper plasmid DNA is essential to embryo survival. This is achieved most optimally by purifying plasmid twice through cesium chloride gradients or a solid-phase anion exchange chromatography column.

These have the advantage of high yields of DNA, but the disadvantage of specialized equipment and long preparation times. Successful transformation has been achieved with plasmids prepared with silica-gel membrane kits from Qiagen Corp. (Valencia, CA, USA), but their successful use has been inconsistent, with failures possibly related to the type of host bacteria and its growth conditions. The Qiagen Endotoxin-free plasmid preparation systems allow additional purity, and this system is routinely used for successful plasmid injection.

Purified plasmid concentration must be titered accurately and verified by gel electrophoresis previous to injection mix preparation. Appropriate amounts of vector and helper plasmid are ethanol precipitated, washed several times in 70% ethanol, and resuspended in injection buffer. Injection buffer has typically been the same as that originally used for *Drosophila* (5 mM KCl, 0.1 mM sodium phosphate, pH 6.8), though this may not be optimal for other insects and embryo survival should be assessed by control injections. Total DNA concentration for injection should not exceed 1 mg ml^{-1} , using two- to fourfold higher concentration of vector to helper (e.g., $600 \text{ ng } \mu\text{l}^{-1}$ vector to $200 \text{ ng } \mu\text{l}^{-1}$ helper). Higher DNA concentrations are inadvisable since they are subject to shearing during injection and may clog the needle, and the nucleic acids and/or contaminants can be toxic to the embryo. High transposase levels may also have a negative effect on transposition, as with the overproduction-inhibition phenomenon observed with *mariner* (Lohe and Hartl, 1996b).

Previous to injection the DNA mixture should be filtered through a $0.45 \mu\text{m}$ membrane, or centrifuged before loading into the injection needle. Typically, DNA is back-filled into the injection needle using a drawn-out silanized $100 \mu\text{l}$ microcapillary, and a microliter of DNA should be sufficient for injecting hundreds of eggs.

10.6.3.1. DNA injection The microinjection of DNA into embryos requires a system that forces a minute amount of DNA through the needle in a highly controllable fashion. Remarkably, many *Drosophila* laboratories simply use a mounted syringe and tubing filled with oil connected to a needle holder, with manual pressure applied. This system is successful due to accumulated expertise and the efficiency of transformation in the species, but would probably be less useful for injecting more sensitive embryos that transform less easily. Regulated air-pressure systems are available that are economical and allow highly controlled and rapid DNA injection. We use the PicoPump from WPI that

is most versatile in allowing positive and negative (with vacuum) pressure, and a hold capability that prevents back flow into the needle resulting in clogging (especially by yolk). A less expensive system can be constructed from Clippard components (Clippard Instrument Laboratory, Inc., Cincinnati, OH, USA) that uses a simple air-pressure regulator and electronic valve and switch (see Handler, 2000). Needle holders from WPI can be used with both systems (MPH-3 and MPH-1, respectively).

All embryo injections are performed on a microscope with a mechanical stage, with the injection needle mounted on a micromanipulator. Microscopes first used for *Drosophila* transformation were inverted or compound microscopes, but the availability of a useful mechanical stage and stage adaptor for the Olympus SZ stereozoom microscopes makes this the most versatile choice (the Olympus stage can be mounted on most stereomicroscopes). The micromanipulator can be free-standing next to the stage or mounted on the microscope base. It allows the precise positioning of the needle at the desired point of entry into the egg, while the actual injection occurs by using the mechanical stage to push the egg onto the needle. Piezo Translators that were developed for rapid and automatic intracellular injection may be more efficient for some embryos, and will obviate the need for a mechanical stage (Peloquin *et al.*, 1997). The WPI MPM20 translator used with the PV820 PicoPump allows a fully automated system for egg penetration, DNA injection, and needle withdrawal.

10.6.4. Postinjection Treatment

After injection the cover slip can be placed in a covered petri dish (but not sealed) with moist filter paper. The use of square dishes with black filter paper seems to be most suitable for up to six cover slips and simple observation of the embryos and hatched larvae. For injected embryos submerged in oil, oxygen concentration may be a limiting factor for development, if not viability. This can be ameliorated by reducing the crowding of eggs on the cover slip, or by incubation in a portable hat-box tissue culture chamber that is humidified and under slight positive pressure with oxygen. For eggs without oil, oxygen saturation without pressure is advisable.

Most helper constructs have the transposase gene under heat shock regulation. The *Drosophila hsp70* promoter is a constitutive promoter that is active in the absence of heat shock (but also responds to anoxia which may occur in embryos under oil), and transformation is possible with most vectors

with or without heat shock treatment. If heat shock is desirable, it should be noted that the optimal temperature varies for different species. For example, *hsp70* responds optimally at 37°C in *Drosophila*, but at 39°C in medfly (Papadimitriou *et al.*, 1998). Injected embryos should be incubated for at least 4–6 h after injection before heat shock, or after overnight incubation. Optimal temperatures for insect development vary, but the lowest temperatures possible can be beneficial to survival, and the injection process can slow development by 50% or more. Thus, larval hatching may be delayed considerably and hatching should be monitored for several days after the expected time before discarding embryos.

Hatched larvae can be placed on normal culture media, though they may be weak and require careful handling and soft diet. Rearing of putative transgenic lines is typically achieved by backcrossing to the parental line in small group matings, or individual mating if a determination of transformation frequency is required. Inbreeding of G₀s can minimize rearing efforts, but this may be complicated by high rates of infertility which is typically close to 50% after fruit fly injections.

10.7. Research Needs for Improved Transgenesis

10.7.1. DNA Delivery

Dramatic progress has been made in transformation technology for non-drosophilid insects, and it appears that the vectors and markers in use should be widely applicable. Nevertheless, transformation of many other insect species will be highly challenging, primarily due to limitations in the delivery of DNA into preblastoderm embryos. As noted, to date all successful non-drosophilid transformations have resulted from embryonic microinjection of DNA, but for many species current injection techniques are likely to result in high levels of lethality or sterility. Experimentation with alternative methods has been reported, though arguably, none has been tested exhaustively for germline transformation, or vector systems were used that are now known to be ineffective. The most promising method is biolistics where eggs are bombarded with micropellets encapsulated by DNA, which was first developed as a ballistics method to transform plant cells (Klein *et al.*, 1987). Ballistics is based upon a “shotgun” technique for bombardment, and it is the only non-injection method successfully used to transform an insect. This was a *P* transformation of *Drosophila*, though only a single transformant line was created

and the technique never gained wide applicability (Baldarelli and Lengyel, 1990). This was most likely due to the high efficiency of *P* transformation of *Drosophila* by microinjection, eliminating the need for an alternative technique. Mosquito eggs are considerably more difficult to inject, and a significant effort was made to modify a biolistics approach to DNA delivery in *A. gambiae*, using a burst of pressurized helium for bombardments (Miahle and Miller, 1994). This technique was effective in introducing plasmid DNA into mosquito eggs, yielding high levels of transient expression of a reporter gene. Biolistics was subsequently used for transient expression in specific tissues, allowing the testing of fibroin gene promoters in the *B. mori* silk gland (Horard *et al.*, 1994; Kravariti *et al.*, 2001). Recent advances have included the use of a rigid macro-carrier in the Bio-Rad PDS/1000-Helium biolistics apparatus, which minimizes the blast effect in soft tissue (Thomas *et al.*, 2001). This allows greater micropellet penetration into insect tissues with improved survival. Despite these advances in delivering DNA into eggs and tissue, biolistics has yet to yield a germline transformant.

The only other method reported for DNA delivery is electroporation, which, like biolistics, has resulted in high levels of transient expression of plasmid-encoded genes in *Drosophila* (Kamdar *et al.*, 1992), as well as in *Helicoverpa zea* and *M. domestica* (Leopold *et al.*, 1996). Though transformation has not been reported, as with biolistics, it is not apparent that this was seriously tested or if functional vectors systems were used (certainly for non-drosophilids). Electroporation techniques have also advanced in recent years, with DNA transferred into many different tissue types from a variety of organisms using new electroporation chamber designs and electric field parameters.

These recent advances with both biolistics and electroporation are highly encouraging that new efforts will have greater chances for success, and they deserve a high priority for testing. Both methods also have the advantage, if successful, of delivering DNA simultaneously to multiple embryos, ranging from hundreds to thousands depending on the species. This would be highly beneficial to all transformation experiments, but especially so for species that transform at low frequencies. These methods could also be used in cellularized embryos after blastoderm formation in insects having embryos that cannot be handled easily or collected in the preblastoderm stage.

Other approaches to DNA delivery can include the incorporation of vector/helper DNA into bacterial or viral carriers, that may be delivered by

maternal injection or feeding. Variations on microinjection that might be required for ovoviviparous insects include maternal injection into ovaries or abdominal hemocoel (Presnail and Hoy, 1994), and the use of liposomes might allow injection into cellularized embryos (Felgner *et al.*, 1987). All of these techniques should be reevaluated with the use of vectors and markers now known to be highly efficient in non-drosophilid systems.

10.7.2. Gene Targeting

The ability to target genes to specific or desired integration sites in the genome would be highly advantageous to the basic and applied uses of transgenic strains. The expression of transgenes in most vector integrations is affected negatively by chromosomal position effects, so target sites known to be devoid of, or insulated from, suppression elements could be utilized for optimal or consistent transgene expression. Target sites positioned in innocuous genomic regions could also eliminate random integrations into genes necessary for viability and fertility, eliminating costs to fitness in host strains. It will also be highly important to gene expression studies to achieve reliable methods for gene replacement or targeted transposition, which is especially important for systems where preexisting null mutations, or gene “knockouts,” do not exist.

Gene targeting can be achieved, generally, in two ways. First is homologous recombination where an endogenous genomic sequence is replaced by recombination with homologous sequences within or surrounding the transgene. This results in targeted transposition which can be used for gene replacement, or for targeting to an innocuous genomic region. This approach has been effective in transforming lower eukaryotes, and plant and vertebrate systems (see Bollag *et al.*, 1989) and has been reported to occur in *Drosophila* (Cherbas and Cherbas, 1997) and mosquito (Eggleston and Zhao, 2000) cell line studies. Homologous recombination can also occur in insects *in vivo*, but this is not routine and thus far, must be facilitated. In *B. mori*, female moths were infected with a modified AcNPV baculovirus that had its polyhedrin gene replaced with fibroin light chain-GFP gene fusion (Yamao *et al.*, 1999). Progeny of the infected moths exhibited stable integration of the gene fusion into the genomic fibroin gene, with resulting GFP expression. This particular method relies on host susceptibility to baculovirus infection, though conceivably other pantropic or species-specific viruses could be used for a wider range of insects.

Homologous recombination was also achieved in *Drosophila* where linearized extrachromosomal

DNA was found to be recombinogenic with homologous sequences in the genome (Rong and Golic, 2000). This was achieved by using the *FRT*-FLP recombination system to create DNA circles, which were linearized at a rare endonuclease recognition site within the *FRT* sequences, resulting in a *yellow* marker gene integrating into its homologous chromosomal site. This system has potential application in any insect species that can be stably transformed, but it requires the integration of three components which can be tedious to achieve and may present fitness costs to the host. Though its use could have major importance to genetic studies in non-drosophilid insects, thus far it is not routinely used in *Drosophila*.

10.7.2.1. Site-specific recombination Site-specific recombination systems such as the *FRT*-FLP system from the 2 μ m circle of yeast (Senecoff *et al.*, 1985) mentioned above, and the bacteriophage *Cre/lox* system (Hoess *et al.*, 1985) can be used for various types of gene targeting and chromosomal manipulation. Both systems function in *Drosophila* in which recombination occurs between specific sequences in the presence of a recombinase enzyme (Golic and Lindquist, 1989; Siegal and Hartl, 1996). For *FRT*, the recombination site consists of two 13 bp inverted repeats separated by an 8 bp spacer that specifically recombines with identical *FRT* sites in the presence of FLP recombinase. Depending upon the orientation of the *FRT* sites, the intervening sequence between them can be inverted or deleted by recombination (Golic and Golic, 1996; Golic *et al.*, 1997). When placed within a vector, such *FRT* rearrangements can allow several types of vector manipulation after genomic integration. Genes or sequences within the vector necessary for the initial transformation or selection, but deleterious to use of the transformed strain, can be deleted or inactivated (Dale and Ow, 1991; see Handler, 2002b). This may include the marker system used for selection (e.g., chemical resistance system) or even a transposase gene used in a single plasmid autonomous vector system. Expression of genes of interest can be similarly manipulated by placing *FRT* sites outside the gene and within an internal noncoding region. Of particular importance to transgene stability would be the rearrangement of vector sequences required for mobility, that typically would include the terminal and subterminal ITRs. If *FRT* site placement between the terminal sequences does not hinder the primary transposon vector integration, then subsequent subterminal ITR deletion could eliminate any secondary vector mobilization. Such mobilization or cross-mobilization would have serious consequences for strain stability and function, as

well as ecological risks due to unintended transmission of the vector into other organisms. Eliminating this possibility would provide a major advancement to the applied use of transgenic insects.

The ultimate use of recombination systems for improved transformant stability and transgene expression would be their development into a second generation of vectors that use an integrated recombination site as a stable chromosomal target. Plasmids having the same recombination site and a marker gene would be used as vectors that integrate by recombination in the presence of recombinase. The expectation is that these systems would be highly stable in eukaryotes, and specific target site loci could be selected that are minimally affected by position effect variegation/suppression. The internal 8 bp spacer sequence within the *FRT* can be varied, but only identical *FRT* sequences will recombine with one another. Thus, multiple independent *FRT* target sites can be incorporated into the same genome. Importantly, manipulations by *FRT* recombination will depend upon a controllable source of FLP recombinase, which can be provided as a separate transgene integration, or exogenously by DNA, RNA, or protein injection.

10.8. Summary

After concerted efforts for more than 30 years to achieve gene transfer in non-drosophilid insects, only in the last decade have these efforts been fruitful. Since 1995 the germline of nearly 20 species in four orders of insects have been transformed, and this number may be only limited by the insects of current experimental and applied interest. Unlike plant and vertebrate animal systems that allow relatively efficient genomic integration of introduced DNA, insect systems have generally relied on vector-mediated integrations, and the only vectors found reliable for germline transformation are those based on transposable elements. Curiously, the two main vector systems developed for routine use in *D. melanogaster*, and originally discovered in that species, *P* and *hobo*, have not been applicable as vectors to any other species. Yet, four other transposons found in non-*melanogaster* or non-drosophilid species are widely functional in insects, and for some, other organisms. Their discovery has been of enormous importance to the wider use of transformation technology, since little progress would have been made if most vector systems were specific to a particular host. Equal in importance to the advancements in vector development, have been concurrent progress in genetic marker discovery and development. This began with the finding

that cloned eye color genes from *Drosophila* could complement existing mutations in other insects, and has continued with the more recent use of several fluorescent protein genes that are widely applicable as markers for transformation and reporters for gene expression.

The advancement of these techniques comes at a fortuitous time when genomics is providing a wealth of genetic information and resources that might be used to create transgenic strains of pest and beneficial insects to control their population size and behavior. As part of these efforts, genetic transformation is also critical to functional genomics studies that will provide information essential to understanding the biological function of genetic material, and relating specific genomic elements to those functions. Techniques such as enhancer traps and transposon tagging, which rely on remobilizable insertional mutagenesis, are only possible with transposon-based vector systems, and other techniques such as RNA interference (RNAi) are greatly facilitated by these systems. Together, routine methods for transposon-mediated germline transformation and genomics analysis should provide the tools for dramatic progress in our understanding and control of insect species.

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References

- Allen, M.L., O'Brochta, D.A., Atkinson, P.W., Levesque, C.S., 2001. Stable, germ-line transformation of *Culex quinquefasciatus* (Diptera: Culicidae). *J. Med. Entomol.* 38, 701–710.
- Allen, M.L., Handler, A.M., Berkebile, D.R., Skoda, S.R., 2004. *piggyBac* transformation of the New World screw-worm, *Cochliomyia hominivorax*, produces multiple distinct mutant strains. *Med. Vet. Entomol.* 18, 1–9.
- Anxolabéhère, D., Kidwell, M., Periquet, G., 1988. Molecular characteristics of diverse populations are consistent with the hypothesis of a recent invasion of *Drosophila melanogaster* by mobile *P* elements. *Mol. Biol. Evol.* 5, 252–269.
- Arca, B., Savakis, C., 2000. Distribution of the transposable element *Minos* in the genus *Drosophila*. *Genetica* 108, 263–267.
- Arca, B., Zabolou, S., Loukeris, T.G., Savakis, C., 1997. Mobilization of a *Minos* transposon in *Drosophila melanogaster* chromosomes and chromatid repair by heteroduplex formation. *Genetics* 145, 267–279.
- Ashburner, M., 1989a. *Drosophila: A Laboratory Handbook*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Ashburner, M., 1989b. *Drosophila: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Ashburner, M., Hoy, M.A., Peloquin, J., 1998. Transformation of arthropods: research needs and long term prospects. *Insect Mol. Biol.* 7, 201–213.
- Atkinson, P.W., Pinkerton, A.C., O'Brochta, D.A., 2001. Genetic transformation systems in insects. *Annu. Rev. Entomol.* 46, 317–346.
- Atkinson, P.W., Warren, W.D., O'Brochta, D.A., 1993. The *hobo* transposable element of *Drosophila* can be cross-mobilized in houseflies and excises like the *Ac* element of maize. *Proc. Natl Acad. Sci. USA* 90, 9693–9697.
- Auge-Gouillou, C., Hamelin, M.-H., Demattei, M.-V., Periquet, G., Bigot, Y., 2001a. The ITR binding domain of the mariner Mos-1 transposase. *Mol. Gen. Genomics* 265, 58–65.
- Auge-Gouillou, C., Hamelin, M.-H., Demattei, M.-V., Periquet, G., Bigot, Y., 2001b. The wild-type conformation of the Mos-1 inverted terminal repeats is suboptimal for transposition in bacteria. *Mol. Gen. Genomics* 265, 51–57.
- Avery, T., Macleod, C.M., McCarty, M., 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. I. Induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. *J. Exp. Med.* 79, 137–158.
- Baldarelli, R.M., Lengyel, J.A., 1990. Transient expression of DNA after ballistic introduction into *Drosophila* embryos. *Nucl. Acids Res.* 18, 5903–5904.
- Bellen, H.J., O'Kane, C.J., Wilson, C., Grossniklaus, U., Pearson, R.K., et al., 1989. *P*-element-mediated enhancer detection: a versatile method to study development in *Drosophila*. *Development* 3, 1288–1300.
- Bender, W., Hudson, A., 2000. *P* element homing in the *Drosophila* bithorax complex. *Development* 127, 3981–3992.
- Benedict, M.Q., Salazar, C.E., Collins, F.H., 1995. A new dominant selectable marker for genetic transformation: *Hsp70-opd*. *Insect Biochem. Mol. Biol.* 25, 1061–1065.
- Berghammer, A.J., Klingler, M., Wimmer, E.A., 1999. A universal marker for transgenic insects. *Nature* 402, 370–371.
- Besansky, N.J., Mukabayire, O., Benedict, M.Q., Rafferty, C.S., Hamm, D.M., et al., 1997. The *Anopheles gambiae* tryptophan oxygenase gene expressed from a baculovirus promoter complements *Drosophila melanogaster vermilion*. *Insect Biochem. Mol. Biol.* 27, 803–805.
- Bigot, Y., Auge-Gouillou, C., Periquet, G., 1996. Computer analyses reveal a *hobo*-like element in the nematode *Caenorhabditis elegans*, which presents a conserved transposase domain common with the *Tc1-mariner* transposon family. *Gene* 174, 265–271.

- Blackman, R.K., Macy, M., Koehler, D., Grimaila, R., Gelbart, W.M., 1989. Identification of a fully functional *hobo* transposable element and its use for germ-line transformation of *Drosophila*. *EMBO J.* 8, 211–217.
- Bollag, R.J., Waldman, A.S., Liskay, R.M., 1989. Homologous recombination in mammalian cells. *Annu. Rev. Genet.* 23, 199–225.
- Brand, A.H., Manoukian, A.S., Perrimon, N., 1994. Ectopic expression in *Drosophila*. *Methods Cell Biol.* 44, 635–654.
- Bryan, G.J., Jacobson, J.W., Hartl, D.L., 1987. Heritable somatic excision of a *Drosophila* transposon. *Science* 235, 1636–1638.
- Bushman, F.D., 1994. Tethering human immunodeficiency virus 1 integrase to a DNA site directs integration to nearby sequences. *Proc. Natl Acad. Sci. USA* 91, 9233–9237.
- Calvi, B.R., Hong, T.J., Findley, S.D., Gelbart, W.M., 1991. Evidence for a common evolutionary origin of inverted repeat transposons in *Drosophila* and plants: *hobo*, *Activator*, and *Tam3*. *Cell* 66, 465–471.
- Campbell, R.E., Tour, O., Palmer, A.E., Steinbach, P.A., Baird, G.S., et al., 2002. A monomeric red fluorescent protein. *Proc. Natl Acad. Sci. USA* 99, 7877–7882.
- Capy, P., Vitalis, R., Langin, T., Higuier, D., Bazin, C., 1996. Relationships between transposable elements based upon the integrase–transposase domains: is there a common ancestor? *J. Mol. Evol.* 42, 359–368.
- Cary, L.C., Goebel, M., Corsaro, H.H., Wang, H.H., Rosen, E., et al., 1989. Transposon mutagenesis of baculoviruses: analysis of *Trichoplusia ni* transposon IFP2 insertions within the FP-Locus of nuclear polyhedrosis viruses. *Virology* 161, 8–17.
- Caspari, E., Nawa, S., 1965. A method to demonstrate transformation in *Ephesia*. *Z. Naturforsch.* 206, 281–284.
- Catteruccia, F., Nolan, T., Blass, C., Muller, H.-M., Crisanti, A., et al., 2000a. Toward *Anopheles* transformation: *Mimos* element activity in anopheline cells and embryos. *Proc. Natl Acad. Sci. USA* 97, 2157–2162.
- Catteruccia, F., Nolan, T., Loukeris, T.G., Blass, C., Savakis, C., et al., 2000b. Stable germline transformation of the malaria mosquito *Anopheles stephensi*. *Nature* 405, 959–962.
- Chalfie, M., Tu, Y., Euskirchen, G., Ward, W., Prasher, D.C., 1994. Green fluorescent protein as a marker for gene expression. *Science* 263, 802–805.
- Cherbas, L., Cherbas, P., 1997. “Parahomologous” gene targeting in *Drosophila* cells: an efficient, homology-dependent pathway of illegitimate recombination near a target site. *Genetics* 145, 349–358.
- Christophides, G.K., Savakis, C., Mintzas, A.C., Komitopoulou, K., 2001. Expression and function of the *Drosophila melanogaster* ADH in male *Ceratitis capitata* adults: a potential strategy for medfly genetic sexing based on gene-transfer technology. *Insect Mol. Biol.* 10, 249–254.
- Chroma, 2004. Chroma Technology Corp., Brattleboro, VT, USA. <http://www.chroma.com>.
- Coates, C.J., Jasinskiene, N., Miyashiro, L., James, A.A., 1998. *Mariner* transposition and transformation of the yellow fever mosquito, *Aedes aegypti*. *Proc. Natl Acad. Sci. USA* 95, 3742–3751.
- Coates, C.J., Johnson, K.N., Perkins, H.D., Howells, A.J., O’Brochta, D.A., et al., 1996. The *hermit* transposable element of the Australian sheep blowfly, *Lucilia cuprina*, belongs to the *hAT* family of transposable elements. *Genetica* 97, 23–31.
- Coates, C.J., Turney, C.L., Frommer, M., O’Brochta, D.A., Atkinson, P.W., 1997. Interplasmid transposition of the *mariner* transposable element in non-drosophilid insects. *Mol. Gen. Genet.* 253, 728–733.
- Cornel, A.J., Benedict, M.Q., Rafferty, C.S., Howells, A.J., Collins, F.H., 1997. Transient expression of the *Drosophila melanogaster cinnabar* gene rescues eye color in the white eye (WE) strain of *Aedes aegypti*. *Insect. Biochem. Mol. Biol.* 27, 993–997.
- Dale, E.C., Ow, D.W., 1991. Gene transfer with subsequent removal of the selection gene from the host genome. *Proc. Natl Acad. Sci. USA* 88, 10558–10562.
- Daniels, S.B., Strausbaugh, L.D., 1986. The distribution of *P*-element sequences in *Drosophila*: the *willistoni* and *saltans* species groups. *J. Mol. Evol.* 23, 138–148.
- Davis, I., Girdham, C.H., O’Farrell, P.H., 1995. A nuclear GFP that marks nuclei in living *Drosophila* embryos; maternal supply overcomes a delay in the appearance of zygotic fluorescence. *Devel. Biol.* 170, 726–729.
- Drabek, D., Zagoraiou, L., deWit, T., Langeveld, A., Roumpaki, C., et al., 2003. Transposition of the *Drosophila hydei Mimos* transposon in the mouse germ line. *Genomics* 81, 108–111.
- Eggleston, P., Zhao, Y., 2000. Targeted transformation of the insect genome. In: Handler, A.M., James, A.A. (Eds.), *Insect Transgenesis: Methods and Applications*. CRC Press, Boca Raton, FL, pp. 29–52.
- Engels, W.R., 1989. *P* elements in *Drosophila melanogaster*. In: Berg, D.E., Howe, M.M. (Eds.), *Mobile DNA*. American Society of Microbiology, Washington, DC, pp. 437–484.
- Engstrom, Y., Schneuwly, S., Gehring, W., 1992. Spatial and temporal expression of an *Antennapedia LacZ* gene construct integrated into the endogenous *Antennapedia* gene of *Drosophila melanogaster*. *Roux’s Arch. Devel. Biol.* 201, 65–80.
- Epicentre, 2004. Epicentre, Madison, WI, USA. <http://www.epicentre.com>.
- Fadool, J.M., Hartl, D.L., Dowling, J.E., 1998. Transposition of the *mariner* element from *Drosophila mauritiana* in zebrafish. *Proc. Natl Acad. Sci. USA* 95, 5182–5186.
- Federoff, N., 1989. Maize transposable elements. In: Berg, D.E., Howe, M.M. (Eds.), *Mobile DNA*. American Society for Microbiology, Washington, DC, pp. 375–411.
- Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., et al., 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl Acad. Sci. USA* 84, 7413–7417.

- ffrench-Constant, R.H., Benedict, M.Q., 2000. Resistance genes as candidates for insect transgenesis. In: Handler, A.M., James, A.A. (Eds.), *Insect Transgenesis: Methods and Applications*. CRC Press, Boca Raton, FL, pp. 109–121.
- ffrench-Constant, R.H., Mortlock, D.P., Shaffer, C.D., MacIntyre, R.J., Roush, R.T., 1991. Molecular cloning and transformation of cyclodiene resistance in *Drosophila*: an invertebrate GABA_A receptor locus. *Proc. Natl Acad. Sci. USA* 88, 7209–7213.
- Finnegan, D.J., 1989. Eucaryotic transposable elements and genome evolution. *Trends Genet.* 5, 103–107.
- Fox, A.S., Duggleby, W.F., Gelbart, W.M., Yoon, S.B., 1970. DNA-induced transformation in *Drosophila*: evidence for transmission without integration. *Proc. Natl Acad. Sci. USA* 67, 1834–1838.
- Fox, A.S., Yoon, S.B., 1966. Specific genetic effects of DNA in *Drosophila melanogaster*. *Genetics* 53, 897–911.
- Fox, A.S., Yoon, S.B., 1970. DNA-induced transformation in *Drosophila*: locus specificity and the establishment of transformed stocks. *Proc. Natl Acad. Sci. USA* 67, 1608–1615.
- Franz, G., Loukeris, T.G., Dialektaki, G., Thompson, C.R.L., Savakis, C., 1994. Mobile *Minos* elements from *Drosophila hydei* encode a two-exon transposase with similarity to the paired DNA-binding domain. *Proc. Natl Acad. Sci. USA* 91, 4746–4750.
- Franz, G., Savakis, C., 1991. *Minos*, a new transposable element from *Drosophila hydei*, is a member of the Tc-1-like family of transposons. *Nucl. Acids Res.* 19, 6646.
- Fraser, M.J., 2000. The TTAA-specific family of transposable elements: identification, functional characterization, and utility for transformation of insects. In: Handler, A.M., James, A.A. (Eds.), *Insect Transgenesis: Methods and Applications*. CRC Press, Boca Raton, FL, pp. 249–268.
- Fraser, M.J., Brusca, J.S., Smith, G.E., Summers, M.D., 1985. Transposon-mediated mutagenesis of a baculovirus. *Virology* 145, 356–361.
- Fraser, M.J., Cary, L., Boonvisudhi, K., Wang, H.G., 1995. Assay for movement of lepidopteran transposon IFP2 in insect cells using a baculovirus genome as a target DNA. *Virology* 211, 397–407.
- Fraser, M.J., Smith, G.E., Summers, M.D., 1983. Acquisition of host-cell DNA sequences by baculoviruses: relationship between host DNA insertions and FP mutants of *Autographa californica* and *Galleria mellonella* nuclear polyhedrosis viruses. *J. Virol.* 47, 287–300.
- Furlong, E.E., Profit, D., Scott, M.P., 2001. Automated sorting of live transgenic embryos. *Nature Biotechnol.* 19, 153–156.
- Garza, D., Medhora, M., Koga, A., Hartl, D.L., 1991. Introduction of the transposable element *mariner* into the germline of *Drosophila melanogaster*. *Genetics* 128, 303–310.
- Germeraad, S., 1976. Genetic transformation in *Drosophila* by microinjection of DNA. *Nature* 262, 229–231.
- Goldberg, D.A., Posakony, J.W., Maniatis, T., 1983. Correct developmental expression of a cloned alcohol dehydrogenase gene transduced into the *Drosophila* germ line. *Cell* 34, 59–73.
- Golic, K.G., Golic, M.M., 1996. Engineering the *Drosophila* genome: chromosome rearrangements by design. *Genetics* 144, 1693–1711.
- Golic, K.G., Lindquist, S.L., 1989. The FLP recombinase of yeast catalyzes site-specific recombination in the *Drosophila* genome. *Cell* 59, 499–509.
- Golic, M.M., Rong, Y.S., Petersen, R.B., Lindquist, S.L., Golic, K.G., 1997. FLP-mediated DNA mobilization to specific target sites in *Drosophila* chromosomes. *Nucl. Acids Res.* 25, 3665–3671.
- Gomez, S.P., Handler, A.M., 1997. A *Drosophila melanogaster* *hobo-white*⁺ vector mediates low frequency gene transfer in *D. virilis* with full interspecific *white*⁺ complementation. *Insect Mol. Biol.* 6, 1–8.
- Gorbunova, V., Levy, A.A., 1997. Circularized *Ac/Ds* transposons: formation, structure and fate. *Genetics* 145, 1161–1169.
- Goryshin, I.Y., Jendrisak, J., Hoffman, L.M., Meis, R., Reznikoff, W.S., 2000. Insertional transposon mutagenesis by electroporation of released *Tn5* transposition complexes. *Nature Biotechnol.* 18, 97–100.
- Goryshin, I.Y., Reznikoff, W.S., 1998. *Tn5* *in vitro* transposition. *J. Biol. Chem.* 273, 7367–7374.
- Green, M.M., 1996. The “Genesis of the White-Eyed Mutant” in *Drosophila melanogaster*: a reappraisal. *Genetics* 142, 329–331.
- Griffith, F., 1928. Significance of pneumococcal types. *J. Hyg., Camb.* 27, 113–159.
- Grossman, G.L., Rafferty, C.S., Clayton, J.R., Stevens, T.K., Mukabayire, O., *et al.*, 2001. Germline transformation of the malaria vector, *Anopheles gambiae*, with the *piggyBac* transposable element. *Insect Mol. Biol.* 10, 597–604.
- Gueiros-Filho, F.J., Beverley, S.M., 1997. Trans-kingdom transposition of the *Drosophila* element *mariner* within the protozoan *Leishmania*. *Science* 276, 1716–1719.
- Guimond, N., Bideshi, D.K., Pinkerton, A.C., Atkinson, P.W., O’Brochta, D.A., 2003. Patterns of *Hermes* transposition in *Drosophila melanogaster*. *Mol. Gen. Genet.* 268, 779–790.
- Hama, C., Ali, Z., Kornberg, T.B., 1990. Region-specific recombination and expression are directed by portions of the *Drosophila engrailed* promoter. *Genes Devel.* 4, 1079–1093.
- Handler, A.M., 2000. An introduction to the history and methodology of insect gene transfer. In: Handler, A.M., James, A.A. (Eds.), *Insect Transgenesis: Methods and Applications*. CRC Press, Boca Raton, FL, pp. 3–26.
- Handler, A.M., 2001. A current perspective on insect gene transfer. *Insect Biochem. Mol. Biol.* 31, 111–128.
- Handler, A.M., 2002a. Use of the *piggyBac* transposon for germ-line transformation of insects. *Insect Biochem. Mol. Biol.* 32, 1211–1220.

- Handler, A.M., 2002b. Prospects for using genetic transformation for improved SIT and new biocontrol methods. *Genetica* 116, 137–149.
- Handler, A.M., 2003. Isolation and analysis of a new *hopper hAT* transposon from the *Bactrocera dorsalis* white eye strain. *Genetica* 118, 17–24.
- Handler, A.M., Gomez, S.P., 1996. The *hobo* transposable element excises and has related elements in tephritid species. *Genetics* 143, 1339–1347.
- Handler, A.M., Gomez, S.P., 1997. A new *hobo*, *Activator*, *Tam3* transposable element, *hopper*, from *Bactrocera dorsalis* is distantly related to *hobo* and *Ac. Gene* 185, 133–135.
- Handler, A.M., Gomez, S.P., O'Brochta, D.A., 1993a. A functional analysis of the *P*-element gene-transfer vector in insects. *Arch. Insect Biochem. Physiol.* 22, 373–384.
- Handler, A.M., Gomez, S.P., O'Brochta, D.A., 1993b. Negative regulation of *P* element excision by the somatic product and terminal sequences of *P* in *Drosophila melanogaster*. *Mol. Gen. Genet.* 237, 145–151.
- Handler, A.M., Harrell, R.A., 1999. Germline transformation of *Drosophila melanogaster* with the *piggyBac* transposon vector. *Insect Mol. Biol.* 8, 449–458.
- Handler, A.M., Harrell, R.A., 2000. Transformation of the Caribbean fruit fly with a *piggyBac* transposon vector marked with polyubiquitin-regulated GFP. *Insect Biochem. Mol. Biol.* 31, 199–205.
- Handler, A.M., Harrell, R.A., 2001. Polyubiquitin-regulated DsRed marker for transgenic insects. *Biotechniques* 31, 820–828.
- Handler, A.M., James, A.A. (Eds.), 2000. *Insect Transgenesis: Methods and Applications*. CRC Press, Boca Raton, FL.
- Handler, A.M., McCombs, S.D., 2000. The *piggyBac* transposon mediates germ-line transformation in the Oriental fruit fly and closely related elements exist in its genome. *Insect Mol. Biol.* 9, 605–612.
- Handler, A.M., McCombs, S.D., Fraser, M.J., Saul, S.H., 1998. The lepidopteran transposon vector, *piggyBac*, mediates germline transformation in the Mediterranean fruitfly. *Proc. Natl Acad. Sci. USA* 95, 7520–7525.
- Handler, A.M., O'Brochta, D.A., 1991. Prospects for gene transformation in insects. *Annu. Rev. Entomol.* 36, 159–183.
- Hartl, D.L., Lohe, A.R., Lozovskaya, E.R., 1997. Modern thoughts on an ancient marinere: function, evolution, regulation. *Annu. Rev. Genet.* 31, 337–358.
- Haymer, D.S., Marsh, J.L., 1986. Germ line and somatic instability of a *white* mutation in *Drosophila mauritiana* due to a transposable element. *Devel. Genet.* 6, 281–291.
- Hazelrigg, T., Levis, R., Rubin, G.M., 1984. Transformation of *white* locus DNA in *Drosophila*: dosage compensation, zeste interaction, and position effects. *Cell* 64, 1083–1092.
- Hazelrigg, T., Liu, N., Hong, Y., Wang, S., 1998. GFP expression in *Drosophila* tissues: time requirements for formation of a fluorescent product. *Devel. Biol.* 199, 245–249.
- Hediger, M., Niessen, M., Wimmer, E.A., Dübendorfer, A., Bopp, D., 2000. Genetic transformation of the housefly *Musca domestica* with the lepidopteran derived transposon *piggyBac*. *Insect Mol. Biol.* 10, 113–119.
- Heinrich, J.C., Li, X., Henry, R.A., Haack, N., Stringfellow, L., et al., 2002. Germ-line transformation of the Australian sheep blowfly *Lucilia cuprina*. *Insect Mol. Biol.* 11, 1–10.
- Higgs, S., Sinkins, D.L., 2000. Green fluorescent protein (GFP) as a marker for transgenic insects. In: Handler, A.M., James, A.A. (Eds.), *Insect Transgenesis: Methods and Applications*. CRC Press, Boca Raton, FL, pp. 93–108.
- Higgs, S., Traul, D., Davis, B.S., Kamrud, K.I., Wilcox, C.L., et al., 1996. Green fluorescent protein expressed in living mosquitoes: without the requirement of transformation. *Biotechniques* 21, 660–664.
- Hoess, R., Wierzbicki, A., Abremski, K., 1985. Formation of small circular DNA molecules via an *in vitro* site-specific recombination system. *Gene* 40, 325–329.
- Horard, B., Mange, A., Pelissier, B., Couble, P., 1994. *Bombyx* gene promoter analysis in transplanted silk gland transformed by particle delivery system. *Insect Mol. Biol.* 3, 261–265.
- Horn, C., Jaunich, B., Wimmer, E.A., 2000. Highly sensitive, fluorescent transformation marker for *Drosophila* transgenesis. *Devel. Genes Evol.* 210, 623–629.
- Horn, C., Schmid, B., Pogoda, F.S., Wimmer, E.A., 2002. Fluorescent transformation markers for insect transgenesis. *Insect Biochem. Mol. Biol.* 32, 1221–1235.
- Horn, C., Wimmer, E.A., 2000. A versatile vector set for animal transgenesis. *Devel. Genes Evol.* 210, 630–637.
- Jacobson, J.W., Medhora, M.M., Hartl, D.L., 1986. Molecular structure of somatically unstable transposable element in *Drosophila*. *Proc. Natl Acad. Sci. USA* 83, 8684–8688.
- Jasinskiene, N., Coates, C.J., Benedict, M.Q., Cornel, A.J., Rafferty, C.S., et al., 1998. Stable transposon mediated transformation of the yellow fever mosquito, *Aedes aegypti*, using the *Hermes* element from the housefly. *Proc. Natl Acad. Sci. USA* 95, 3743–3747.
- Jasinskiene, N., Coates, C.J., James, A.A., 2000. Structure of *Hermes* integrations in the germline of the yellow fever mosquito, *Aedes aegypti*. *Insect Mol. Biol.* 9, 11–18.
- Kamdar, P., Von Allmen, G., Finnerty, V., 1992. Transient expression of DNA in *Drosophila* via electroporation. *Nuc. Acids Res.* 11, 3526.
- Kaminski, J.M., Huber, M.R., Summers, J.B., Ward, M.B., 2002. Design of a nonviral vector for site-selective, efficient integration into the human genome. *FASEB J.* 16, 1242–1247.
- Kapetanaki, M.G., Loukeris, T.G., Livadaras, I., Savakis, C., 2002. High frequencies of *Minos* transposon mobilization are obtained in insects by using *in vitro*

- synthesized mRNA as a source of transposase. *Nucl. Acids Res.* 30, 3333–3340.
- Karess, R.E., 1985. *P* element mediated germ line transformation of *Drosophila*. In: Glover, D.M. (Ed.), DNA Cloning, vol. 2. A Practical Approach. IRL Press, Oxford, pp. 121–141.
- Karess, R.E., Rubin, G.R., 1984. Analysis of *P* transposable element functions in *Drosophila*. *Cell* 38, 135–146.
- Kassis, J.A., Noll, E., VanSickle, E.P., Odenwald, W.F., Perrimon, N., 1992. Altering the insertional specificity of a *Drosophila* transposable element. *Proc. Natl Acad. Sci. USA* 89, 1919–1923.
- Kaufman, P.K., Doll, R.F., Rio, D.C., 1989. *Drosophila P* element transposase recognizes internal *P* element DNA sequences. *Cell* 59, 359–371.
- Kidwell, M.G., Kidwell, J.F., Sved, J.A., 1977. Hybrid dysgenesis in *Drosophila melanogaster*: a syndrome of aberrant traits including mutation, sterility, and male recombination. *Genetics* 86, 813–833.
- Klein, T.M., Wolf, E.D., Wu, R., Sanford, J.C., 1987. High-velocity microprojectiles for delivering nucleic acids into living cells. *Nature* 327, 70–73.
- Klemenz, R., Weber, U., Gehring, W.J., 1987. The white gene as a marker in a new *P*-element vector for gene transfer in *Drosophila*. *Nucl. Acids Res.* 15, 3947–3959.
- Klinakis, A.G., Loukeris, T.G., Pavlopoulos, A., Savakis, C., 2000. Mobility assays confirm the broad host-range activity of the *Minos* transposable element and validate new transformation tools. *Insect Mol. Biol.* 9, 269–276.
- Kokoza, V., Ahmed, A., Wimmer, E.A., Raikhel, A.S., 2001. Efficient transformation of the yellow fever mosquito *Aedes aegypti* using the *piggyBac* transposable element vector pBac[3xP3-EGFP afm]. *Insect Biochem. Mol. Biol.* 31, 1137–1143.
- Kravariti, L., Thomas, J., Sourmeli, S., Rodakis, G.C., Mauchamp, B., et al., 2001. The biolistic method as a tool for testing the differential activity of putative silkworm chorion gene promoters. *Insect Biochem. Mol. Biol.* 31, 473–479.
- Lampe, D.J., Akerley, B.J., Rubin, E.J., Mekalanos, J.J., Robertson, H.M., 1999. Hyperactive transposase mutants of the *Himar1 mariner* transposon. *Proc. Natl Acad. Sci. USA* 96, 11428–11433.
- Lampe, D.J., Churchill, M.E., Robertson, H.M., 1996. A purified *mariner* transposase is sufficient to mediate transposition *in vitro*. *EMBO J.* 15, 5470–5479.
- Lampe, D.J., Grant, T.E., Robertson, H.M., 1998. Factors affecting transposition of the *Himar1 mariner* transposon *in vitro*. *Genetics* 149, 179–187.
- Lampe, D.J., Walden, K.K.O., Sherwood, J.M., Robertson, H.M., 2000. Genetic engineering of insects with *mariner* transposons. In: Handler, A.M., James, A.A. (Eds.), *Insect Transgenesis: Methods and Applications*. CRC Press, Boca Raton, FL, pp. 237–248.
- Lee, H., Simon, J.A., Lis, J.T., 1988. Structure and expression of ubiquitin genes of *Drosophila melanogaster*. *Mol. Cell. Biol.* 8, 4727–4735.
- Leopold, R.A., Hughes, K.J., DeVault, J.D., 1996. Using electroporation and a slot cuvette to deliver plasmid DNA to insect embryos. *Genet. Anal.* 12, 197–200.
- Lerat, E., Brunet, F., Bazin, C., Capy, P., 1999. Is the evolution of transposable elements modular? *Genetica* 107, 15–25.
- Li, X., Heinrich, J.C., Scott, M.J., 2001a. *piggyBac*-mediated transposition in *Drosophila melanogaster*: an evaluation of the use of constitutive promoters to control transposase gene expression. *Insect Mol. Biol.* 10, 447–456.
- Li, X., Lobo, N., Bauser, C.A., Fraser, M.J., Jr., 2001b. The minimum internal and external sequence requirements for transposition of the eukaryotic transformation vector *piggyBac*. *Mol. Genet. Genomics* 266, 190–198.
- Lidholm, D.-A., Gudmundsson, G.H., Boman, H.G., 1991. A highly repetitive, *mariner*-like element in the genome of *Hyalophora cecropia*. *J. Biol. Chem.* 266, 11518–11521.
- Lidholm, D.-A., Lohe, A.R., Hartl, D.L., 1993. The transposable element *mariner* mediates germline transformation in *Drosophila melanogaster*. *Genetics* 134, 859–868.
- Lis, J.T., Simon, J.A., Sutton, C.A., 1983. New heat shock puffs and β -galactosidase activity resulting from transformation of *Drosophila* with an *hsp70-LacZ* hybrid gene. *Cell* 35, 403–410.
- Lobo, N.F., Hua-Van, A., Li, X., Nolen, B.M., Fraser, M.J., Jr., 2002. Germ line transformation of the yellow fever mosquito, *Aedes aegypti*, mediated by transpositional insertion of a *piggyBac* vector. *Insect Mol. Biol.* 11, 133–913.
- Lobo, N., Li, X., Hua-Van, A., Fraser, M.J., Jr., 2001. Mobility of the *piggyBac* transposon in embryos of the vectors of Dengue fever (*Aedes albopictus*) and La Crosse encephalitis (*Ae. triseriatus*). *Mol. Genet. Genomics* 265, 66–71.
- Lohe, A.R., Hartl, D.L., 1996a. Germline transformation of *Drosophila virilis* with the transposable element *mariner*. *Genetics* 143, 365–374.
- Lohe, A.R., Hartl, D.L., 1996b. Autoregulation of *mariner* transposase activity by overproduction and dominant-negative complementation. *Mol. Biol. Evol.* 13, 549–555.
- Lohe, A.R., Hartl, D.L., 2002. Efficient mobilization of *mariner in vivo* requires multiple internal sequences. *Genetics* 160, 519–526.
- Lohe, A.R., Lidholm, D.-A., Hartl, D.L., 1995. Genotypic effects, maternal effects and grandmaternal effects of immobilized derivatives of the transposable element *mariner*. *Genetics* 140, 183–192.
- Lorenzen, M.D., Berghammer, A.J., Brown, S.J., Denell, R.E., Klingler, M., et al., 2003. *piggyBac*-mediated germline transformation in the beetle *Tribolium castaneum*. *Insect Mol. Biol.* 12, 433–440.
- Lorenzen, M.D., Brown, S.J., Denell, R.E., Beeman, R.W., 2002. Cloning and characterization of the *Tribolium castaneum* eye-color genes encoding tryptophan

- oxygenase and kynurenine 3-monooxygenase. *Genetics* 160, 225–234.
- Loukeris, T.G., Arca, B., Livadras, I., Dialektaki, G., Savakis, C., 1995a. Introduction of the transposable element *Minos* into the germ line of *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA* 92, 9485–9489.
- Loukeris, T.G., Livadaras, I., Arca, B., Zabalou, S., Savakis, C., 1995b. Gene transfer into the Medfly, *Ceratitis capitata*, with a *Drosophila hydei* transposable element. *Science* 270, 2002–2005.
- Lozovskaya, E.R., Nurminsky, D.I., Hartl, D.L., Sullivan, D.T., 1996. Germline transformation of *Drosophila virilis* mediated by the transposable element *hobo*. *Genetics* 142, 173–177.
- Lozovsky, E.R., Nurminsky, D., Wimmer, E.A., Hartl, D.L., 2002. Unexpected stability of *mariner* transgenes in *Drosophila*. *Genetics* 160, 527–535.
- Mamoun, C.B., Guzman, I.Y., Beverly, S.M., Goldberg, D.E., 2000. Transposition of the *Drosophila* element *mariner* within the human malaria parasite *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* 110, 405–407.
- Matz, M.V., Fradkov, A.F., Labas, Y.A., Savitsky, A.P., Zarausky, A.G., et al., 1999. Fluorescent proteins from nonbioluminescent *Anthozoa* species. *Nature Biotechnol.* 17, 969–973.
- Matz, M.V., Lukyanov, K.A., Lukyanov, S.A., 2002. Family of the green fluorescent protein: journey to the end of the rainbow. *BioEssays* 24, 953–959.
- McGrane, V., Carlson, J.O., Miller, B.R., Beaty, B.J., 1988. Microinjection of DNA into *Aedes triseriatus* ova and detection of integration. *Am. J. Trop. Med. Hyg.* 39, 502–510.
- Medhora, M.M., MacPeck, A.H., Hartl, D.L., 1988. Excision of the *Drosophila* transposable element *mariner*: identification and characterization of the *Mos* factor. *EMBO J.* 7, 2185–2189.
- Miahle, E., Miller, L.H., 1994. Biolistic techniques for transfection of mosquito embryos (*Anopheles gambiae*). *Biotechniques* 16, 924–931.
- Michel, K., Atkinson, P.W., 2003. Nuclear localization of the *Hermes* transposase depends on basic amino acid residues at the N-terminus of the protein. *J. Cell. Biochem.* 89, 778–790.
- Michel, K.A., O'Brochta, D.A., Atkinson, P.W., 2002. Does the proposed DSE motif form the active center in the *Hermes* transposase? *Gene* 298, 141–146.
- Michel, K., O'Brochta, D.A., Atkinson, P.W., 2003. The C-terminus of the *Hermes* transposase contains a protein multimerization domain. *Insect Biochem. Mol. Biol.* 33, 959–970.
- Michel, K., Stamenova, A., Pinkerton, A.C., Franz, G., Robinson, A.S., et al., 2001. *Hermes*-mediated germline transformation of the Mediterranean fruit fly *Ceratitis capitata*. *Insect Mol. Biol.* 10, 155–162.
- Miller, L.H., Sakai, R.K., Romans, P., Gwadz, R.W., Kantoff, P., et al., 1987. Stable integration and expression of a bacterial gene in the mosquito *Anopheles gambiae*. *Science* 237, 779–781.
- Morris, A.C., 1997. Microinjection of mosquito embryos. In: Crampton, J.M., Beard, C.B., Louis, C. (Eds.), *Molecular Biology of Insect Disease Vectors: A Methods Manual*. Chapman and Hall, London, pp. 423–429.
- Morris, A.C., Eggleston, P., Crampton, J.M., 1989. Genetic transformation of the mosquito *Aedes aegypti* by micro-injection of DNA. *Med. Vet. Entomol.* 3, 1–7.
- Mullins, M.C., Rio, D.C., Rubin, G.M., 1989. Cis-acting DNA sequence requirements for *P*-element transposition. *Cell* 3, 729–738.
- Nawa, S., Sakaguchi, B., Yamada, M.A., Tsujita, M., 1971. Hereditary change in *Bombyx* after treatment with DNA. *Genetics* 67, 221–234.
- Nawa, S., Yamada, S., 1968. Hereditary change in *Ephesia* after treatment with DNA. *Genetics* 58, 573–584.
- Nolan, T., Bower, T.M., Brown, A.E., Crisanti, A., Catteruccia, F., 2002. *piggyBac*-mediated germline transformation of the malaria mosquito *Anopheles stephensi* using the red fluorescent protein dsRED as a selectable marker. *J. Biol. Chem.* 277, 8759–8762.
- O'Brochta, D.A., Atkinson, P.W., Lehane, M.J., 2000. Transformation of *Stomoxys calcitrans* with a *Hermes* gene vector. *Insect Mol. Biol.* 9, 531–538.
- O'Brochta, D.A., Gomez, S.P., Handler, A.M., 1991. *P* element excision in *Drosophila melanogaster* and related drosophilids. *Mol. Gen. Genet.* 225, 387–394.
- O'Brochta, D.A., Handler, A.M., 1988. Mobility of *P* elements in drosophilids and nondrosophilids. *Proc. Natl Acad. Sci. USA* 85, 6052–6056.
- O'Brochta, D.A., Warren, W.D., Saville, K.J., Atkinson, P.W., 1994. Interplasmid transposition of *Drosophila hobo* elements in non-drosophilid insects. *Mol. Gen. Genet.* 244, 9–14.
- O'Brochta, D.A., Warren, W.D., Saville, K.J., Atkinson, P.W., 1996. *Hermes*, a functional non-drosophilid insect gene vector. *Genetics* 142, 907–914.
- O'Hare, K., Rubin, G.M., 1983. Structures of *P* transposable elements and their sites of insertion and excision in the *Drosophila melanogaster* genome. *Cell* 34, 25–35.
- Papadimitriou, E., Kritikou, D., Mavroidis, M., Zacharopoulou, A., Mintzas, A.C., 1998. The heat shock 70 gene family in the Mediterranean fruit fly *Ceratitis capitata*. *Insect Mol. Biol.* 7, 279–290.
- Peloquin, J.J., Thibault, S.T., Schouest, L.P., Jr., Miller, T.A., 1997. Electromechanical microinjection of pink bollworm *Pectinophora gossypiella* embryos increases survival. *Biotechniques* 22, 496–499.
- Peloquin, J.J., Thibault, S.T., Staten, R., Miller, T.A., 2000. Germ-line transformation of pink bollworm (Lepidoptera: Gelechiidae) mediated by the *piggyBac* transposable element. *Insect Mol. Biol.* 9, 323–333.
- Perera, O.P., Harrell, R.A., Handler, A.M., 2002. Germ-line transformation of the South American malaria vector, *Anopheles albimanus*, with a *piggyBac*/EGFP transposon vector is routine and highly efficient. *Insect Mol. Biol.* 11, 291–297.
- Phillips, J.P., Xin, J.H., Kirby, K., Milne, C.P., Krell, P., et al., 1990. Transfer and expression of an

- organophosphate insecticide degrading gene from *Pseudomonas* in *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA* 87, 8155–8159.
- Pinkerton, A.C., Michel, K., O'Brochta, D.A., Atkinson, P.W., 2000. Green fluorescent protein as a genetic marker in transgenic *Aedes aegypti*. *Insect Mol. Biol.* 9, 1–10.
- Pinkerton, A.C., Whyard, S., Mende, H.M., Coates, C.J., O'Brochta, D.A., *et al.*, 1999. The Queensland fruit fly, *Bactrocera tryoni*, contains multiple members of the *hAT* family of transposable elements. *Insect Mol. Biol.* 8, 423–434.
- Pirrotta, V., 1988. Vectors for *P*-mediated transformation in *Drosophila*. In: Rodriguez, R.L., Denhardt, D.T. (Eds.), *Vectors: A Survey of Molecular Cloning Vectors and their Uses*. Butterworth, Oxford, pp. 437–456.
- Pirrotta, V., Steller, H., Bozzetti, M.P., 1985. Multiple upstream regulatory elements control the expression of the *Drosophila white* gene. *EMBO J.* 4, 3501–3508.
- Plautz, J.D., Day, R.N., Dailey, G.M., Welsh, S.B., Hall, J.C., *et al.*, 1996. Green fluorescent protein and its derivatives as versatile markers for gene expression in living *Drosophila melanogaster*, plant and mammalian cells. *Gene* 173, 83–87.
- Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G., Cormier, M.J., 1992. Primary structure of the *Aequorea victoria* green fluorescent protein. *Gene* 111, 229–233.
- Presnail, J.K., Hoy, M.A., 1994. Transmission of injected DNA sequences to multiple eggs of *Metaseiulus occidentalis* and *Amblyseius finlandicus* (Acari: Phytoseiidae) following maternal microinjection. *Exp. Appl. Acarol.* 18, 319–330.
- Reznikoff, W.S., 2000. *Tn5* transposition. In: Craig, N.L., Craige, R., Gellert, M., Lambowitz, A.M. (Eds.), *Mobile DNA II*. American Society for Microbiology, Washington, DC, pp. 403–422.
- Reznikoff, W.S., Bhasin, A., Davies, D.R., Goryshin, I.Y., Mahnke, L.A., *et al.*, 1999. *Tn5*: a molecular window on transposition. *Biochem. Biophys. Res. Commun.* 266, 729–734.
- Rio, D.C., Laski, F.A., Rubin, G.M., 1986. Identification and immunochemical analysis of biologically active *Drosophila P* element transposase. *Cell* 44, 21–32.
- Rio, D.C., Rubin, G.M., 1988. Identification and purification of a *Drosophila* protein that binds to the terminal 31-base-pair inverted repeats of the transposable element *P*. *Proc. Natl Acad. Sci. USA* 85, 8929–8933.
- Robertson, H.M., 1993. The *mariner* transposable element is widespread in insects. *Nature* 362, 241–245.
- Robertson, H.M., 2000. Evolution of DNA transposons in eukaryotes. In: Craig, N.L., Craige, R., Gellert, M., Lambowitz, A.M. (Eds.), *Mobile DNA II*. American Society for Microbiology, Washington, DC, pp. 1093–1110.
- Robertson, H.M., Lampe, D.J., 1995a. Distribution of transposable elements in arthropods. *Annu. Rev. Entomol.* 40, 333–357.
- Robertson, H.M., Lampe, D.J., 1995b. Recent horizontal transfer of a *mariner* element between Diptera and Neuroptera. *Mol. Biol. Evol.* 12, 850–862.
- Robertson, H.M., MacLeod, E.G., 1993. Five major subfamilies of *mariner* transposable elements in insects, including the Mediterranean fruit fly, and related arthropods. *Insect Mol. Biol.* 2, 125–139.
- Robertson, H.M., Zumpano, K.L., 1997. Molecular evolution of an ancient *mariner* transposon, *Hsmar1*, in the human genome. *Gene* 205, 203–217.
- Rong, Y.S., Golic, K.G., 2000. Gene targeting by homologous recombination in *Drosophila*. *Science* 288, 2013–2018.
- Rowan, K., Orsetti, J., Atkinson, P.W., O'Brochta, D.A., 2004. *Tn5* as an insect gene vector. *Insect Biochem. Molec. Biol.* 34, 695–705.
- Rubin, E.J., Akerley, B.J., Novik, V.N., Lampe, D.J., Husson, R.N., *et al.*, 1999. *In vivo* transposition of *mariner*-based elements in enteric bacteria and mycobacteria. *Proc. Natl Acad. Sci. USA* 96, 1645–1650.
- Rubin, G.M., Kidwell, M.G., Bingham, P.M., 1982. The molecular basis of *P-M* hybrid dysgenesis: the nature of induced mutations. *Cell* 29, 987–994.
- Rubin, G.M., Spradling, A.C., 1982. Genetic transformation of *Drosophila* with transposable element vectors. *Science* 218, 348–353.
- Sarkar, A., Coates, C.J., Whyard, S., Willhoeft, U., Atkinson, P.W., *et al.*, 1997a. The *Hermes* element from *Musca domestica* can transpose in four families of cyclorrhaphan flies. *Genetica* 99, 15–29.
- Sarkar, A., Collins, F.C., 2000. Eye color genes for selection of transgenic insects. In: Handler, A.M., James, A.A. (Eds.), *Insect Transgenesis: Methods and Applications*. CRC Press, Boca Raton, FL, pp. 79–91.
- Sarkar, A., Yardley, K., Atkinson, P.W., James, A.A., O'Brochta, D.A., 1997b. Transposition of the *Hermes* element in embryos of the vector mosquito, *Aedes aegypti*. *Insect Biochem. Mol. Biol.* 27, 359–363.
- Saville, K.J., Warren, W.D., Atkinson, P.W., O'Brochta, D.A., 1999. Integration specificity of the *hobo* element of *Drosophila melanogaster* is dependent on sequences flanking the target site. *Genetica* 105, 133–147.
- Scavarda, N.J., Hartl, D.L., 1984. Interspecific DNA transformation in *Drosophila*. *Proc. Natl Acad. Sci. USA* 81, 7515–7519.
- Scholnick, S.B., Morgan, B.A., Hirsh, J., 1983. The cloned Dopa decarboxylase gene is developmentally regulated when reintegrated into the *Drosophila* genome. *Cell* 34, 37–45.
- Senecoff, J.F., Bruckner, R.C., Cox, M.M., 1985. The FLP recombinase of the yeast 2-micron plasmid: characterization of its recombination site. *Proc. Natl Acad. Sci. USA* 82, 7270–7274.
- Sheng, G., Thouvenot, E., Schmucker, D., Wilson, D.S., Desplan, C., 1997. Direct regulation of rhodopsin 1 by *Pax-6/eyeless* in *Drosophila*: evidence for a conserved function in photoreceptors. *Genes Devel.* 11, 1122–1131.

- Sherman, A., Dawson, A., Mather, C., Gilhooley, H., Li, Y., *et al.*, 1998. Transposition of the *Drosophila* element *mariner* into the chicken germ line. *Nature Biotechnol.* 16, 1050–1053.
- Siegal, M.L., Hartl, D.L., 1996. Transgene coplacement and high efficiency site-specific recombination with the *Cre/loxP* system in *Drosophila*. *Genetics* 144, 715–726.
- Simmons, M.J., Haley, K.J., Grimes, C.D., Raymond, J.D., Niemi, J.B., 2002. A *hobo* transgene that encodes the *P*-element transposase in *Drosophila melanogaster*: autoregulation and cytotype control of transposase activity. *Genetics* 161, 195–204.
- Spradling, A.C., 1986. *P* element-mediated transformation. In: Roberts, D.B. (Ed.), *Drosophila: A Practical Approach*. Oxford, IRL Press, pp. 174–197.
- Steller, H., Pirrotta, V., 1985. A transposable *P* vector that confers selectable G418 resistance to *Drosophila* larvae. *EMBO J.* 4, 167–171.
- Steller, H., Pirrotta, V., 1986. *P* transposons controlled by the heat shock promoter. *Mol. Cell Biol.* 6, 1640–1649.
- Sumitani, M., Yamamoto, D.S., Oishi, K., Lee, J.M., Hatakeyama, M., 2003. Germline transformation of the sawfly, *Athalia rosae* (Hymenoptera: Symphyta), mediated by a *piggyBac*-derived vector. *Insect Biochem. Mol. Biol.* 33, 449–458.
- Sundararajan, P., Atkinson, P.W., O'Brochta, D.A., 1999. Transposable element interactions in insects: cross-mobilization of *hobo* and *Hermes*. *Insect Mol. Biol.* 8, 359–368.
- Sundaresan, V., Freeling, M., 1987. An extrachromosomal form of the *Mu* transposon of maize. *Proc. Natl Acad. Sci. USA* 84, 4924–4928.
- Taillebourg, E., Dura, J.M., 1999. A novel mechanism for *P* element homing in *Drosophila*. *Proc. Natl Acad. Sci. USA* 96, 6856–6861.
- Tamura, T., Thibert, T., Royer, C., Kanda, T., Eappen, A., *et al.*, 2000. A *piggyBac* element-derived vector efficiently promotes germ-line transformation in the silkworm *Bombyx mori* L. *Nature Biotechnol.* 18, 81–84.
- Thibault, S.T., Luu, H.T., Vann, N., Miller, T.A., 1999. Precise excision and transposition of *piggyBac* in pink bollworm embryos. *Insect Mol. Biol.* 8, 119–123.
- Thomas, J.L., Bardou, J., Lhoste, S., Mauchamp, B., Chavancy, G., 2001. A helium burst biolistic device adapted to penetrate fragile insect tissues. *J. Insect Sci.* 1, 9.
- Thomas, J.L., Da Rocha, M., Besse, A., Mauchamp, B., Chavancy, G., 2002. 3xP3-EGFP marker facilitates screening for transgenic silkworm *Bombyx mori* L. from the embryonic stage onwards. *Insect Biochem. Mol. Biol.* 32, 247–253.
- Tosi, L.R.O., Beverly, S.M., 2000. *Cis* and *trans* factors affecting *Mos1 mariner* evolution and transposition *in vitro*, and its potential for functional genomics. *Nucl. Acids Res.* 28, 784–790.
- Uhlirva, M., Asahina, M., Riddiford, L.M., Jindra, M., 2002. Heat-inducible transgenic expression in the silkworm *Bombyx mori*. *Devel. Genes Evol.* 212, 145–151.
- Walker, V.K., 1990. Gene transfer in insects. *Adv. Cell Culture* 7, 87–124.
- Wang, H.H., Fraser, M.J., 1993. TTAA serves as the target site for TFP3 lepidopteran transposon insertions in both nuclear polyhedrosis virus and *Trichoplusia ni* genomes. *Insect Mol. Biol.* 1, 1–7.
- Wang, H.H., Fraser, M.J., Cary, L.C., 1989. Transposon mutagenesis of baculoviruses: analysis of TFP3 lepidopteran insertions at the FP locus of nuclear polyhedrosis viruses. *Gene* 81, 97–108.
- Wang, W., Swevers, L., Iatrou, K., 2000. *Mariner* (*Mos1*) transposase and genomic integration of foreign gene sequences in *Bombyx mori* cells. *Insect Mol. Biol.* 9, 145–155.
- Warren, W.D., Atkinson, P.W., O'Brochta, D.A., 1994. The *Hermes* transposable element from the house fly, *Musca domestica*, is a short inverted repeat-type element of the *hobo*, *Ac*, and *Tam3* (*hAT*) element family. *Genet. Res.* 64, 87–97.
- White, L.D., Coates, C.J., Atkinson, P.W., O'Brochta, D.A., 1996. An eye color gene for the detection of transgenic non-drosophilid insects. *Insect Biochem. Mol. Biol.* 26, 641–644.
- Wilson, C., Pearson, R.K., Bellen, H.J., O'Kane, C.J., Grossniklaus, U., *et al.*, 1989. *P*-element-mediated enhancer detection: an efficient method for isolating and characterizing developmentally regulated genes in *Drosophila*. *Genes Devel.* 3, 1301–1313.
- Wilson, R., Orsetti, J., Klocko, A.D., Aluvihare, C., Peckham, E., *et al.*, 2003. Post-integration behavior of a *Mos1* gene vector in *Aedes aegypti*. *Insect Biochem. Mol. Biol.* 33, 853–863.
- Yamao, M., Katayama, N., Nakazawa, H., Yamakawa, M., Hayashi, Y., *et al.*, 1999. Gene targeting in the silkworm by use of a baculovirus. *Genes Devel.* 13, 511–516.
- Zhang, J.K., Pritchett, M.S., Lampe, D.J., Robertson, H.M., Metcalf, W.W., 2000. *In vivo* transposon mutagenesis of the methanogenic archeon *Methanosarcina acetivorans* C2A using a modified version of the insect *mariner*-family transposable element *Himar1*. *Proc. Natl Acad. Sci. USA* 97, 9665–9670.
- Zhang, H., Shinmyo, Y., Hirose, A., Mito, T., Inoue, Y., *et al.*, 2002. Extrachromosomal transposition of the transposable element *Minos* in embryos of the cricket *Gryllus bimaculatus*. *Devel. Growth Differ.* 44, 409–417.
- Zhang, L., Sankar, U., Lampe, D.J., Robertson, H.M., Graham, F.L., 1998. The *Himar1 mariner* transposase cloned in a recombinant adenovirus vector is functional in mammalian cells. *Nucl. Acids Res.* 26, 3687–3693.
- Zwiebel, L.J., Saccone, G., Zacharapoulou, A., Besansky, N.J., Favia, G., *et al.*, 1995. The *white* gene of *Ceratitidis capitata*: a phenotypic marker for germline transformation. *Science* 270, 2005–2008.

11 Chitin Metabolism in Insects

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11.1. Introduction

“Chitin Metabolism in Insects” was the title of one of the chapters in the original edition of *Comprehensive Insect Physiology, Biochemistry, and Pharmacology* series published in 1985 (Kramer *et al.*, 1985). Since that time substantial progress in gaining an understanding of this topic has occurred, primarily through the application of techniques of molecular genetics and biotechnology to assorted studies on insect chitin metabolism. Several other reviews have also been published, which have reported on some of the advances that have taken place (Kramer and Koga, 1986; Cohen, 1987, 2001; Koga *et al.*, 1999; Fukamizo, 2000). Thus, in this chapter we will highlight some of the more important findings since 1985, with an emphasis on results obtained from studies conducted on the two enzymes primarily responsible for chitin synthesis and degradation, namely chitin synthase (CHS) and chitinase (CHI).

11.2. Chitin Structure and Occurrence

Chitin is widely distributed in animals and represents the skeletal polysaccharide of several phyla such as the Arthropoda, Annelida, Mollusca, and Coelenterata. In several groups of fungi, chitin replaces cellulose as the structural polysaccharide. In insects, it is found in the body wall, gut lining, cuticle, salivary glands, trachea, mouth parts, and muscle attachment points.

In the course of evolution, insects have made excellent use of the rigidity and chemical stability of the polymeric chitin to assemble extracellular structures such as the cuticle (exoskeleton) and gut lining (peritrophic membrane (PM)), both of which enable insects to be protected from the environment while allowing growth, mobility, respiration, and communication. Several genes and gene products are involved in chitin metabolism in insects. In general there are two primary extracellular structures in

which chitin deposition occurs. Those are the cuticle and the PM where both synthesis and degradation of chitin take place at different developmental stages.

Chitin is the major polysaccharide present in insects and many other invertebrates and several microbes. Structurally, it is the simplest of the glycosaminoglycans, being a β (1 \rightarrow 4) linked linear homopolymer of *N*-acetylglucosamine (GlcNAc, $(C_8H_{13}O_5N)_{n \gg 1}$). It is usually synthesized as the old endocuticle and PM are resorbed and the digested materials are recycled. Because of the intractable nature of insect sclerotized structures such as cuticle, there was very little quantitative data available about chemical composition until recently when solid-state nuclear magnetic resonance (NMR) was utilized for analyses. The cuticle and PM are composed primarily of a mixture of protein and chitin, with the former usually predominating (Kramer *et al.*, 1995). Chitin contents vary substantially depending on the type of cuticle. For example, in the sclerotized puparial cuticle from the housefly, *Musca domestica*, the chitin content is approximately 45% of the wet weight, whereas in the mineralized puparial cuticle of the face fly, *Musca autumnalis*, the chitin content is only about 19% (Roseland *et al.*, 1985; Kramer *et al.*, 1988). In larval, pupal, and adult cuticles of the tobacco hornworm, *Manduca sexta*, the chitin content is approximately 14%, 25%, and 7%, respectively (Kramer *et al.*, 1995). In newly ecdysed pupal cuticle, there is only about 2% chitin prior to sclerotization, but that amount increases more than 10-fold after sclerotization. When cuticular protein and chitin are mixed, they form a matrix in which the components of lower abundance, such as water, catechols, lipids, and minerals, are interspersed. The PM of the tobacco hornworm is made up primarily of protein (60%) and chitin (40%) (Kramer *et al.*, 1995). Although primarily composed of poly-GlcNAc, chitin also can contain a small percentage of unsubstituted (or *N*-deacetylated) glucosamine (GlcN) residues (Fukamizo *et al.*, 1986). When the epidermal and gut cells synthesize and secrete a particular form of chitin consisting of antiparallel chains, α -chitin, the chains are formed into sheets. As layers are added, the sheets become cross-oriented to one another, which can contribute to the formation of an extremely strong plywood-like material. The origin of proteins in the cuticle is unknown, but some hemolymph proteins are deposited in cuticle. Thus, apparently the epidermal cells do not need to supply all of the component parts of the exoskeleton. The cells lining the gut produce some of the PM-associated proteins and these proteins are referred

to as the peritrophins (Tellam *et al.*, 1999; Wang and Granados, 2000a; Bolognesi *et al.*, 2001; Eisemann *et al.*, 2001). Analysis of expressed sequence tags in the cat flea, *Ctenocephalides felis*, demonstrated that some peritrophins are produced exclusively by hindgut and Malpighian tubule tissues (Gaines *et al.*, 2002).

The last step in cuticle formation, tanning, involves modification of the free amino acid tyrosine that is sequestered as a conjugate with glucose in the hemolymph. Tyrosine is first hydroxylated to 3,4-dihydroxyphenylalanine (DOPA), and then decarboxylated to 3,4-dihydroxyphenethylamine (dopamine) (Hopkins and Kramer, 1992). Dopamine is *N*-acylated with acetate or β -alanine in the epidermal cells and sequestered in the hemolymph as conjugates with glucose, sulfate, or another hydrophilic compound. The *N*-acylated dopamine conjugates then are delivered through pore canals to the epicuticle where the conjugates are hydrolyzed and then converted by phenoloxidases to very highly reactive quinones and quinone methides. These transient compounds then cross-link proteins to form tanned proteins in a process known as sclerotization. These cross-linked proteins and chitin make up most of the exocuticle. Chitin chains also may become cross-linked with cuticular proteins, but the evidence for that is not definitive.

Chitin oligosaccharides that are produced during degradation of chitin by chitinases appear to play an important role in insect immunity towards microorganisms. The basic immune strategy against microbial infection in insects appears to be similar to the strategy used by plants against fungal infection. These oligosaccharides are known to activate chitinase genes in plants, which are actively involved in the plant defense response against fungal infection (Nichols *et al.*, 1980). In the silkworm, *Bombyx mori*, chitin oligomers trigger expression of three different antibacterial proteins – cecropin, attacin, and lebecin – in the fat body and hemocytes (Furukawa *et al.*, 1999).

11.3. Chitin Synthesis

Relatively little additional biochemical data on the enzymes of the chitin biosynthetic pathway have been generated since the previous review was published (Kramer *et al.*, 1985). The paucity of information concerning the biochemical properties of these enzymes is due to the inability to obtain soluble preparations of CHSs and the instability of the glutamine-fructose-6-phosphate aminotransferase (GFAT), the enzyme that provides

the GlcN precursor of the chitin biosynthetic pathway. However, CHSs have been identified in a variety of organisms, including nematodes, fungi, and insects. Amino acid sequence similarities have been the principal tools used for identifying CHSs, which form a subfamily within a larger group (family GT2) of the glycosyltransferases that catalyze the transfer of a sugar moiety from an activated sugar donor onto saccharide or nonsaccharide acceptors (Coutinho and Henrissat, 1999; Coutinho *et al.*, 2003; CAZY, 2004). During the past 3 years, there has been a sudden increase in research in the area of chitin synthesis. The impetus for this enhanced interest has come predominantly from cloning of genes for the two key enzymes of the pathway, GFAT and CHS, from insects.

CHS has not been an easy enzyme to assay, which has made its study rather difficult. Traditionally, CHS activity was measured by a radioactive assay using [¹⁴C]UDP-GlcNAc as the substrate followed by quantification of insoluble ¹⁴C-labeled chitin after acid precipitation. Recently, however, a high throughput nonradioactive assay has been developed (Lucero *et al.*, 2002). The procedure involves binding of synthesized chitin to a wheat germ agglutinin (WGA)-coated surface followed by detection of the polymer with a horseradish peroxidase–WGA conjugate. This nonradioactive assay should facilitate greater progress in CHS studies in the future.

11.3.1. Precursors of the Chitin Biosynthetic Pathway

Early studies on chitin synthesis using whole insects or isolated tissues demonstrated that in addition to whole animals, a variety of tissues including larval and pupal epidermis, abdomen, integument, gut, imaginal discs, leg regenerates, hypodermis, and oocytes were capable of synthesizing chitin (review: Kramer *et al.*, 1985). An assortment of compounds, including glycogen, glucose, glucosamine, fructose, and GlcNAc could serve as biosynthetic precursors of chitin in these tissues. These early studies also identified several compounds that inhibited the pathway. This list includes substrate analogs such as tunicamycin, polyoxin D, nikkomycin, and uridine diphosphate (UDP), as well as several compounds belonging to the benzoylphenylurea class of insect growth regulators whose exact mode of action has not yet been established. Results of these studies also indicated that ecdysone may influence chitin synthesis either directly or indirectly. However, the details of such a regulation remain unclear.

11.3.2. Sites of Chitin Biosynthesis

The epidermis and the midgut are two major tissues where chitin synthesis occurs in insects. Epidermal cells are responsible for the deposition of new cuticle during each molt and the midgut cells are generally associated with the formation of the PM during feeding. Chitin is associated with other tissues as well, including the foregut, hindgut, trachea, wing hinges, salivary gland, and mouth parts of adults and/or larvae (Wilson and Cryan, 1997). In general, it is assumed that the cells closest to the site where chitin is found are responsible for its biosynthesis. However, this interpretation is complicated by the fact that assembly of chitin microfibrils occurs in the extracellular space and is influenced by the presence or absence of associated proteins. This is particularly true in the gut where some cells around the cardia may be contributing to chitin synthesis and secretion, whereas other cells in different parts of the gut may be responsible for synthesis of PM-associated proteins (Wang and Granados, 2000a). Visible PM may appear at sites remote from the original site of synthesis of either chitin or PM proteins.

11.3.3. Light and Electron Microscopic Studies of Peritrophic Membrane Synthesis

The most detailed picture of chitin synthesis and its association with proteins to form the composite PM has emerged from observations using light microscopy as well as transmission and scanning electron microscopy (SEM) of PM synthesis in the three lepidopteran insects, *Ostrinia nubilalis* (European corn borer), *Trichoplusia ni* (cabbage looper), and *M. sexta* (Harper and Hopkins, 1997; Harper *et al.*, 1998; Harper and Granados, 1999; Wang and Granados, 2000a; Hopkins and Harper, 2001). The presence of chitin in nascent PM can be followed by staining with gold-labeled WGA, which binds to GlcNAc residues in chitin and glycoproteins. This method was used to show that chitin-containing fibrous material appears first at the tips of the microvilli of the midgut epithelial cells of *O. nubilalis* just past the stomadeal valves and is rapidly assimilated into a thin PM surrounding the food bolus (Harper and Hopkins, 1997). The PM becomes thicker and multilayered in the middle and posterior regions of the mesenteron. The orthogonal lattice of chitin meshwork is slightly larger than the diameter of the microvilli. SEM and light microscopic studies revealed that the PM delaminates from the tips of the microvilli. This observation suggests that microvilli serve as sites and possibly as templates for the organization of the PM by laying down a matrix of chitin microfibrils onto

which some PM proteins are deposited. A similar pattern of delamination of PM containing both chitin and intestinal mucins was demonstrated in larvae of *T. ni* (Harper and Granados, 1999; Wang and Granados, 2000a).

Incorporating WGA into the diet can interrupt formation of the PM. WGA-fed *O. nubilalis* larvae had an unorganized PM, which was multilayered and thicker than the normal PM (Hopkins and Harper, 2001). WGA was actually associated with the PM as well as with the microvillar surface as revealed by immunostaining with antibodies specific for WGA. Because there was very little WGA within the epithelial cells, the action of WGA appears to be extracellular. Presumably, WGA interferes with the formation of the organized chitin network and/or the association of PM proteins with the chitin network, leading to a reduced protein association with the PM (Harper *et al.*, 1998). There was also extensive disintegration of the microvilli and the appearance of dark inclusion bodies as well as apparent microvillar fragments within the thickened multilayered PM. Insects such as *M. sexta*, which secrete multiple and thickened PMs that are somewhat randomly organized, tolerated WGA better and sequestered large amounts of WGA within the multilayered PM (Hopkins and Harper, 2001).

11.3.4. *In Situ* Hybridization and Immunological Studies

In situ hybridizations with a DNA probe for the catalytic domain of a CHS revealed that high levels of transcripts for this gene are present in apical regions of the columnar cells of the anterior midgut of *M. sexta* larvae (Zimoch and Merzendorfer, 2002). Lesser amounts of CHS transcripts were detected in the posterior midgut. An antibody to the catalytic domain of *M. sexta* CHS also detected the enzyme in midgut brush border membranes at the extreme apical ends of microvilli, suggestive of some special compartment or possibly apical membrane-associated vesicles. Staining was also seen in apical membranes of tracheal and salivary gland cells. Materials reacting with CHS antibody also were detected underneath the epidermal cuticle, even though it could not be specifically assigned to the apical membrane of epidermal cells due to loss of structural integrity of these cells during cryosectioning. These *in situ* hybridization and immunological studies are in agreement with earlier observations about chitin synthesis in *Calpodex ethlius* (larger canna leafroller), which indicated the involvement of specialized structures called plasma

membrane plaques found in apical portions of epidermal cells (Locke and Huie, 1979). Comparable electron microscope (EM) and immunological localization of CHS associated with epidermis during cuticle deposition have not been reported primarily because of technical difficulties with the handling of cuticular samples. In *Drosophila melanogaster* the chitin synthase gene (*kkv*) is expressed predominantly in developmental stages 13-14 in the embryonic ventral and dorsal epidermis, foregut and in the larval tracheal system (see the "Patterns of gene expression in *Drosophila* embryogenesis" at the Berkeley *Drosophila* Genome Project (BDGP)).

11.3.5. Chitin Biosynthetic Pathway

It has been assumed that the pathway of chitin biosynthesis in insects would be similar or identical to the pathway that has been worked out extensively in fungi and other microbes (Figure 1). This appears to be the case except for some minor details (Palli and Retnakaran, 1999). The source of the sugar residues for chitin synthesis can be traced to fat body glycogen, which is acted upon by glycogen phosphorylase. Glucose-1-P produced by this reaction is converted to trehalose, which is released into the hemolymph. Trehalose, the extracellular source of sugar in many insects, is acted upon by a trehalase, which is widely distributed in insect tissues including the epidermis and gut to yield intracellular glucose (Becker *et al.*, 1996). The conversion of glucose to fructose-6-P needed for chitin synthesis involves two glycolytic enzymes present in the cytosol. These enzymes are hexokinase and glucose-6-P isomerase, which convert glucose to fructose-6-P. From the latter, the chitin biosynthetic pathway branches off, with the first enzyme catalyzing this branch being GFAT, which might be thought of as the first committed step in amino sugar biosynthesis. The conversion of fructose-6-P to GlcNAc phosphate involves amination, acetyl transfer, and an isomerization step, which moves the phosphate from C-6 to C-1 (phosphoacetylglucoamine mutase). The conversion of this compound to the nucleotide sugar derivative follows the standard pathway and leads to the formation of a UDP-derivative of GlcNAc, which serves as the substrate for CHS. The entire chitin biosynthetic pathway is outlined in Figure 1.

The involvement of dolichol-linked GlcNAc as a precursor for chitin was proposed quite some time ago (Horst, 1983), but it has received very limited experimental support (Quesada-Allue, 1982). At this point, this possibility remains unproven. Similarly, the requirement for a primer to which the

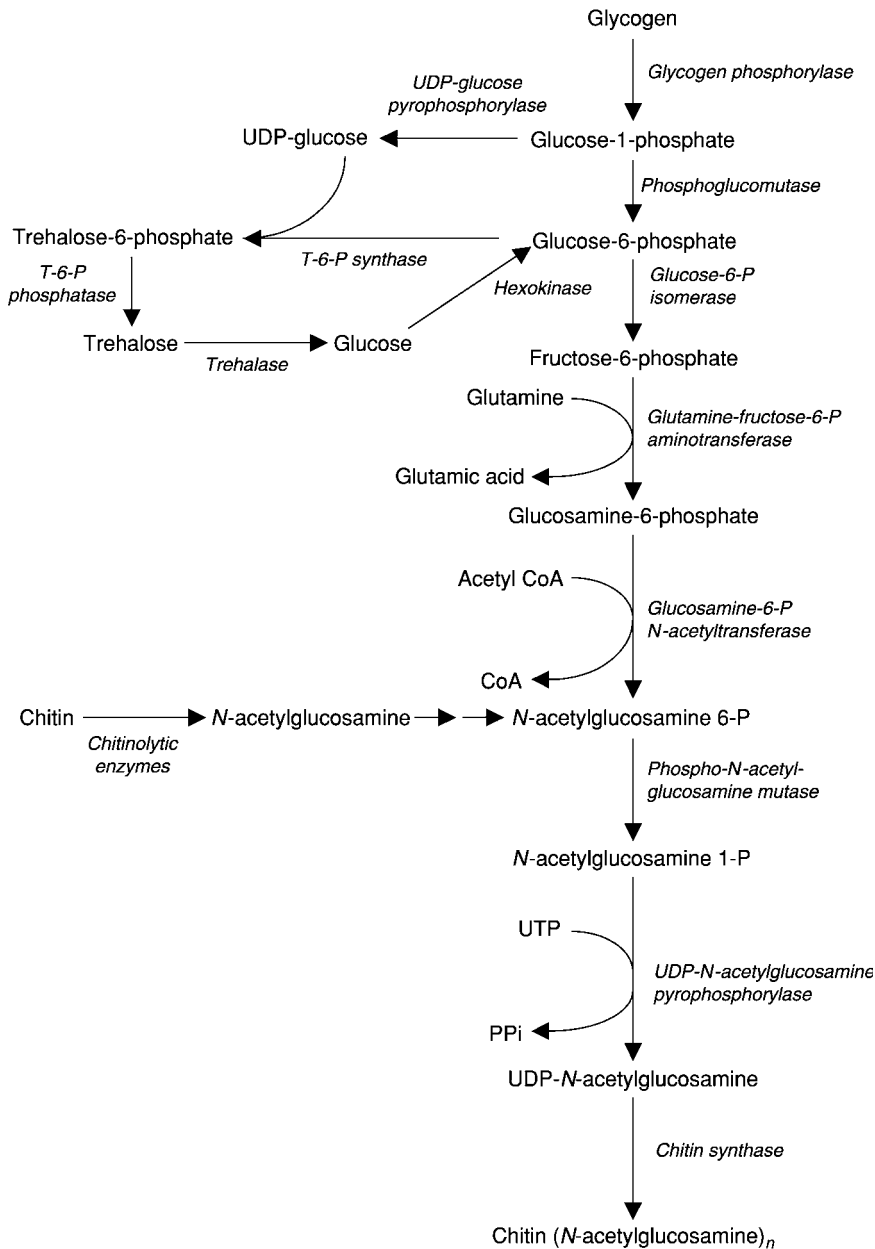


Figure 1 Biosynthetic pathway for chitin in insects starting from glycogen, trehalose, and recycled chitin.

GlcNAc residues can be transferred also remains speculative. Based on the model for glycogen biosynthesis, which requires glycogenin as the primer (Gibbons *et al.*, 2002), CHS or an associated protein may fulfill this priming function. Because each sugar residue in chitin is rotated $\sim 180^\circ$ relative to the preceding sugar, which requires CHS to accommodate a alternating “up/down” configuration, another precursor, UDP-chitobiose, has been proposed to be a disaccharide donor during biosynthesis (Chang *et al.*, 2003). However, evaluation of radiolabeled UDP-chitobiose as a CHS substrate in yeast revealed

that it was not a viable one. Even at elevated concentrations, no incorporation of radioactivity above background was observed using membranous preparations of CHS from the yeast *Saccharomyces cerevisiae* (Chang *et al.*, 2003).

11.3.5.1. Key enzymes The biosynthetic pathway of chitin can be thought of as consisting of two segments. The first set of reactions leads to the formation of the amino sugar, GlcNAc, and the second set of reactions leads to the synthesis of the polymeric chitin from the amino sugar. The

rate-limiting enzyme in the first segment appears to be GFAT (also known as glucosamine-fructose-6-phosphate aminotransferase (GFAT, EC 2.6.1.16), which is found in the cytosol. The critical enzyme in the second segment is CHS (EC 2.4.1.16), which is localized in the plasma membrane. Not surprisingly, these two enzymes appear to be major sites of regulation of chitin synthesis.

11.3.5.2. Regulation of glutamine-fructose-6-phosphate aminotransferase synthesis

11.3.5.2.1. *Drosophila* GFAT Two genes encoding GFAT (*Gfat1* and *Gfat2*) have been identified in *Drosophila* (Adams *et al.*, 2000; Graack *et al.*, 2001). Both of these genes are on chromosome 3, but they are at different locations. Their intron-exon organizations are different as are the amino acid sequences of the encoded proteins. GFAT consists of two separate domains, an N-terminal domain that has both glutamine binding and aminotransferase motifs identified in GFATs from other sources and a C-terminal domain with both fructose-6-phosphate binding and isomerase motifs. *Gfat1* is expressed in embryos in the developing trachea and in cuticle-forming tissues including the chitinous mouth armature of the developing first instar larva. In the last larval stadium, *Gfat1* is expressed in the corpus cells of salivary glands, but this synthesis may be related to the production of the highly glycosylated Sgs glue proteins (Graack *et al.*, 2001). The major regulation of GFAT1 appears to be posttranslational. When *Gfat1* was expressed in yeast cells, the resulting enzyme was feedback inhibited by UDP-GlcNAc and was stimulated by protein kinase A. Even though it has not been demonstrated that there is a phosphorylated form of GFAT1 that is susceptible to feedback inhibition by UDP-GlcNAc, this possibility remains viable. The expression and regulation of the other GFAT isozyme (GFAT2) has not yet been reported.

11.3.5.2.2. *Aedes aegypti* GFAT The gene and cDNA for the mosquito *Aedes aegypti* GFAT1 have been cloned (Kato *et al.*, 2002). The mosquito gene has no introns and the promoter appears to contain sequences related to ecdysteroid response elements (EcRE) as well as E74 and Broad complex Z4 elements. E74 and Broad complex Z4 proteins are transcription factors known to be upregulated by ecdysone (Thummel, 1996). Two *Gfat1* transcripts with different sizes were observed in Northern blot analyses of RNA from adult females and their levels increased further after blood-feeding (Kato *et al.*, 2002). Since ecdysteroid titers increase following blood-feeding, it is possible that this gene

is under the control of ecdysteroid either directly or indirectly. Feedback inhibition by UDP-GlcNAc has not been reported, but the *Aedes* enzyme is likely to be regulated in a manner similar to the *Drosophila* enzyme by this effector and possibly by a phosphorylation/dephosphorylation mechanism as well.

11.3.5.3. CHS gene number and organization

CHS genes from numerous fungi have been isolated and characterized (Munrow and Gow, 2001). However, the complete sequence of a cDNA clone for an insect CHS (sheep blowfly, *Lucilia cuprina*) was reported only recently (Tellam *et al.*, 2000). Since then, the sequences of several other full-length cDNAs and genes for CHSs from other insects and nematodes have been reported. The nematode CHSs were from two filarial pathogens, *Brugia malayi*, and *Dirofilaria immitis*, and the plant parasite *Meloidogyne artiellia* (Harris *et al.*, 2000; Veronico *et al.*, 2001; Harris and Fuhrman, 2002). The other insect species from which CHS cDNAs have been isolated are *A. aegypti* (Ibrahim *et al.*, 2000), *M. sexta* (Zhu *et al.*, 2002) and the red flour beetle, *Tribolium castaneum* (Arakane *et al.*, 2004). DNA sequencing of polymerase chain reaction (PCR)-amplified fragments encoding a highly conserved region in the catalytic domains of insect CHSs indicates a high degree of sequence conservation (Tellam *et al.*, 2000). In addition, a search of the databases in light of the sequence data from these cDNAs has allowed identification of open reading frames (ORFs) from CHS genes from *Drosophila*, *Anopheles*, *Aedes* and the nematode *Caenorhabditis elegans* (Tellam *et al.*, 2000; Gagou *et al.*, 2002; Arakane *et al.*, 2004). **Table 1** lists the properties of insect CHSs encoded by these genes/cDNAs. Insect species typically have two genes for CHSs. Among the nematodes, the *C. elegans* genome contains two CHS genes, but so far there is evidence for only one gene in the plant parasitic nematode *M. artiellia*, and in the filarial nematodes *B. malayi* and *D. immitis* (Harris *et al.*, 2000; Veronico *et al.*, 2001; Harris and Fuhrman, 2002). Fungi, on the other hand, exhibit a wide range in the number of genes for CHS (Munrow and Gow, 2001).

The two *Tribolium* CHS genes, *TcCHS1* and *TcCHS2*, have ten and eight exons, respectively (Arakane *et al.*, 2004). The organizations of the two genes in *Tribolium* are quite different, with some introns occurring in identical positions in both genes, whereas others are at variable positions. The introns ranged in length from 46 bp to more than 3000 bp. The most interesting difference between the two genes was the presence of two

Table 1 Properties of insect chitin synthases and their genes

Species	Number of amino acids	Expressed in	Alt. Exon	Coiled-coil	CHS class	GI no.	Reference
<i>Lucilia cuprina</i>	1592	Epidermis	Yes ^a	Yes	A	9963823	Tellam <i>et al.</i> (2000)
<i>Drosophila melanogaster</i>	1615	Epidermis/gut/tracheal	Yes	Yes	A	24644218	Adams <i>et al.</i> (2000);
	1674		Yes	Yes	A	24644220	Fly base – http://www.flybase.bio.indiana.edu ; Berkeley
	1416	ND	No	No	B	24668460	Drosophila genome project (Drosophila EST database) – http://www.fruitfly.org
<i>Anopheles gambiae</i>	1578		Yes	Yes	A		
	1583		No	No	B		
<i>Aedes aegypti</i>	1564	Midgut	No	No	B	22773456	Ibrahim <i>et al.</i> (2000)
<i>Tribolium castaneum</i>	1558	ND	Yes	Yes	A		
	1558	ND	Yes	Yes	A		Arakane <i>et al.</i> (2004)
	1464	ND	No	No	B		
<i>Manduca sexta</i>	1563	Epidermis/gut	Yes	Yes	A	24762312	Zhu <i>et al.</i> (2002)
	1563	Epidermis/gut	Yes	Yes	A		H. Merzendorfer (unpublished data)
	1524	Gut	No	No	B		D. Hogenkamp <i>et al.</i> (unpublished data)

^aPredicted.

ND, not determined.

nonidentical copies of exon 8 (named 8a and 8b) in *TcCHS1*, whereas *TcCHS2* has only one copy of this region as a part of exon 6. An analysis of genomic sequences from the *D. melanogaster* and *Anopheles gambiae* genome projects, partial sequencing of cDNAs available as separate sequence files submitted to GenBank, and “TBLASTN” queries were used to determine the organization of CHS genes in these insects (Figure 2). These analyses revealed that the sequences and organization of CHS genes of *D. melanogaster* (Tellam *et al.*, 2000) and *A. gambiae* were similar to those of *TcCHS1* and *TcCHS2* (Arakane *et al.*, 2004). One major difference between the two exons that are alternately spliced is that all of the B forms code for segments that have a site for N-linked glycosylation just before the transmembrane helix, whereas none of the A forms do. The physiological significance of alternate exon usage and potential glycosylation in CHS expression is unknown even though it is clear that there is developmental regulation of alternate exon usage (see Section 11.3.5.6).

11.3.5.4. Modular structure of chitin synthases

CHSs are members of family GT2 of the glycosyltransferases (Coutinho *et al.*, 2003), which generally utilize a mechanism where inversion of the anomeric configuration of the sugar donor occurs. The protein fold (termed GT-A) for this family is considered to be two associated $\beta/\alpha/\beta$ domains that form a continuous central sheet of at least eight β -strands.

The GT-A enzymes share a common ribose/metal ion-coordinating motif (termed DxD motif) as well as another carboxylate residue that acts as a catalytic base. The general organization of CHSs has been deduced from a comparison of amino acid sequences of these enzymes from several insects, nematodes and yeasts (Zhu *et al.*, 2002; Arakane *et al.*, 2004). These enzymes have three distinguishable domains: an N-terminal domain with moderate sequence conservation among different species and containing several transmembrane segments, a middle catalytic domain that is highly conserved even among CHSs from different kingdoms, and a C-terminal module with multiple transmembrane segments (Figure 3). The catalytic domain contains several stretches of highly conserved amino acid sequences including the following: CATMWHXT at the beginning of the catalytic domain, FEYAIGHW and VQYDDQGEDRW in the middle of the catalytic domain, and the presumed catalytic site, EFYNQRRRW, at the end of the catalytic domain. While the transmembrane segments in the N-terminal domain show different patterns among different insect species, the transmembrane segments in the C-terminal domain are remarkably conserved both with respect to their location and the spacing between adjacent transmembrane segments. Particularly striking is the fact that five such transmembrane segments are found in a cluster immediately following the catalytic domain and two more segments are located closer to the

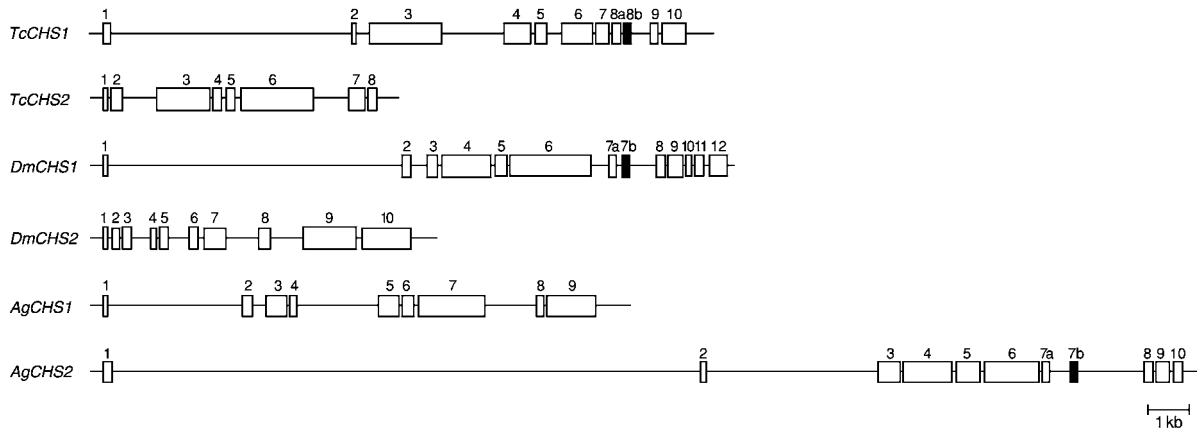


Figure 2 Schematic diagram of the organization of the *TcCHS1*, *TcCHS2*, *DmCHS1*, *DmCHS2*, *AgCHS1*, and *AgCHS2* genes. Boxes indicate exons. Lines indicate introns. The second of the two alternative exons (8b) of *TcCHS1*, *DmCHS1* (7b), and *AgCHS2* (6b) are indicated as closed boxes. About 9 kb of the *TcCHS1* and *TcCHS2* gDNA sequences were compared to their respective cDNA sequences to define the exons and introns. The exon–intron organization of the other four CHS genes was deduced partially from comparisons of available cDNA and genomic sequences. (Reprinted with permission from Arakane, Y., Hogenkamp, D., Zhu, Y.C., Kramer, K.J., Specht, C.A., *et al.*, 2004. Chitin synthase genes of the red flour beetle, *Tribolium castaneum*: characterization, expression, linkage mapping and alternate exon usage. *Insect Biochem. Mol. Biol.* 34, 291–304.)

C-terminus. The 5-transmembrane cluster, known as 5-TMS, has been suggested to be involved in the extrusion of the polymerized chitin chains across the plasma membrane to the exterior of the cell as proposed for extrusion of cellulose (Richmond, 2000).

The CHSs of insects characterized so far can be broadly grouped into two classes, A and B, based on amino acid sequence identities. The class A proteins were predicted to have a coiled-coil region immediately following the 5-TMS region (Zhu *et al.*, 2002; Arakane *et al.*, 2004). Also, all of the genes encoding the class A CHSs have two alternate exons (corresponding to alternate exon 7 of *D. melanogaster*, exon 8 of *T. castaneum*, exon 6 of *A. gambiae*, and an unnumbered exon of *M. sexta* CHS-A gene) (see Table 1). The alternate exons are located on the C-terminal side of the 5-TMS region and encode the next transmembrane segment and flanking sequences. The alternate exon-encoded regions of the CHS proteins differ in sequence by as much as 30% and most of these differences are in the regions flanking the transmembrane segment. This finding suggests that the proteins may differ in their ability to interact with cytosolic or extracellular proteins, which might regulate chitin synthesis and/or

transport. An attractive hypothesis is that these flanking sequences may influence the plasma membrane location of a CHS by interacting with cytoskeletal elements or perhaps by generation of extracellular vesicles involved in chitin assembly.

11.3.5.5. Regulation of chitin synthase gene expression The two insect genes encoding CHSs appear to have different patterns of expression during development. The high degree of sequence identity of the catalytic domains and the absence of antibodies capable of discriminating between the two isoforms have complicated the interpretation of experimental data to some extent. In some cases, the technical difficulties associated with isolation of specific tissues free of other contaminating tissues have precluded unambiguous assignment of tissue specificity of expression. Nonetheless, the following conclusions can be reached from the analyses of expression of CHS genes in several insect species. CHS genes are expressed at all stages of insect growth including embryonic, larval, pupal, and adult stages. *CHS1* genes (coding for class A CHS proteins) are expressed over a wider range of developmental stages (Tellam *et al.*, 2000; Zhu *et al.*,

Figure 3 Alignment of deduced amino acid sequences of *TcCHS1*, *TcCHS2*, *DmCHS1*, *DmCHS2*, *AgCHS1*, and *AgCHS2* using ClustalW software. Transmembrane regions predicted using TMHMM software (v. 2.0) are shaded. Shaded arrowheads indicate the positions in the protein sequences of *TcCHS1* and *TcCHS2* where coding regions are interrupted by introns. Intron 1 of *TcCHS1* lies in the 5'-UTR region two nucleotides 5' of the translation start site and is not indicated in this figure. The putative catalytic domains are boxed. Symbols below the aligned amino acid sequences indicate identical (*), highly conserved (:), and conserved residues (·). The regions in *TcCHS1* and *TcCHS2* corresponding to the PCR probe made from two degenerate primers representing two highly conserved sequences in CHSs are underlined. (Reprinted with permission from Arakane, Y., Hogenkamp, D., Zhu, Y.C., Kramer, K.J., Specht, C.A., *et al.*, 2004. Chitin synthase genes of the red flour beetle, *Tribolium castaneum*: characterization, expression, linkage mapping and alternate exon usage. *Insect Biochem. Mol. Biol.* 34, 291–304.)

2002). *CHS2* genes (coding for class B CHSs) are not expressed in the embryonic or pupal stages but are expressed in the larval stages, especially during feeding in the last instar and in the adults including blood-fed mosquitoes (Ibrahim *et al.*, 2000; Zimoch and Merzendorfer, 2002; Arakane *et al.*, 2004). The finding that both classes of CHS genes are expressed at high levels 3 h after pupariation in *Drosophila* suggests that both enzymes are required for postpupal development (Gagou *et al.*, 2002).

CHS genes also show tissue-specific expression patterns. In *L. cuprina*, *CHS1* (coding for a class A CHS) is expressed only in the carcass (larva minus internal tissues) and trachea but not in salivary gland, crop, cardia, midgut or hindgut (Tellam *et al.*, 2000). In blood-fed female mosquitoes, a CHS gene encoding a class B CHS is expressed in the epithelial cells of the midgut (Ibrahim *et al.*, 2000). In *M. sexta*, *CHS1* (coding for a class A CHS) is expressed in the epidermal cells of larvae and pupae (Zhu *et al.*, 2002). Transcripts specific for class B CHS were detected only in the gut tissue (D. Hogenkamp *et al.*, unpublished data). As discussed above, in *Drosophila*, both classes of CHS genes were shown to be upregulated after the ecdysone pulse had ceased in the last larval instar, but the tissue specificity of expression of each gene was not determined. In *T. castaneum*, the *CHS1* gene (coding for a class A CHS) was expressed in embryos, larvae and pupae, and in young adults, but not in mature adults (Arakane *et al.*, 2004). Even though unequivocal data are not available for each of these insect species, the following generalizations may be made. Class A CHS proteins are synthesized by epidermal cells when cuticle deposition occurs in embryos, larvae, pupae, and young adults, whereas the class B CHS proteins are expressed by the midgut columnar epithelial cells facing the gut lumen in the larval and adult stages and is probably limited to feeding stages.

11.3.5.6. Developmental control of alternate exon usage Insect CHS genes characterized so far have eight or more exons. The genes encoding *Drosophila*, *Anopheles*, *Tribolium*, and *Manduca* class A CHSs, but not the genes encoding class B CHSs, have two alternate exons, each encoding a 59 amino acid long segment following the 5-TMS region (Table 1). This segment contains a 20 amino acid long transmembrane region and flanking sequences. In addition, the presence of a predicted coiled-coil region immediately following the 5-TMS region in the CHSs encoded by those genes that have the alternate exons suggests a link between these two structural features and the possibility of regulation

of alternate exon usage. In agreement with this idea, transcripts containing either one of these exons have been detected in *T. castaneum* and *M. sexta* (Arakane *et al.*, 2004; D. Hogenkamp *et al.*, unpublished data). In *T. castaneum* embryos, transcripts with either exon 8a or 8b were detected, whereas in last instar larvae and prepupae, only exon 8a transcripts were present. By the pupal stage, however, transcripts with exon 8a or exon 8b were abundant along with trace amounts of a transcript with both exons. In mature adults, none of these transcripts was detectable, whereas *TcCHS2* transcripts were easily detected especially in females (Arakane *et al.*, 2004). In *Drosophila*, transcripts containing either exon 7a or both exons 7a and 7b (but not those containing exon 7b alone) have been reported (*Drosophila* EST Database).

It appears that the *TcCHS1* with the exon 8b-encoded segment is needed during cuticle deposition in the pupal and embryonic stages but not at other stages of development. Similar results were observed with fifth instar *M. sexta* larvae (Hogenkamp *et al.*, unpublished data). The biochemical basis for a specific requirement of the *TcCHS1* with the exon 8b-encoded segment is unknown.

11.3.6. Chitin Synthesis during Development

11.3.6.1. Effect of chitin inhibitors Chitin synthesis occurs during embryonic, larval, pupal, and adult stages for cuticle deposition and for production of the PM in larvae and adults. The inhibition of chitin synthesis using chemical inhibitors or by introduction of mutations affects insect development at different developmental stages and to varying degrees. Studies with “chitin inhibitors” have provided some insights concerning the role of chitin in development and its biological function. The use of lufenuron, a member of the class of insecticides known as benzoylphenylureas, has provided substantial information on chitin synthesis during *Drosophila* development (Wilson and Cryan, 1997). The effects of this insect growth regulator were complex and variable depending on the developmental stage and dose at which the insects were exposed to this agent. When newly hatched larvae were reared on a diet containing very low concentrations of lufenuron, the larvae did not die until the second or third instar and usually pupariated even though the pupae were abnormally compressed. Pharate adults either failed to eclose or died shortly after emergence and had deformed legs. The flight ability of the emerged adults was also affected when the larvae were exposed to very low concentrations of lufenuron. First and second instar larvae fed higher concentrations of lufenuron

had normal growth and physical activity for several hours, but the insects died at about the time of the next ecdysis. Third instar larvae fed high concentrations of lufenuron underwent pupariation, but the puparia had an abnormal appearance. The anterior spiracles failed to evert. Thus, insect development is affected by lufenuron at all stages when chitin synthesis occurs. Another aspect of insect development affected by this compound was egg hatching even though oviposition was normal. The embryos completed development but failed to rupture the vitelline membrane. These results indicated that maternally derived lufenuron can affect egg hatching, which requires the use of chitinous mouth parts by the newly ecdysed larvae. The adults showed no mortality and had no flight disability even when fed high levels of lufenuron, indicating that once all chitin-containing structures had been formed, this “chitin inhibitor” had very little effect on adult morphology and function. However, the benzoylphenylureas may not be affecting CHS activity directly because diflubenzuron did not inhibit incorporation of UDP-GlcNAc into chitin microfibrils in an *in vitro* assay using a microsomal preparation from *T. castaneum* (Cohen and Casida, 1980). It is more likely that the benzoylphenylurea class of insecticides interferes with a step in the assembly of the cuticle and/or PM rather than chitin synthesis *per se*.

11.3.6.2. Genetics of chitin synthesis Several *Drosophila* genes involved in controlling cuticle morphology have been characterized (Jurgens *et al.*, 1984; Nusslein-Volhard *et al.*, 1984; Wiechaus *et al.*, 1984; Ostrowski *et al.*, 2002). These genes are *krotzkopf verkehrt* (*kkv*), *knickkopf* (*knk*), *grainy head* (*grh*), *retroactive* (*rtv*), and *zepellin* (*zep*). All of these mutations result in poor cuticle integrity and reversal of embryo orientation in the egg to varying degrees. The homozygous mutant embryos failed to hatch. When these mutant embryos were mechanically devitellinized, the cuticles became grossly enlarged, yielding the “blimp” phenotype. Ostrowski *et al.* (2002) characterized the *kkv* gene and identified it as a CHS-like gene. Interestingly, embryos derived from wild-type females treated with high concentrations of lufenuron displayed a similar “blimp” phenotype when devitellinized, indicating that either genetic or chemical disruption of chitin deposition leads to this phenotype. The *knk* gene codes for a protein with sequence similarity to a protein component of the nuclear spindle matrix and is located on chromosome 3 close to the *kkv* gene near the centromere. The *knk* and *kkv* functions are not additive

and *kkv* appears to be epistatic to *knk*, which is expressed at very low levels compared to the *kkv* gene as indicated by mRNA levels. The *knk* and *zep* genes appear to function in the epidermis prior to cuticle deposition because they exacerbate the effect of a heterozygous *shotgun* (*shg*) mutation, which codes for an E-cadherin-like protein. The *shg* gene is recessive, but in a *knk/knk* or *zep/zep* background, the cuticle is fragmented suggesting that the protein products of these genes interact with cadherin to reinforce the cuticle by promoting adhesion of the epithelia. Thus, products of all of the “blimp” class of genes, including *kkv*, control the integrity of the embryonic cuticle. It is also possible that some of these genes, whose functions have not been identified yet, may be involved directly or indirectly in extrusion or polymerization of chitin microfibrils. Alternatively, these proteins may reinforce chitin–chitin or chitin–protein interactions. For example, the *grh* gene encodes a GATA family transcription factor that regulates the expression of a DOPA decarboxylase needed for the production of precursors of cuticular protein cross-linking agents (Bray and Kafatos, 1991). It is also possible that some of these proteins are involved in vesicular trafficking and/or targeting CHS to plasma membrane plaques that are associated with chitin synthesis (Locke and Huie, 1979).

11.4. Chitin Degradation

Chitinases are among a group of proteins that insects use to digest the structural polysaccharide in their exoskeletons and gut linings during the molting process (Kramer *et al.*, 1985; Kramer and Koga, 1986; Kramer and Muthukrishnan, 1997; Fukamizo, 2000). Chitin is digested in the cuticle and PM to GlcNAc by a binary enzyme system composed of a chitinase (CHI) and a β -N-acetylglucosaminidase (Fukamizo and Kramer, 1985; Filho *et al.*, 2002). The former enzyme from molting fluid hydrolyzes chitin into oligosaccharides, whereas the latter, which is also found in the molting fluid, further degrades the oligomers to the monomer from the nonreducing end. This system also probably operates in the gut during degradation of chitin in the PM or in digestion of chitin-containing prey.

Chitinase (EC 3.2.1.14, endochitinase) is defined as an enzyme that catalyzes the random hydrolysis of N-acetyl- β -D-glucosaminide β -1,4-linkages in chitin and chitodextrins. Chitinases are found in a variety of organisms besides insects including bacteria, fungi, plants, and marine and land animals (Watanabe and Kono, 2002). Many genes encoding chitinolytic enzymes including several from insects

(Table 2) have been cloned and characterized. Some chitinases are now being used for biotechnological applications in agriculture and healthcare (Patil *et al.*, 2000).

Chitinases are members of the superfamily of O-glycoside hydrolases, which hydrolyze the glycosidic bond in polysaccharides or between a sugar and a noncarbohydrate moiety. The International Union for Biochemistry and Molecular Biology enzyme nomenclature of glycoside hydrolases is based on their substrate specificity and occasionally based on their molecular mechanism. Such a classification, however, does not reflect the structural features of these enzymes. Another classification of glycoside hydrolases into families is based on amino acid sequence similarities. This classification is expected to: (1) reflect the structural features of these enzymes better than their sole substrate specificity; (2) help to reveal the evolutionary relationships between these enzymes; and (3) provide a convenient tool to derive mechanistic information (Henrissat and Bairoch, 1996). There are 91 families of glycosylhydrolases and to date all mechanistically characterized insect chitinases belong to family 18 (Coutinho and Henrissat, 1999; CAZY, 2004). Unlike family 19 chitinases that are found almost exclusively in plants, members of family 18 have been found in a wide variety of sources including bacteria, yeasts and other fungi, nematodes, arthropods, and even vertebrates such as mice, chickens, and humans (Nagano *et al.*, 2002). The vertebrate proteins probably function as defensive proteins against chitin-containing pathogenic organisms.

11.4.1. Insect *N*-Acetylglucosaminidases

Beta-*N*-acetylglucosaminidases (EC 3.2.1.30) have been defined as enzymes that release β -*N*-acetylglucosamine residues from the nonreducing end of chitooligosaccharides and from glycoproteins with terminal *N*-acetylglucosamine. Insect β -*N*-acetylglucosaminidases are members of family 20 of the glycosylhydrolases (Coutinho and Henrissat, 1999; CAZY, 2004). These enzymes have been detected in the molting fluid, hemolymph, integument, and gut tissues of several species of insects (Kramer and Koga, 1986 and references therein). A β -*N*-acetylglucosaminidase also has been detected in the gut of *A. aegypti*, where its activity increased dramatically after blood feeding (Filho *et al.*, 2002). Beta-*N*-acetylglucosaminidases also hydrolyze synthetic substrates such as *p*-nitrophenyl *N*-acetylglucosamine and 4-methylumbelliferyl oligo- β -*N*-acetylglucosamines. These two substrates have proven to be very useful in assays of these enzymes.

During development, β -*N*-acetylglucosaminidase activities are the highest in hemolymph a few days prior to larval or pupal ecdysis and in molting fluid from pharate pupae (Kimura, 1976, 1977; Turner *et al.*, 1981). Two different enzymes with different physical and kinetic properties have been purified from the lepidopterans *B. mori* and *M. sexta*. The first enzyme (EI), which is found in larval and pharate pupal molting fluid and in pupal hemolymph, is probably involved in the turnover of chitobiose and possibly chitooligosaccharides because it has a lower K_m for these substrates than does the second (EII) enzyme. EII is found in larval and pupal hemolymph and has a lower K_m for pNpGlcNAc. The role of the enzyme (EII) is unclear, but its natural substrates may be glycoproteins containing terminal *N*-acetylglucosamines. However, this specificity remains to be proven.

11.4.2. Catalytic Mechanism of Insect *N*-Acetylglucosaminidases

N-acetylglucosaminidases have lower K_m values for substrates containing *N*-acetylglucosamine than those with *N*-acetylgalactosamine residues. They release monosaccharides from the nonreducing end by an exocleavage mechanism. Two ionizable groups with pKa values of 3.8 and 8.1 are involved in catalysis (Koga *et al.*, 1982). Studies with competitive inhibitors such as δ -lactone derivatives of *N*-acetylglucosamine and *N*-acetylgalactosamine suggested that the active site of enzyme EI consists of subsites that bind larger substrates than does the active site of the EII enzyme. EI has a lower K_m than EII for the chitooligosaccharides and a larger K_m for pNp β GlcNAc, properties that are consistent with the two enzymes having different endogenous substrate specificities.

11.4.3. Cloning of cDNAs for Insect *N*-Acetylglucosaminidases

cDNAs for epidermal β -*N*-acetylglucosaminidases of *B. mori* (GenBank accession no. S77548), *B. mandarina* (accession no. AAG48701), *T. ni* (accession no. AAL82580), and *M. sexta* (accession no. AY368703) have been isolated and characterized (Nagamatsu *et al.*, 1995; Zen *et al.*, 1996; Goo *et al.*, 1999). A search of the *Drosophila* and *Anopheles* genome databases also revealed the presence of closely related genes encoding β -*N*-acetylglucosaminidases. These genes encode closely related proteins (70-75% amino acid sequence identity between the *Manduca* and *Bombyx* enzymes) of approximately 68 kDa. The conceptual proteins contain leader peptides of 22-23 amino acids followed by stretches of

Table 2 Properties of insect chitinases

<i>Species</i>	<i>Common name</i>	<i>Tissue source</i>	<i>Number of amino acids</i>	<i>Domain structure^a</i>	<i>GI no.</i>	<i>Reference</i>
<i>Aedes aegypti</i>	Yellow fever mosquito	ND	574	Cat-linker-ChBD 3ChBDs-3Cats	2564719	de la Vega <i>et al.</i> (1998)
<i>Anopheles gambiae</i>	Malaria mosquito	Gut	525	Cat-linker-ChBD	2654602	Shen and Jacobs-Lorena (1997)
<i>Bombyx mori</i>	Silkworm	Epidermis/gut	565	Cat-linker-ChBD	1841851, 10119784	Kim <i>et al.</i> (1998), Mikitani <i>et al.</i> (2000), Abdel-Banat and Koga (2001)
<i>Chelonus</i> sp. venom	Wasp	Venom gland	483	Cat-linker-ChBD	1079185	Krishnan <i>et al.</i> (1994)
<i>Chironomus tentans</i>	Midge	Cell line	475	Cat	2113832	Feix <i>et al.</i> (2000)
<i>Choristoneura fumiferana</i>	Spruce budworm	Epidermis/fat body	557	Cat-linker-ChBD	21913148	Zheng <i>et al.</i> (2002)
<i>Drosophila melanogaster</i>	Fruit fly	ND	508	Cat	17647257	de la Vega <i>et al.</i> (1998), Adams <i>et al.</i> (2000)
		ND	484	Cat	24655584	
		ND	458	ChBD-Cat	17647259	
<i>Glossina morsitans</i>	Tsetse fly	Fat body	460	Cat-ChBD	18201665	Yan <i>et al.</i> (2002)
<i>Hyphantria cunea</i>	Fall webworm	Epidermis	553	Cat-linker-ChBD	1841853	Kim <i>et al.</i> (1998)
<i>Lutzomyia longipalpis</i>	Sand fly	Midgut	474	Cat-linker-ChBD	28863959	Ramalho-Ortigão and Traub-Csekö (2003)
<i>Manduca sexta</i>	Tobacco hornworm	Epidermis/gut	554	Cat-linker-ChBD	1079015	Kramer <i>et al.</i> (1993), Choi <i>et al.</i> (1997)
<i>Phaedon cochleariae</i>	Mustard beetle	Gut	405	Cat	4210812	Girard and Jouanin (1999)
<i>Spodoptera litura</i>	Common cutworm	Epidermis	552	Cat-linker-ChBD	9971609	Shinoda <i>et al.</i> (2001)
<i>Tenebrio molitor</i>	Yellow mealworm	ND	2838	5 Cats+5 linkers+4 ChBDs+2 Mucs	21038943	Royer <i>et al.</i> (2002)

^aCat, catalytic domain; linker, linker region; ChBD, chitin-binding domain; Muc, mucin-like domain.

ND, not determined.

mature N-terminal amino acid sequences experimentally determined from *N*-acetylglucosaminidases purified from either the molting fluid or integument of these two species. The amino acid sequences include two regions that are highly conserved among *N*-acetylglucosaminidases from a variety of sources including bacteria, yeast, mouse, and humans (Zen *et al.*, 1996). The *M. sexta* gene was expressed most abundantly in epidermal and gut tissues prior to metamorphosis and was induced by 20-hydroxyecdysone. The inductive effect of molting hormone was suppressed by juvenoids (Zen *et al.*, 1996).

11.4.4. Cloning of Genes Encoding Insect Chitinases

A chitinase from *M. sexta*, which is a 535 amino acid long glycoprotein (Chi535), as well as the cDNA and gene that encode it (*MsCHI*, accession no. AAC04924) were the first insect chitinase and gene to be isolated and characterized (Koga *et al.*, 1983; Kramer *et al.*, 1993; Choi *et al.*, 1997; Kramer and Muthukrishnan, 1997). They represent the most extensively studied chitinase enzyme–gene system in any insect species and they have become a model for study of other insect chitinases and their genes. Since the cloning of the *M. sexta* gene in 1993, cDNAs or genomic clones for several other insect chitinases have been isolated and sequenced (Table 2). The organization of most of these genes is very similar to that of *M. sexta* and most of the proteins display a domain architecture consisting of catalytic, linker, and/or chitin-binding domains similar to *MsCHI*. These genes/enzymes include epidermal chitinases from the silkworm, *B. mori* (Kim *et al.*, 1998; Abdel-Banat and Koga, 2001), the fall webworm, *Hyphantria cunea* (Kim *et al.*, 1998), wasp venom (*Chelonus* sp.) (Krishnan *et al.*, 1994), the common cutworm, *Spodoptera litura* (Shinoda *et al.*, 2001), a molt-associated chitinase from the spruce budworm, *Choristoneura fumiferana* (Zheng *et al.*, 2002), and midgut-associated chitinases from the malaria mosquito, *A. gambiae* (Shen and Jacobs-Lorena, 1997), yellow fever mosquito, *A. aegypti* (de la Vega *et al.*, 1998), the beetle *Phaedon cochleariae* (Girard and Jouanin, 1999), and the sand fly, *Lutzomyia longipalpis* (Ramalho-Ortigão and Traub-Csekö, 2003), and several deduced from the *Drosophila* genome data. A smaller linkerless fatbody-specific chitinase from the tsetse fly, *Glossina morsitans* (Yan *et al.*, 2002) and a very large epidermal chitinase with five copies of the catalytic-linker-chitin binding domain from the yellow mealworm, *Tenebrio molitor* (Royer *et al.*, 2002) have also been described.

Recently, a gene encoding another type of chitinase from the silkworm, *BmChi-h*, has been reported (Daimon *et al.*, 2003). The encoded chitinase shared extensive similarities with microbial and baculoviral chitinases (73% amino acid sequence identity to *Serratia marcescens* chitinase and 63% identity to *Autographa californica* nuclear polyhedrosis virus chitinase). Even though this enzyme had the signature sequence characteristic of family 18 chitinases, it had a rather low percentage of sequence identity with the family of insect chitinases listed in Table 2. It was suggested that an ancestral species of *B. mori* acquired this chitinase gene via horizontal gene transfer from *Serratia* or a baculovirus. Unlike the chitinases listed in Table 2, which typically have a leader peptide, catalytic domain, a serine/threonine(S/T)-rich domain and a C-terminal chitin-binding domain, *BmChi-h* chitinase has a leader peptide, one copy of module w1 domain that is found only in bacterial and baculoviral chitinases (Perrakis *et al.*, 1994; Henrissat, 1999), and a catalytic domain. Apparently, *B. mori* is not alone among insects possessing such a chitinase of bacterial origin. A protein in the molting fluid of *M. sexta*, which cross-reacted with an antibody to *M. sexta* *N*-acetylglucosaminidase, was found to have an N-terminal amino acid sequence closely resembling that of *Serratia* chitinase (Zen *et al.*, 1996). The N-terminal sequence of this protein was identical to that of *BmChi-h* up to the 25th amino acid residue, which strongly suggested that an ortholog of this chitinase gene exists in *M. sexta* as well. It will be interesting to investigate in the future whether this enzyme is widespread and found in other insect species. A search of the *Drosophila* and *Anopheles* genome databases, however, failed to identify any chitinase-like protein with an amino acid sequence identity to *BmChi-h* of greater than 40% (S. Muthukrishnan *et al.*, unpublished data).

Reports of multiple forms of insect chitinases, which can be generated by several mechanisms, have appeared. Some of these proteins are no doubt products of multiple genes as described in the previous paragraph. Others are likely the result of posttranslational modifications that are caused by glycosylation and/or proteolysis, which can lead to larger glycosylated forms and smaller truncated forms (Koga *et al.*, 1983; Wang *et al.*, 1996; Gopalakrishnan *et al.*, 1995; Arakane *et al.*, 2003). Another cause can be alternative splicing of mRNA. In *B. mori*, alternative splicing of the primary transcript from a single chitinase gene generates heterogeneity within the products (Abdel-Banat and Koga, 2002). Larger chitinase-like proteins have been observed in the mosquito *Anopheles* and

it has been proposed that these zymogenic proteins are activated via proteolysis by trypsin (Shen and Jacobs-Lorena, 1997). However, Filho *et al.* (2002) found no evidence for such activation in the mosquito *Aedes* because high levels of chitinase activity were observed early after a blood meal and even in the guts of unfed insects. Putative zymogenic forms have been reported in other insects as well (Koga *et al.*, 1992; Bhatnagar *et al.*, 2003). However, the existence of a chitinase zymogen is still speculative in most cases because all of the fully characterized cDNAs encoding full-length insect chitinases apparently have the mature catalytic domains immediately following their leader peptides and there is no indication of the presence of pre-proproteins (Table 2). Preliminary evidence suggests that most, if not all, of the larger proteins reacting with chitinase antibodies are multimeric forms that are enzymatically inactive and produced as a result of intermolecular disulfide pairing. These larger forms appear after long periods of storage of the monomeric enzyme and they can be reconverted to enzymatically active monomeric forms by treatment with thiol reagents (Y. Arakane *et al.*, unpublished data).

11.4.5. Modular Structure of Insect Chitinases

A multidomain structural organization is generally observed in polysaccharide-degrading enzymes where one or more domains are responsible for hydrolysis and other domains are responsible for associating with the solid polysaccharide substrate. In addition, there usually are linker regions between the two types of domains, which also may be responsible, at least in part, for some functional properties of the enzymes. For example, the first chitinases shown to contain catalytic, linker, and chitin-binding or fibronectin-like domains were isolated from the bacterium *Bacillus circulans* (Watanabe *et al.*, 1990), the yeast *S. cerevisiae* (Kuranda and Robbins, 1991), and the parasitic nematode *B. malayi* (Venegas *et al.*, 1996). Insect chitinases possess a similar structural organization, as do some other nematode, microbial, and plant chitinases as well as fungal cellulases. Observed in all of these enzymes is a multidomain architecture that may include a signal peptide and one or more of the following domains: catalytic domains, cysteine-rich chitin-binding domains, fibronectin-like domains, mucin-like domains, and S/T-rich linker domains, with the latter usually being rather heavily glycosylated (Tellam, 1996; Henrissat, 1999; Suzuki *et al.*, 1999). For example, chitinases from the bacterium *S. marcescens*, fall into three classes with sizes ranging from 36 to 52 kDa, which are

composed of different combinations of catalytic domains, fibronectin type-III-like domains, and N- or C-terminal chitin-binding domains (Suzuki *et al.*, 1999). A novel multidomain structure exhibited by an insect chitinase is that of the yellow mealworm beetle, *T. molitor* (Royer *et al.*, 2002). This protein is unusually large, with a calculated molecular mass of approximately 320 kDa. It contains five catalytic domains, five S/T-rich linker domains, four chitin-binding domains, and two mucin-like domains. Gene duplication and domain deletion mechanisms have probably generated the diversity and multiplicity of chitinase genes in insects, as was demonstrated previously in bacteria (Saito *et al.*, 2003).

The structure of the catalytic domain of insect chitinase is a $(\beta\alpha)_8$ TIM (triose phosphate isomerase) barrel fold, which is one of the most common folds found in proteins (Nagano *et al.*, 2001, 2002). During protein evolution, domain shuffling has allowed this fold to acquire a large number of specific catalytic functions such as enzymes with a glycosidase activity like insect chitinase. The presence of additional domains such as linker and chitin-binding domains appears to further enhance the catalytic properties of these enzymes.

Figure 4 shows a phylogenetic tree of 16 insect chitinases inferred from an amino acid sequence alignment. All five of the lepidopteran enzymes and only one dipteran chitinase reside in the upper portion of the tree, whereas the other seven dipteran, one hymenopteran, and two coleopteran enzymes appear in the lower part.

Manduca sexta CHI is much smaller than the *Tenebrio* enzyme and much less complex in domain structure with only a single N-terminal catalytic domain (376 amino acids long), a linker domain (about 100 amino acids long), and a C-terminal chitin-binding domain (ChBD, 58 amino acids long) (Arakane *et al.*, 2003). Alternate domain arrangements occur in other glycosylhydrolases. For example, class I, class IV, and class VII plant chitinases contain an N-terminal ChBD and a G/P-rich linker preceding the catalytic domain (Raikhel *et al.*, 1993; Neuhaus, 1999), whereas fungal cellulases, like insect chitinase, possess a threonine/serine/proline-rich linker between the N-terminal catalytic domain and the C-terminal cellulose-binding domain (Srisodsuk *et al.*, 1993). The *Manduca* CHI linker region that is rich in T and S residues is also rich in P, D, and E residues, which qualifies it as a PEST sequence-containing protein according to Rogers *et al.* (1986). That composition suggested that insect chitinase might be rapidly degraded via the intracellular ubiquitin-conjugating enzymes/proteasome system, which recognizes the PEST

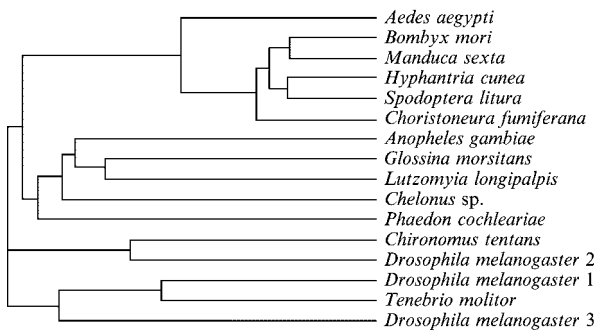


Figure 4 Phylogenetic tree of insect chitinases inferred from an amino acid sequence alignment of 16 enzymes from *Aedes aegypti*, *Bombyx mori*, *Manduca sexta*, *Hyphantria cunea*, *Spodoptera litura*, *Choristoneura fumiferana*, *Anopheles gambiae*, *Glossina morsitans*, *Lutzomyia longipalpis*, *Chelonus sp.*, *Phaeton cochleariae*, *Chironomus tentans*, *Drosophila melanogaster 2*, *Drosophila melanogaster 1*, *Tenebrio molitor*, and *Drosophila melanogaster 3*. The GI numbers are listed in **Table 2**. Multiple sequence alignment was performed using Clustal W software (Thompson *et al.*, 1994) and the tree was built using the neighbor-joining method (Saitou and Nei, 1987).

sequence so that proteosomes can digest the conjugated protein when it is localized intracellularly. However, since insect chitinase is a secreted protein, it would be exposed to intracellular proteases or the ubiquitin-conjugating system only for a relatively short period of time. Instead, the linker apparently helps to optimize interactions with the insoluble substrates and to stabilize proteins, and perhaps also helps to protect protease-susceptible bonds in the catalytic domains from hydrolysis. Recombinant chitinases that contain this linker region were more stable in the presence of midgut digestive proteases than recombinant proteins lacking the linker region (Arakane *et al.*, 2003). The linker domain also may have another function involving protein trafficking. Recombinant forms of *Manduca* CHI lacking amino acid residues beyond position 376 accumulated intracellularly during expression in the baculovirus-insect cell line, whereas all of the forms that had an additional ten amino acids or longer stretches of the linker domain were secreted into the media (Arakane *et al.*, 2003). We concluded, therefore, that for secretion of recombinant protein to the outside of the insect cells to occur, the N-terminal portion of the linker region (residues 377–386) must be present, in addition to the 19 amino acid long N-terminal leader peptide. For secretion, the linker region may also need to be O-glycosylated because when glycosylation was inhibited by the addition of tunicamycin, insect chitinase accumulated intracellularly in an insect cell line (Gopalakrishnan *et al.*, 1995). Some of the critical residues for secretion/glycosylation, therefore, may involve residues between amino acids 376 and

386 (which includes two threonines) because the truncated Chi376 accumulated intracellularly, whereas Chi386 was secreted. Site-directed mutagenesis of these residues might help to answer the question about what residues in the linker region are required for secretion.

Peptides linking protein domains are very common in nature and some, unlike the insect chitinase linker, are believed to join domains rather passively without disturbing their function or affecting their susceptibility to cleavage by host proteases (Argos, 1990; Gilkes *et al.*, 1991). Linker peptides with G, T, or S residues are most common, perhaps because those residues are relatively small with G providing flexibility and T and S being uncharged but polar enough to interact with solvent or by their ability to hydrogen bond to water or to the protein backbone to achieve conformational and energetic stability. The interdomain linker peptide of a fungal cellobiohydrolase apparently has a dual role in providing the necessary distance between the two functional domains and also facilitating the dynamic adsorption process led by the cellulose-binding domain (Srisodsuk *et al.*, 1993). Solution conformation studies of a fungal cellulase with two domains revealed that its linker exhibited an extended conformation leading to maximum distance between the two domains and that heterogeneous glycosylation of the linker was likely a key factor defining its extended conformation (Receveur *et al.*, 2002). Since the domain structure of *M. sexta* CHI is similar to that of this fungal cellulase, these two enzymes may have similar global structural characteristics. Circular dichroism (CD) spectra of the wild-type and truncated insect chitinases were consistent with the hypothesis that whereas the catalytic and ChBDs possess secondary structure, the linker region itself does not (Arakane *et al.*, 2003).

Mammalian chitinase is similar in structure to *M. sexta* chitinase in both the catalytic domain and ChBD, but it lacks a linker domain (Tjoelker *et al.*, 2000). The absence of the ChBD does not affect the ability of the human enzyme to hydrolyze soluble oligosaccharides but does abolish hydrolysis of the insoluble substrate, a result consistent with the hypothesis that the function of the ChBD is to facilitate heterogeneous catalysis on insoluble substrates. One of the basic functions of carbohydrate-binding domains (CBD) is thought to be to help localize the enzyme on the insoluble substrate to enhance the efficiency of degradation (Linder and Teeri, 1997). These domains aid in recognition and hydrolysis of substrates that can exist in several physical states, i.e., contain both crystalline and noncrystalline forms. In general, for many glycosylhydrolases,

the binding specificity of the carbohydrate-binding domain mirrors that of the catalytic domain and these two domains are usually in relatively close association. Such is not the case for *Manduca* CHI, which has a very long linker of over several hundred angstroms.

Like their cognate catalytic domains, CBDs are classified into families of related amino acid sequences. The ChBD of insect chitinases belongs to carbohydrate-binding module family 14, which consists of approximately 70 residues (Coutinho and Henrissat, 1999; CAZY, 2004). Only three subfamilies of chitin-binding modules have been identified to date and the ChBD of *M. sexta* CHI is a member of subfamily 1 (Henrissat, 1999). Such a carbohydrate-binding function has been demonstrated in several other carbohydrases and carbohydrate-binding proteins. Other CBD families, family 17 and family 28, both of which recognize cellulose, have been found to act in a cooperative manner either by modifying the action of the catalytic module or by targeting the enzyme to areas of cellulose that differ in susceptibility to hydrolysis (Boraston *et al.*, 2003). ChBDs may play a similar role in chitinases. These domains are attached not only to catalytic domains but also to chitinase-like proteins devoid of enzyme activity. The ChBDs can be either N- or C-terminal and may be present as a single copy or as multiple repeats. They are cysteine-rich and have several highly conserved aromatic residues (Shen and Jacobs-Lorena, 1999). The cysteine residues help to maintain protein folding by forming disulfide bridges and the aromatic residues interact with saccharides in the ligand-binding pocket. The PM proteins, mucins, which have affinity for chitin, also have a six-cysteine-containing peritrophin-A/mucin consensus sequence that is similar to ChBD sequences in chitinases (Tellam *et al.*, 1999; Morlais and Severson, 2001).

When fused with the catalytic domain of *M. sexta* CHI, both insect and rice ChBDs promoted the binding to and hydrolysis of chitin (Arakane *et al.*, 2003). The influence of extra substrate-binding domains has been examined previously using a fungal chitinase that was constructed to include plant and fungal carbohydrate-binding domains (Limón *et al.*, 2001). The addition of those domains increased the substrate-binding capacity and specific activity of the enzyme toward insoluble substrates of high molecular mass such as ground chitin or chitin-rich fungal cell walls. On the other hand, removal or addition of cellulose-binding domains can reduce or enhance, respectively, the ability of cellulases to degrade crystalline cellulose (Chhabra and Kelly, 2002). When a second cellulose-binding

domain was fused to *Trichoderma reesei* cellulase, the resulting protein had a much higher affinity for cellulose than the protein with only a single binding domain (Linder *et al.*, 1996). Likewise, the *M. sexta* CHI catalytic domain fused with two ChBDs associated with chitin more strongly than any of the single ChBD-containing proteins or the protein devoid of a ChBD (Arakane *et al.*, 2003). This domain apparently helps to target the secreted enzyme to its insoluble substrate.

The chitin-binding domain of insect chitinase not only has the function of associating with insoluble chitin, but it may also help to direct the chitin chain into the active site of the catalytic domain in a manner similar to the processive hydrolysis mechanism proposed for *S. marcescens* chitinase A (ChiA), which has a very short ChBD (Uchiyama *et al.*, 2001). However, whether such an extended linker like that of *M. sexta* chitinase can direct the substrate into the active site in a manner similar to that proposed for a shorter linker is unknown. Catalytically, the full-length *M. sexta* CHI was two- to fourfold more active in hydrolyzing insoluble colloidal chitin than any of the other truncated enzymes with an intact catalytic domain, but all of the enzymes were comparable in turnover rate when two soluble substrates, carboxymethyl-chitin-remazol-brilliant-violet (CM-chitin-RBV), which is a chromogenic chitin derivative that is O-carboxymethylated, and MU-(GlcNAc)₃, a fluorogenic oligosaccharide substrate, were hydrolyzed (Arakane *et al.*, 2003). A moderate increase in catalytic efficiency of hydrolysis of insoluble substrate was observed when the catalytic domain was fused with the ChBD. When the C-terminal ChBD was deleted from a bacterial chitinase (*Aeromonas caviae*), this truncated chitinase was active also, but it liberated longer oligosaccharide products than did the full-length enzyme (Zhou *et al.*, 2002). Thus, as was observed with other carbohydrases such as xylanases (Gill *et al.*, 1999), the ChBD of insect chitinase facilitates hydrolysis of insoluble, but not soluble, substrates, and also influences the size of the oligosaccharide products generated. The linker region also can influence the functionality of the carbohydrate-binding domain. When a fungal cellulose-binding domain was fused with a fungal S/T-rich linker peptide, the fusion protein adsorbed to both crystalline and amorphous cellulose. However, deletion of the linker peptide caused a decrease in cellulose adsorption and a higher sensitivity to protease digestion (Quentin *et al.*, 2002). The addition of a carbohydrate-binding module to a catalytic domain via a linker domain may increase the catalytic efficiency for degradation of the insoluble

polysaccharide and may modify the finely tuned binding specificity of the enzyme (McLean *et al.*, 2002; Lehtio *et al.*, 2003).

Figure 5 shows a theoretical model structure for *M. sexta* chitinase that is complexed with chitin oligosaccharides in both the catalytic domain and ChBD at a time subsequent to hydrolysis of a larger oligosaccharide. What is perhaps most striking is the very long linker (>200 Å) between the other domains. Apparently, the enzyme is tethered to the cuticle by the ChBD, which anchors the catalytic domain to the insoluble substrate and localizes the hydrolysis of chitin to an area with a radius of several hundred angstroms. The use of such a tethered enzyme would help to prevent diffusion of the soluble enzyme from the insoluble polysaccharide. In the case of *Tenebrio* chitinase, which consists of five catalytic, five linker, and six chitin-binding domains (Royer *et al.*, 2002), one could envision a situation where a much wider area of the chitin-protein matrix undergoes intensive degradation by a much larger tethered enzyme.

11.4.6. Mechanism of Catalysis

Insect chitinases are members of family 18 of the glycosylhydrolases (CAZY, 2004), which generally utilize a mechanism where retention of the anomeric configuration of the sugar donor occurs via a

substrate-assisted catalysis, rather than a mechanism similar to lysozyme, which involves a proton donor and an electrostatic stabilizer (Fukamizo, 2000). However, a recent kinetic study of bacterial family 18 chitinases demonstrated that substrates lacking the *N*-acetyl group and thus incapable of anchimeric assistance were nevertheless hydrolyzed, suggesting that the reaction mechanism of family 18 chitinases cannot be fully explained by the substrate-assisted catalysis model (Honda *et al.*, 2003). Therefore, additional studies are still required to understand fully the reaction mechanism of family 18 chitinases.

The interaction of insect chitinases with insoluble chitin in the exoskeleton and PM is rather complex and believed to be a dynamic process that involves adsorption via a substrate-binding domain, hydrolysis, desorption, and repositioning of the catalytic domain on the surface of the substrate. This degradative process apparently requires a coordinated action of multiple domains by a mechanism that is not well understood. In addition to the catalytic events, the mechanism of binding of the enzyme onto the heterogeneous surface of native chitin is poorly characterized. Hydrolysis of chitin to GlcNAc is accomplished by a binary enzyme system composed of a chitinase and a β -*N*-acetylglucosaminidase (Fukamizo and Kramer, 1985;

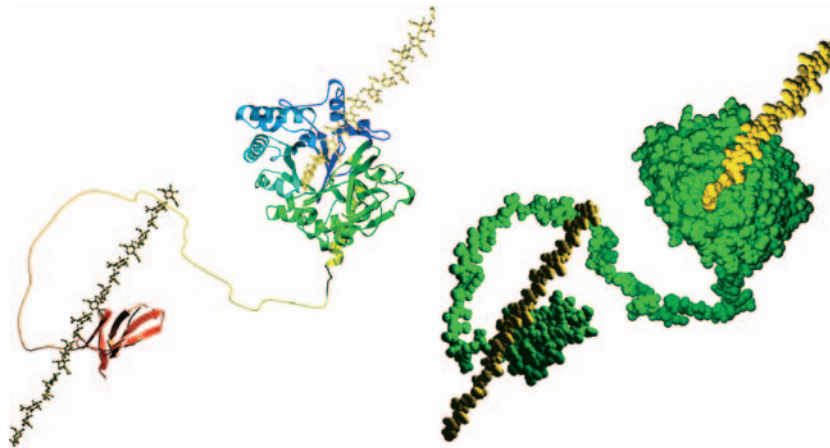


Figure 5 Ribbon (left) and space-filling (right) model structures of *Manduca sexta* chitinase with the catalytic and chitin-binding domains shown in complexes with chitin oligosaccharides (yellow). In the ribbon representation, the polypeptide chain is color-coded, beginning with blue at the N-terminus and proceeding through the rainbow to red at the C-terminus. The catalytic domain structure (top) was modeled using the program SOD (Kleywegt *et al.*, 2001) with human chitotriosidase (PDB entry code 1LG1) (Fusetto *et al.*, 2002) serving as the template. The chitin-binding domain (bottom) was similarly obtained using tachychitin (PDB entry 1DQC) (Suetake *et al.*, 2000) as the template. The linker region is shown as a random coil as predicted by secondary structure prediction software and supported by circular dichroism data. The oligosaccharides are shown as stick models (left) and space-filling models (right). Substrate binding to the catalytic domain was modeled using the available structures of complexes from glycosyl hydrolase family 18, while binding to the chitin-binding domain was modeled based on sequence conservation within the subfamily. *M. sexta* chitinase is a glycoprotein that is glycosylated especially in the linker region; however, no carbohydrate is shown in the model. (The model was constructed by Wimal Ubhayasekera and Dr. Sherry Mowbray, Swedish University of Agricultural Sciences, Uppsala.)

Filho *et al.*, 2002). The former enzyme hydrolyzes the insoluble polymer into soluble oligosaccharides, whereas the latter further degrades the oligomers to the monomer from the nonreducing end. Mechanistically, chitinases of family 18 hydrolyze chitin with retention of the anomeric configuration at the cleavage site, involving a double-displacement mechanism where a substrate-assisted catalysis occurs (Tomme *et al.*, 1995; Henrissat, 1999; Zechel and Withers, 2000; Brameld *et al.*, 2002). *B. mori* chitinase utilizes a retaining mechanism, yielding products that retain the β -anomeric configuration (Abdel-Banat *et al.*, 1999). All of the enzymes of this family are inhibited by allosamidin, a transition state analog inhibitor which apparently is diagnostic for enzymes that utilize the retaining mechanism (Koga *et al.*, 1987; Bortone *et al.*, 2002; Brameld *et al.*, 2002; Lu *et al.*, 2002). Analysis of the products from the hydrolysis of chitin oligosaccharides by the family 18 chitinase from *S. marcescens* revealed variable subsite binding preferences, anomeric selectivity, and the importance of individual binding sites for the processing of short oligosaccharides compared to the cumulative recognition and processive hydrolysis mechanism used to digest the polysaccharide (Aronson *et al.*, 2003).

Polysaccharide-hydrolyzing enzymes are known to exhibit nonideal kinetic behavior because they often are susceptible to inhibition by both substrates and products (Väljamäe *et al.*, 2001). All insect chitinases examined were found to be susceptible to inhibition by oligosaccharide substrates but to varying extents (Fukamizo and Kramer, 1985; Fukamizo *et al.*, 1995; Fukamizo, 2000). Apparently, the oligosaccharide substrate molecules can bind to these enzymes in such a manner that none of the target bonds is properly exposed to the functional groups of catalytic amino acids or the substrate may bind in only noncatalytic subsites of the larger active site, forming nonproductive instead of productive complexes. Cellulose is also degraded by the synergistic action of cellulolytic enzymes, which also display this characteristic substrate inhibition (Väljamäe *et al.*, 2001). Site-directed mutagenesis studies involving amino acids present in the putative catalytic site of *M. sexta* CHI have identified residues required for catalysis (Huang *et al.*, 2000; Lu *et al.*, 2002; Zhang *et al.*, 2002). Aspartic acids 142 and 144, tryptophan 145, and glutamic acid 146 were identified as residues very important for catalysis and also for extending the pH range of enzyme activity into the alkaline pH range. Acidic and aromatic residues in other family 18 chitinases also are important for substrate binding and catalysis

(Watanabe *et al.*, 1993, 1994; Uchiyama *et al.*, 2001; Bortone *et al.*, 2002). Some of these residues are essential only for crystalline chitin hydrolysis, whereas others are important not only for crystalline chitin hydrolysis but for other substrates as well (Watanabe *et al.*, 2003).

11.4.7. Glycosylation of Insect Chitinases

Manduca sexta CHI is moderately *N*-glycosylated in the catalytic domain and heavily *O*-glycosylated in the linker region (Arakane *et al.*, 2003). The insect cell line TN-5B1-4 (Hi 5), which is routinely used for expression of recombinant foreign glycoprotein, synthesizes proteins with both *N*- and *O*-linked oligosaccharides (Davidson *et al.*, 1990; Davis and Wood, 1995; Jarvis and Finn, 1995; Hsu *et al.*, 1997). Results of experiments investigating the effects of the *N*-glycosylation inhibitor tunicamycin on recombinant expression of insect chitinases in these cells indicated that the proteins were glycosylated prior to being secreted by the cells (Gopalakrishnan *et al.*, 1995; Zheng *et al.*, 2002). Direct chemical and enzymatic analyses confirmed that *M. sexta* CHI was both *N*- and *O*-glycosylated. Prolonged deglycosylation with a mixture of *N*- and *O*-glycosidases resulted in a protein that was smaller by about 6 kDa accounting for about 30 sugar residues per mole of protein (Arakane *et al.*, 2003). Because *N*-linked oligosaccharides in insects typically have six or seven residues, two of which are GlcNAc (Paulson, 1989; Kubelka *et al.*, 1995), the best estimate of the distribution of *N*-glycosylation indicated a single or possibly two sites of *N*-glycosylation in the catalytic domain and *O*-glycosylation of between 10 and 20 serine or threonine residues in the linker region. *O*-glycosylation may involve mainly addition of galactose and *N*-acetylgalactosamine.

The chitinase from *B. mori* also is probably glycosylated because this protein and its breakdown product (65 kDa) stain with periodic acid-Schiff reagent. Further, the apparent mobility of the protein in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is 88 kDa, whereas the molecular weight of the mature protein predicted from the cDNA sequence is only 60 kDa (Koga *et al.*, 1997). This protein has an S/T-rich linker similar to the *M. sexta* chitinase. On the other hand, the chitinase from wasp venom which has only a short linker region and is low in serine and threonine has nearly the same molecular weight as the one predicted from the cDNA sequence, suggesting that this protein may not be glycosylated (Krishnan *et al.*, 1994). Thus, there is a good

correlation between the presence of an S/T-rich linker and extensive glycosylation (predominantly O-glycosylation) of the chitinolytic proteins.

Glycosylation of the linker region may help to prevent proteolytic cleavage(s) at sites between the catalytic and chitin-binding domains. Such a functional role of glycosylated regions has been observed in some bacterial cellulases (Langsford *et al.*, 1987). The full-length and near full-length O-glycosylated forms of *M. sexta* CHI were the most stable proteins when incubated with the midgut proteinases of the hornworm (Arakane *et al.*, 2003). Protein modeling studies using the crystal structures of other family 18 glycosylhydrolases as templates suggested that the catalytic domain of *M. sexta* CHI has a ($\beta\alpha$)₈-triose phosphate isomerase (TIM) barrel structure (Kramer and Muthukrishnan, 1997; Nagano *et al.*, 2002). The ChBD probably exhibits a multistranded β -sheet structure based on similarity to tachycitin (Suetake *et al.*, 2000). We know of no structures computed or proposed for linker domains, which may be very hydrophilic and rather flexible as well as potentially susceptible to proteolytic degradation unless they are protected by glycosylation. The CD spectrum of the linker domain was consistent with the lack of any secondary structure in this domain (Figure 5). It is conceivable that during the developmental period of maximum chitinase activity, the enzyme is fully glycosylated. When required, a glycosidase(s) could be produced that would remove sugar residues, thus exposing several more peptide bonds for proteolytic cleavage. Alternatively, proteolytic cleavage may be reduced because of glycosylation. Consistent with this notion is the finding that analysis of molting fluid from *M. sexta* and *B. mori* revealed the presence of truncated forms of catalytically active chitinases with sizes ranging from 50 to 60 kDa (Kramer and Koga, 1986; Koga *et al.*, 1997; Abdel-Banat *et al.*, 1999). We also detected similar truncated forms in our insect cell recombinant chitinase expression system, especially several days subsequent to infection with the recombinant baculovirus (Gopalakrishnan *et al.*, 1995).

11.4.8. Antigenicity of Insect Chitinases

Invertebrate chitinases have been reported to elicit allergies in mammals. For example, a high prevalence of IgE antibodies to a tick chitinase was identified in canine atopic dermatitis with the chitinase formally designated Der f 15 (McCall *et al.*, 2001). In ticks, this chitinase was localized in the proventriculus and intestine, indicating that it has a digestive, rather than molting-related, function. Like

insect chitinase, tick chitinase is extensively O-glycosylated on multiple sites along the 84 amino acid long S/T-rich sequence in the molecule. The transmission blocking antibody MF1 from the blood of gerbils infected with the nematode *B. malayi* was found to be directed against a microfilarial chitinase (Fuhrman *et al.*, 1992). This antibody mediates the clearance of peripheral microfilaremia in gerbils, indicating that chitinase is indeed a potent antigen. Even though it is unclear which region of the nematode chitinase is highly antigenic, the most probable one is the S/T-rich region known to be O-glycosylated.

The primary epitope recognized by antibodies elicited by *Manduca* chitinases is the highly glycosylated S/T-rich linker region (Arakane *et al.*, 2003). Other highly immunogenic insect proteins that also are extensively O-glycosylated in S/T-rich domains similar to the linker region of *Manduca* CHI are peritrophins-55 and -95 from the sheep blowfly, *L. cuprina* (Tellam *et al.*, 2000, 2003). The sera of sheep vaccinated with these peritrophins exhibited a strong immune response that also inhibited growth of blowfly larvae (Casu *et al.*, 1997; Tellam *et al.*, 2003).

11.4.9. Other Possible Enzymes of Chitin Metabolism

Chitin deacetylases and chitosanases are two other enzymes that play major roles in chitin catabolism in other types of organisms. Chitin deacetylase catalyzes the removal of acetyl groups from chitin. This enzyme is widely distributed in microorganisms and may have a role in cell wall biosynthesis and in counteracting plant defenses (Tsigos *et al.*, 2000). There is one report of an insect chitin deacetylase in physogastric queens of the termite *Macrotermes estherae* (Sundara Rajulu *et al.*, 1982). However, there have been no follow-up studies about this enzyme in other insect species. To our knowledge, there are no reports of chitosanases present in insects.

11.5. Nonenzymatic Proteins That Bind to Chitin

There are approximately 32 families of CBDs that are defined as contiguous amino acid sequences within a carbohydrate-active enzyme or noncatalytic analogs, which exhibit a discrete fold having carbohydrate-binding activity (CAZY, 2004). One or more members in families 1, 2, 3, 5, 12, 14, 16, 18, and 19 are reported to bind chitin. Most, if not all, of the insect ChBDs, however, belong only to family 14.

Several chitinase-related proteins have been identified in insects, which are catalytically inactive because they are missing an amino acid residue critical for hydrolytic activity but nonetheless are carbohydrate-binding proteins with either a single copy or multiple repeats of ChBDs. These proteins may act as growth factors or play a defensive function as anti-inflammatory proteins. A chitinase homolog glycoprotein HAIP (hemolymph aggregation inhibiting protein) occurs in hemolymph of the lepidopteran *M. sexta*, which inhibits hemocyte aggregation (Kanost *et al.*, 1994). A similar immunoreactive protein was detected in hemolymph of three other lepidopterans, *B. mori*, *Heliothis zea*, and *Galleria mellonella*. These proteins may have a role in modulating adhesion of hemocytes during defensive responses. Another glycoprotein, Ds47, which is produced *in vitro* by a *Drosophila* embryo-derived cell line and by fat body and hemocytes, may play a role in promoting the growth of imaginal discs (Kirkpatrick *et al.*, 1995; Bryant, 2001). Another chitinase-related protein is induced together with a chitinase and β -N-acetylglucosaminidase by ecdysteroid in the anterior silk gland of *B. mori* at molting and at metamorphosis (Takahashi *et al.*, 2002). The former is rather large in size and has a novel structure consisting of tandemly repeated catalytic domain-like plus linker sequences, but it has only one ChBD located in the middle of the protein. All of these proteins are evolutionarily related to chitinases, but they apparently have acquired a new growth-promoting or infection-resistance function that does not require catalytic activity. Evidently, chitinases have evolved into these lectin-like proteins by mutation of key residues in the active site, which abolishes enzyme activity and fine tunes the ligand-binding specificity.

Chitin-binding proteins in vertebrates, invertebrates, and plants share a common structural motif composed of one to eight disulfide bonds and several aromatic residues, apparently the result of convergent evolution (Shen and Jacobs-Lorena, 1999; Suetake *et al.*, 2000). A chitin-binding antifungal peptide from the coconut rhinoceros beetle, *Oryctes rhinoceros*, scarabaecin, is only 36 residues in length and contains only one disulfide bond (Hemmi *et al.*, 2003). It shares significant tertiary structural similarity with ChBDs of other invertebrates and plants that have multiple disulfide bonds, even though there is no overall sequence similarity. Other invertebrate proteins that contain one or more ChBDs include the peritrophins (Tellam *et al.*, 1999), mucins (Casu *et al.*, 1997; Wang and Granados, 1997; Tellam *et al.*, 1999; Rayms-Keller *et al.*,

2000; Sarauer *et al.*, 2003), and tachycitin (Suetake *et al.*, 2000).

Other proteins that bind to chitin include several lectins and cuticular proteins (see Chapter 12). The lectins are related to ChBDs found in PM and chitinases. Many insect cuticular proteins contain an amino acid sequence motif of approximately 35 residues known as the R&R consensus sequence (Rebers and Willis, 2001). This sequence, however, has no similarity to the cysteine-rich ChBDs found in chitinases, some PM proteins, and lectins. There are no or very few cysteine residues in the cuticular protein ChBDs (noncysChBD). Thus, there are two distinct classes of invertebrate ChBDs, those with the chitin-binding domain found in lectins, chitinases, and PM proteins (cysChBDs) and those with the cuticular protein chitin-binding domain (noncysChBDs). Homology modeling of insect cuticle proteins using the bovine plasma retinol binding protein as a template predicted an antiparallel β -sheet half-barrel structure as the basic folding motif where an almost flat surface consisting of aromatic amino acid side chains interacts with the polysaccharide chains of chitin (Hamodrakas *et al.*, 2002).

In mammals there are several nonenzymatic members of the chitinase protein family. Oviduct-specific glycoprotein (OGP), a member of this family, is believed to be involved in the process of fertilization such as sperm function and gamete interactions (Araki *et al.*, 2003). However, OGP was not essential for *in vitro* fertilization in mice, and so the functionality of OGP remains unknown. The human cartilage protein HCgp-39 is a chitin-specific lectin (Renkema *et al.*, 1998; Houston *et al.*, 2003) that is overexpressed in articular chondrocytes and certain cancers. It is thought to be an anti-inflammatory-response protein and/or to play a role in connective tissue remodeling. In contrast to chitinases, which bind and hydrolyze chitin oligosaccharides but do not undergo large conformational changes, HCgp-39 exhibits a large conformational change upon ligand binding, which appears to signal the presence of chitinous pathogens such as fungi and nematodes (van Aalten, 2003). The murine *Ym1* gene belongs to a family of mammalian genes encoding nonenzymatic proteins that are homologous to the chitinases from lower organisms, such as insects, nematodes, bacteria, and plants (Sun *et al.*, 2001). YKL-40 is a nonenzymatic member of the mammalian family 18 glycosylhydrolases, which is a growth factor for connective tissue cells and stimulates migration of endothelial cells (Johansen *et al.*, 2003). It is secreted in large amounts by

human osteosarcoma cells and murine mammary tumors, and it is also elevated in patients with metastatic breast cancer and colorectal cancer. These homologous mammalian proteins have no demonstrable chitinase activity and, therefore, cannot be considered chitinases. The biological functions of these proteins remain obscure. However, these proteins likely function through binding to carbohydrate polymers and since they are secreted from activated hemocytes, they may have a function in immunity such as a hemocyte inhibition (Falcone *et al.*, 2001). Sequence comparison of these nonenzymatic and enzymatic proteins indicates that the enzymatic proteins have evolved into these lectins by the mutation of key residues in the active site and optimization of the substrate-binding specificity (Fusetti *et al.*, 2002).

11.6. Regulation of Chitin Degradation

The *M. sexta* chitinase and *N*-acetylglucosaminidase genes were shown to be upregulated by ecdysteroid (see Chapter 7) and down-regulated by the juvenile hormone mimic (see Chapter 8), phenoxycarb, in larval abdomens cut off from their hormonal sources (Fukamizo and Kramer, 1987; Koga *et al.*, 1991; Kramer *et al.*, 1993; Zen *et al.*, 1996). Differential display was used to show that chitinase expression was regulated not only by ecdysteroid but also by juvenile hormone in the beetle *T. molitor* (Royer *et al.*, 2002). Northern blot analysis of RNA from epidermis and 20-hydroxyecdysone-injected pupae showed that chitinase transcripts were correlated with molting hormone levels during metamorphosis. In addition, topical application of a juvenile hormone (JH) analog indirectly induced expression of chitinase mRNA. Thus, the *Tenebrio* chitinase gene is an early direct ecdysteroid-responsive one at the transcriptional level, but unlike *M. sexta* chitinase, it is apparently a direct target of JH as well. In the former case, the level at which JH regulates chitinase mRNA levels remains to be determined. The 20-hydroxyecdysone agonist, tebufenozide, induced expression of *C. fumiferana* chitinase when it was injected into mature larvae. The enzyme was produced 24 h post treatment in both the epidermis and molting fluid (Zheng *et al.*, 2003).

11.7. Chitin Metabolism and Insect Control

Chitinases have been used in a variety of ways for insect control and other purposes (Kramer *et al.*, 1997; Gooday, 1999). Several chitinase inhibitors

with biological activity have been identified based on natural products chemistry (Spindler and Spindler-Barth, 1999), such as allosamidin (Rao *et al.*, 2003) which mimics the carbohydrate substrate, and cyclic peptides (Houston *et al.*, 2002). Although useful for biochemical studies, none of these chitin catabolism inhibitors have been developed for commercial use primarily because of their high cost of production and potential side effects. As we learn more details about chitinase catalysis, it might become more economically feasible to develop and optimize chitinase inhibitors for insect pest management.

Additional uncharacterized steps in chitin synthesis and/or assembly of chitin microfibrils, on the other hand, have proved to be important for developing control chemicals that act selectively on economically important groups of insect pests (Verloop and Ferrell, 1977; Ishaaya, 2001). The benzoylphenylureas have been developed as commercial compounds for controlling agricultural pests. These antimolting insecticides are relatively nontoxic to mammals due to their strong protein binding and extensive metabolization to less toxic compounds (Bayoumi *et al.*, 2003). Studies using imaginal discs and cell-free systems indicated that benzoylphenylureas inhibit ecdysteroid-dependent GlcNAc incorporation into chitin (Mikolajczyk *et al.*, 1994; Oberlander and Silhacek, 1998). Those results suggest that benzoylphenylureas affect ecdysone-dependent sites, which leads to chitin inhibition. However, the site of action of the benzoylphenylureas still is not well known. Recently, several heteryl nucleoside nonhydrolyzable transition state analogs of UDP-GlcNAc were synthesized and evaluated for fungicidal activity, but they were not assayed for insecticidal activity (Behr *et al.*, 2003).

Entomopathogens secrete a plethora of extracellular proteins with potential activity in insect hosts. One of these proteins is chitinase, which is used by fungi such as *Metarhizium anisopliae* to help penetrate the host cuticle and render host tissues suitable for consumption (St. Leger *et al.*, 1996; Krieger de Moraes *et al.*, 2003). Among the 10 most frequent transcripts in a strain of *M. anisopliae* are three encoding chitinases and one a chitosanase, presumably reflecting a greater propensity to produce chitinases for host cuticle penetration (Freimoser *et al.*, 2003a). Expressed sequence tag analysis of *M. anisopliae* may hasten gene discovery to enhance development of improved mycoinsecticides. However, when *M. anisopliae* was transformed to overexpress its native chitinase, the pathogenicity to the tobacco hornworm was unaltered, suggesting that

wild-type levels of chitinase are not limiting for cuticle penetration (Screen *et al.*, 2001). Another fungal species, *Conidiobolus coronatus*, also produces both endo- and exo-acting chitinolytic enzymes during growth on insect cuticle (Freimoser *et al.*, 2003b). Apparently, both *M. anisopliae* and *C. coronatus* produce a chitinolytic enzyme system to degrade cuticular components.

Both microbial and insect chitinases have been shown to enhance the toxicity of the entomopathogenic bacterium *Bacillus thuringiensis* (Bt) (Regev *et al.*, 1996; Tantimavanich *et al.*, 1997; Ding *et al.*, 1998; Sampson and Gooday, 1998; Wiwat *et al.*, 2000). For example, when the chitinolytic activities of several strains of *B. thuringiensis* were compared with their insecticidal activity, it was determined that the enzyme could enhance the toxicity of Bt to *Spodoptera exigua* larvae by more than twofold (Liu *et al.*, 2002). Microbial chitinases have been used in mixing experiments to increase the potency of entomopathogenic microorganisms (review: Kramer *et al.*, 1997). Synergistic effects between chitinolytic enzymes and microbial insecticides have been reported as early as the 1970s. Bacterial chitinolytic enzymes were first used to enhance the activity of Bt and a baculovirus. Larvae of *C. fumiferana* died more rapidly when exposed to chitinase–Bt mixtures than when exposed to the enzyme or bacterium alone (Smirnoff and Valero, 1972; Morris, 1976; Lysenko, 1976). Mortality of gypsy moth, *Lymantria dispar*, larvae was enhanced when chitinase was mixed with Bt relative to a treatment with Bt alone in laboratory experiments (Dubois, 1977). The toxic effect was correlated positively with enzyme levels (Gunner *et al.*, 1985). The larvicidal activity of a nuclear polyhedrosis virus toward *L. dispar* larvae was increased about fivefold when it was administered with a bacterial chitinase (Shapiro *et al.*, 1987). Chitin synthesis-inhibiting antifungal agents such as flufenoxuron and nikkomycin were used to promote the infection of silkworms with *B. mori* nucleopolyhedrovirus (Arakawa, 2002, 2003; Arakawa and Sugiyama, 2002; Arakawa *et al.*, 2002). The mechanism of viral infection enhancement by these agents is not established, but it may involve destruction of PM structure, which would facilitate tissue invasion.

Inducible chitinolytic enzymes from bacteria cause insect mortality under certain conditions. These enzymes may compromise the structural integrity of the PM barrier and improve the effectiveness of Bt toxin by enhancing contact of the toxin molecules with their epithelial membrane receptors. For example, five chitinolytic bacterial strains isolated from midguts of *Spodoptera littoralis*

induced a synergistic increase in larval mortality when combined with Bt spore-crystal suspensions relative to either an individual bacterial strain or a Bt suspension alone (Sneh *et al.*, 1983). An enhanced toxic effect toward *S. littoralis* also resulted when a combination of low levels of a truncated recombinant Bt toxin and a bacterial endochitinase was incorporated into a semisynthetic insect diet (Regev *et al.*, 1996). Crude chitinase preparations from *B. circulans* enhanced the toxicity of Bt *kurstaki* toward diamondback moth larvae (Wiwat *et al.*, 1996). Liu *et al.* (2002) recently reported that several strains of Bt produced their own chitinases, which had synergistic larvicidal activity with the endotoxins.

In biopesticide development research, we used a family 18 insect chitinase as an enhancer protein for baculovirus toxicity and as a host plant resistance factor in transgenic plants. Introduction of an insect chitinase cDNA into *A. californica* multiple nuclear polyhedrosis viral (AcMNPV) DNA accelerated the rate of killing of fall armyworm compared to the wild-type virus (Gopalakrishnan *et al.*, 1995). Baculoviral chitinases themselves play a role in liquefaction of insect hosts (Hawtin *et al.*, 1997; Thomas *et al.*, 2000). A constitutively expressed exochitinase from *B. thuringiensis* potentiated the insecticidal effect of the vegetative insecticidal protein Vip when they were fed to neonate larvae of *S. litura* (Arora *et al.*, 2003). Some granuloviruses, on the other hand, do not utilize chitinases in a similar manner, which helps to explain why some granulovirus-infected insects do not lyse at the end of the infection process (Wormleaton *et al.*, 2003). Mutagenesis of the AcMNPV chitinase gene resulted in cessation of liquefaction of infected *T. ni* larvae, supporting a role of chitinase in virus spread (Thomas *et al.*, 2000). However, the insecticidal activity of insect chitinase was not substantial enough for commercial development. We have attempted with little success to improve the catalytic efficiency and stability of this enzyme so that its pesticidal activity would be enhanced (Lu *et al.*, 2002; Zhang *et al.*, 2002; Arakane *et al.*, 2003). Nevertheless, tobacco budworms were killed when reared on transgenic tobacco expressing a truncated, enzymatically active form of insect chitinase (Ding *et al.*, 1998). We also discovered a synergistic interaction between insect chitinase expressed in transgenic tobacco plants and Bt (applied as a spray at sublethal levels) using the tobacco hornworm as the test insect. In contrast to results obtained with the tobacco budworm, studies with the hornworm revealed no consistent differences in larval growth or foliar damage when the insects were reared on

first-generation transgenic chitinase-positive tobacco plants as compared to chitinase-negative control plants. When Bt toxin was applied at levels where no growth inhibition was observed on control plants, chitinase-positive plants had significantly less foliar damage and lower larval biomass production. These results indicated that the insect chitinase transgene did potentiate the effect of sublethal doses of Bt toxin and vice versa (Ding *et al.*, 1998). Tomato plants have been transformed with fungal chitinase genes with concomitant enhancement in resistance to insect pests (Gongora *et al.*, 2001). Effects observed include reduced growth rates and increased mortality, as well as a decrease in plant height and flowering time with an increase in the number of flowers and fruits (Gongora and Broadway, 2002). Chitinase-secreting bacteria have been used to suppress herbivorous insect pests. A chitinase gene-transformed strain of *Enterobacter cloacae* digested the chitinous membranes of phytophagous ladybird beetles, *Epilachna vigintioctopunctata*, and also suppressed leaf-feeding and oviposition when the beetles ingested transformed bacteria entrapped in alginate microbeads sprayed on tomato seedlings (Otsu *et al.*, 2003).

Several GlcNAc-specific lectins from plants have been evaluated for insect toxicity (Harper *et al.*, 1998; Macedo *et al.*, 2003). These proteins appear to disrupt the integrity of the PM by binding to chitin or glycan receptors on the surface of cells lining the insect gut. They also may bind to glycosylated digestive enzymes and inhibit their activity. Another type of plant chitin-binding protein is the seed storage protein, vicilin, which is actually a family of oligomeric proteins with variable degrees of glycosylation (Macedo *et al.*, 1993; Shutov *et al.*, 1995). Some vicilins are insecticidal to bruchid beetles and stalk borers (Sales *et al.*, 2001; Mota *et al.*, 2003). Apparently, these proteins bind to the PM, causing developmental abnormalities and reduced survival rates. To date no carbohydrate-binding protein derived from an insect has been evaluated for biocidal activity. A novel approach has been proposed to develop strategies for insect control by utilizing chitin-binding molecules to specifically target formation of the PM. Calcofluor, a chemical whitener with chitin-binding properties, was used as a model compound in the diet to inhibit PM formation in *T. ni* and also to increase larval susceptibility to baculovirus infection (Wang and Granados, 2000b). It also was effective in suppressing PM formation in *Spodoptera frugiperda* and at the same time in preventing the establishment of a decreasing gradient of proteinases along the midgut tissue (Bolognesi *et al.*, 2001).

Another type of hydrolytic enzyme with a ChBD has been shown to exhibit insecticidal activity in plants. Maize accumulates a 33 kDa cysteine protease containing a ChBD in response to insect feeding (Perchan *et al.*, 2002). This enzyme apparently damages the insect's PM by utilizing the ChBD to localize itself at the chitin-protein-rich PM, where the PM proteins are digested, rendering the PM dysfunctional. Another protease with a chitin-binding domain has been described from *A. gambiae*, which may be involved in insect defense (Danielli *et al.*, 2000). This 147 kDa protein, sp22D, is expressed in a variety of tissues, most strongly in hemocytes, and is secreted into the hemolymph. Upon bacterial infection, the transcripts for this protein increase by about twofold suggesting a role in insect defense. This protein has a multidomain organization that includes two copies of an N-terminal ChBD, a C-terminal protease domain, and additional receptor domains. It binds strongly to chitin and undergoes complex proteolytic processing during pupal to adult metamorphosis. It has been proposed that exposure of this protease to chitin may regulate its activity during tissue remodeling or wounding.

Recently, two synthetic peptides were found to inhibit *A. gambiae* midgut chitinase and also to block sporogonic development of the human malaria parasite, *Plasmodium falciparum*, and avian malaria parasite, *P. gallinaceum*, when the peptides were fed to infected mosquitoes (Bhatnagar *et al.*, 2003). The design of these peptides was based on the putative proregion sequence of mosquito midgut chitinase. The results indicated that expression of chitinase inhibitory peptides in transgenic mosquitoes might alter the vectorial capacity of mosquitoes to transmit malaria.

11.8. Concluding Remarks

Although chitin was discovered nearly two centuries ago, it remains a biomaterial in waiting because, unlike other natural materials such as collagen and hyaluronic acid, very few technological uses have been developed (Khor, 2002; Tharanathan and Kittur, 2003). There are many unanswered questions about chitin morphology and chitin deposition in the insect cuticle and PM. We do not know how or whether chitin forms covalent interactions with other components in these extracellular matrices. Chitosan, on the other hand, does react with quinones (Muzzarelli and Muzzarelli, 2002; Muzzarelli *et al.*, 2003). Thus, if there were any free amino groups in insect chitin, C–N linkages between chitin and catechols would be expected (Schaefer *et al.*,

1987). We do not yet understand how factors such as metal ions affect chitin metabolism. In fungi, ions such as zinc were found to alter chitin deposition and morphology (Lanfranco *et al.*, 2002). Perhaps, in insects there is an ionic effect on differential expression of CHS isozymes.

We know much more about insect chitinolytic enzymes than about insect chitin biosynthetic enzymes. Many questions remain about the biosynthesis of insect chitin, not the least of which are why insects have multiple genes for CHS, how many CHSs are required to make an insect, at what developmental stages are the various CHSs produced, and what are the unique properties and functions of each CHS. Of particular interest is the role of alternate splicing in generating different isoforms of CHSs from the same gene. The developmental cues that control alternate splicing and how they affect chitin synthesis and/or deposition will be subjects of future studies. The cloning of CHS genes should soon lead to availability of large amounts of recombinant enzymes or subdomains thereof using appropriate expression systems. Studies with pure proteins and the availability of molecular probes will provide a better understanding of the chitin biosynthetic pathway and its regulation in the future.

Two other major questions about insect chitin biosynthesis are: what is the mechanism of the initiation phase and is there an autocatalytic initiator. Like glycogen synthesis, chitin synthesis probably includes both initiation and elongation phases. As the initiator of glycogen synthesis, glycogenin transfers glucose from UDP-glucose to itself to form an oligosaccharide-protein primer for elongation (Gibbons *et al.*, 2002). Like chitin synthase, glycogenin is a glycosyltransferase, which raises the question of whether chitin synthase has an autocatalytic function similar to glycogenin and whether there is a chitinogenin-like protein. Another possibility is the participation of a lipid primer for chitin synthesis. Recently, cellulose synthesis in plants was found to involve the transfer of lipid-linked cellodextrins to a growing glucan chain (Read and Bacic, 2002). The lipid in this case was sitosterol- β -glucoside.

Little is known about the catalytic mechanism of any insect CHS. Once insect CHS-related recombinant proteins are obtained, site-directed mutagenesis can be used to probe for essential residues in the catalytic and regulatory domains. It is likely that acidic amino acids play critical roles in CHS catalysis in a manner comparable to those identified in other glycosyltransferases (Hefner and Stockigt, 2003) and in yeast chitin synthases (Nagahashi *et al.*, 1995).

Chitinolytic enzymes are gaining importance for their biotechnological applications in agriculture and healthcare (Patil *et al.*, 2000). Additional success in using chitinases for different applications depends on a better understanding of their biochemistry and regulation so that their useful properties can be optimized through genetic and biochemical engineering. Reasons for the rather high multiplicity of domain structures for insect and other chitinases are not fully understood. So far little success has occurred in using chitinase in pest control applications, but it may prove more useful as an enhancer protein in a cocktail with other biopesticides targeted at the cuticle or gut. Also, only a few catalytic domains or chitin-binding domains or various combinations thereof have been evaluated for biocidal activity and thus, further toxicological experimentation is warranted.

Although substantial progress in studies of insect chitin metabolism has occurred since the first edition of *Comprehensive Insect Physiology, Biochemistry, and Pharmacology* was published in 1985, we still do not know much about how chitin is produced and transported across the membrane so that it can interact perfectly with other components for assembly of the supramolecular extracellular structures called the exoskeleton and PM. These materials are still very much biochemical puzzles in which we do not understand well how the various components come together during morphogenesis or are digested apart during the molting process. Hopefully, this chapter will stimulate more effort to understand how insects utilize chitin metabolism for growth and development, and to develop materials that may perturb insect chitin metabolism for pest management purposes.

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References

- Abdel-Banat, B.M.A., Kameyama, Y., Yoshioka, T., Koga, D., 1999. Purification and characterization of a 54 kDa chitinase from *Bombyx mori*. *Insect Biochem. Mol. Biol.* 30, 107–117.
- Abdel-Banat, B.M.A., Koga, D., 2001. A genomic clone for a chitinase gene from the silkworm, *Bombyx mori*: structural organization identifies functional motifs. *Insect Biochem. Mol. Biol.* 31, 497–508.
- Abdel-Banat, B.M.A., Koga, D., 2002. Alternative splicing of the primary transcript generates heterogeneity within the products of the gene for *Bombyx mori* chitinase. *J. Biol. Chem.* 277, 30524–30534.
- Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., et al., 2000. The genome sequence of *Drosophila melanogaster*. *Science* 287, 2185–2195.
- Arakane, Y., Hogenkamp, D., Zhu, Y.C., Kramer, K.J., Specht, C.A., et al., 2004. Chitin synthase genes of the red flour beetle, *Tribolium castaneum*: characterization, expression, linkage mapping and alternate exon usage. *Insect Biochem. Mol. Biol.* 34, 291–304.
- Arakane, Y., Zhu, Q., Matsumiya, M., Muthukrishnan, S., Kramer, K.J., 2003. Properties of catalytic, linker and chitin-binding domains of insect chitinase. *Insect Biochem. Mol. Biol.* 33, 631–648.
- Arakawa, T., 2002. Promotion of nucleopolyhedrovirus infection in larvae of the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae) by flufenoxuron. *Appl. Entomol. Zool.* 37, 7–11.
- Arakawa, T., 2003. Chitin synthesis inhibiting antifungal agents promote nucleopolyhedrovirus infection in silkworm, *Bombyx mori* (Lepidoptera: Bombycidae) larvae. *J. Invert. Pathol.* 83, 261–263.
- Arakawa, T., Furuta, Y., Miyazawa, M., Kato, M., 2002. Flufenoxuron, an insect growth regulator, promotes peroral infection by nucleopolyhedrovirus (BmNPV) budded particles in the silkworm, *Bombyx mori* L. *J. Virol. Methods* 100, 141–147.
- Arakawa, T., Sugiyama, M., 2002. Promotion of nucleopolyhedrovirus infection in larvae of the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae) by an antibiotic, nikkomycin Z. *Appl. Entomol. Zool.* 37, 393–397.
- Araki, Y., Nohara, M., Yoshida-Komiya, H., Kuramochi, T., Ito, M., et al., 2003. Effect of a null mutation of the oviduct-specific glycoprotein gene on mouse fertilization. *Biochem. J.* 374, 551–557.
- Argos, P., 1990. An investigation of oligopeptides linking domains in protein tertiary structures and possible candidates for general gene fusion. *J. Mol. Biol.* 211, 943–958.
- Aronson, N.N., Halloran, B.A., Alexyev, M.F., Amable, L., Madura, J.D., et al., 2003. Family 18 chitinase-oligosaccharide substrate interaction: subsite preference and anomer selectivity of *S. marcescens* chitinase A. *Biochem. J.* 376, 87–95. Immediate Publication, doi:10.1042/BJ20030273.
- Arora, N., Ahmad, T., Rajagopal, R., Bhatnagar, R.K., 2003. A constitutively expressed 36 kDa exochitinase from *Bacillus thuringiensis* HD-1. *Biochem. Biophys. Res. Commun.* 307, 620–625.
- Bayoumi, A.E., Rerez-Pertejo, Y., Zidan, H.Z., Balana-Fouce, R., Ordonez, C., et al., 2003. Cytotoxic effects of two antimolting insecticides in mammalian CHO-K1 cells. *Ecotoxicol. Environ. Safety* 55, 19–23.
- Becker, A., Schloder, P., Steele, J.E., Wegener, G., 1996. The regulation of insect metabolism in insects. *Experientia* 52, 433–439.
- Behr, J.B., Gourlain, T., Helimi, A., Guillerm, G., 2003. Design, synthesis and biological evaluation of heteryl-nucleoside derivatives as inhibitors of chitin synthase. *Bioorgan. Medicinal Chem. Lett.* 13, 1713–1716.
- Bhatnagar, R.K., Arora, N., Sachidanand, S., Shahabuddin, M., Keister, D., et al., 2003. Synthetic propeptide inhibits mosquito midgut chitinase and blocks sporogonic development of malaria parasite. *Biochem. Biophys. Res. Commun.* 304, 783–787.
- Bolognesi, R., Ribeiro, A.F., Terra, W.R., Terra, C., 2001. The peritrophic membrane of *Spodoptera frugiperda*: secretion of peritrophins and role in immobilization and recycling digestive enzymes. *Arch. Insect Biochem. Physiol.* 47, 62–75.
- Boraston, A.B., Kwan, E., Chiu, P., Warren, A.J., Kilburn, D.G., 2003. Recognition and hydrolysis of noncrystalline cellulose. *J. Biol. Chem.* 278, 6120–6127.
- Bortone, K., Monzingo, A.F., Ernst, S., Robertus, J.D., 2002. The structure of an allosamidin complex with the *Coccidioides immitis* chitinase defines a role for a second acid residue in substrate-assisted mechanism. *J. Mol. Biol.* 320, 293–302.
- Brameld, K.A., Shrader, W.D., Imperiali, B., Goddard, W.A., 2002. Substrate assistance in the mechanism of family 18 chitinases: theoretical studies of potential intermediates and inhibitors. *J. Mol. Biol.* 280, 913–923.
- Bray, S.J., Kafatos, F.C., 1991. Developmental function of *Elf-1*: an essential transcription factor during embryogenesis in *Drosophila*. *Genes Devel.* 5, 1672–1683.
- Bryant, P., 2001. Growth factors controlling imaginal disc growth in *Drosophila*. In: Bock, G., Cardew, G., Goode, J.A. (Eds.), *The Cell Cycle and Development*. John Wiley, New York, pp. 182–199.
- Casu, R., Eisemann, C., Pearson, R., Riding, G., East, I., et al., 1997. Antibody-mediated inhibition of the growth of larvae from an insect causing cutaneous myiasis in a mammalian host. *Proc. Natl Acad. Sci. USA* 94, 8939–8944.
- CAZY, 2004. Carbohydrate-active enzymes family server: <http://afmb.cnrs.mrs.fr/~pedro/CAZY/db.html>.
- Chang, R., Yeager, A.R., Finney, N.S., 2003. Probing the mechanism of fungal glycosyltransferase essential for cell wall synthesis: UDP-Chitobiose is not a substrate for chitin synthase. *Org. Biomol. Chem.* 1, 39–41.
- Chhabra, S.R., Kelly, R.M., 2002. Biochemical characterization of *Thermotoga maritima* endoglucanase Cel74

- with and without a carbohydrate-binding module (CBM). *FEBS Lett.* 531, 375–380.
- Choi, H.K., Choi, K.H., Kramer, K.J., Muthukrishnan, S., 1997. Isolation and characterization of a genomic clone for the gene of an insect molting enzyme, chitinase. *Insect Biochem. Mol. Biol.* 27, 37–47.
- Cohen, E., 1987. Chitin biochemistry: synthesis and inhibition. *Annu. Rev. Entomol.* 32, 71–93.
- Cohen, E., 2001. Chitin synthesis and inhibition: a revisit. *Pest Mgt Sci.* 57, 946–950.
- Cohen, E., Casida, J.E., 1980. Inhibition of *Tribolium* gut chitin synthetase. *Insecticide Biochem. Physiol.* 13, 129–136.
- Coutinho, P.M., Deleury, E., Davies, G.J., Henrissat, B., 2003. An evolving hierarchical family classification for glycosyltransferases. *J. Mol. Biol.* 328, 307–317.
- Coutinho, P.M., Henrissat, B., 1999. Carbohydrate-active enzymes: an integrated database approach. In: Gilbert, H.H., Davies, G.J., Henrissat, H., Svensson, B. (Eds.), *Recent Advances in Carbohydrate Bio-engineering*. Royal Society of Chemistry, Cambridge, pp. 3–12.
- Daimon, T., Hamada, K., Mita, K., Okano, K., Suzuki, M.G., et al., 2003. A *Bombyx mori* gene, *BmChi-h*, encodes a protein homologous to bacterial and baculovirus chitinases. *Insect Biochem. Mol. Biol.* 33, 749–759.
- Danielli, A., Loukeris, T.G., Lagueux, M., Muller, H.M., Richman, A., et al., 2000. A modular chitin-binding protease associated with hemocytes and hemolymph in the mosquito *Anopheles gambiae*. *Proc. Natl Acad. Sci. USA* 97, 7136–7141.
- Davidson, D.J., Fraser, M.J., Castellino, F.J., 1990. Oligosaccharide processing in the expression of human plasminogen cDNA by lepidopteran insect (*Spodoptera frugiperda*) cells. *Biochemistry* 19, 5584–5590.
- Davis, T.R., Wood, H.A., 1995. Intrinsic glycosylation potentials of insect cell lines and insect larvae. *In Vitro Cell. Devel. Biol. Anim.* 31, 659–663.
- de la Vega, H., Specht, C.A., Liu, Y., Robbins, P.W., 1998. Chitinases are a multi-gene family in *Aedes*, *Anopheles* and *Drosophila*. *Insect Mol. Biol.* 7, 233–239.
- Ding, X., Gopalakrishnan, B., Johnson, L., White, F.F., Wang, X., et al., 1998. Insect resistance of transgenic tobacco expressing an insect chitinase gene. *Transgenic Res.* 7, 77–84.
- Dubois, N.R., 1977. Pathogenicity of selected resident microorganisms of *Lymantria dispar* after induction for chitinase. Ph.D. Dissertation, Univ. Massachusetts, Amherst.
- Eisemann, C., Wijffels, G., Tellam, R.L., 2001. Secretion of type 2 peritrophic matrix protein, peritrophin-15, from the cardia. *Arch. Insect Biochem. Physiol.* 47, 76–85.
- Falcone, F.H., Loke, P., Zang, X., MacDonald, A.S., Maizels, R.M., et al., 2001. A *Brugia malayi* homolog of macrophage migration inhibitory factor reveals an important link between macrophages and eosinophil recruitment during nematode infection. *J. Immunol.* 167, 5348–5354.
- Feix, M., Gloggl, S., Londershausen, M., Weidemann, W., Spindler, K.D., et al., 2000. A cDNA encoding a chitinase from the epithelial cell line of *Chironomus tentans* (Insecta, Diptera) and its functional expression. *Arch. Insect Biochem. Physiol.* 45, 24–36.
- Filho, B.P.D., Lemos, F.J.A., Secundino, N.F.C., Pascoa, V., Pereira, S.T., et al., 2002. Presence of chitinase and beta-N-acetylglucosaminidase in the *Aedes aegypti* chitinolytic system involving peritrophic matrix formation and degradation. *Insect Biochem. Mol. Biol.* 32, 1723–1729.
- Fly Base, 2004. <http://www.flybase.bio.indiana.edu/>
- Freimoser, F.M., Screen, S., Bagga, S., Hu, G., St. Leger, R.J., 2003a. Expressed sequence tag (EST) analysis of two subspecies of *Metarhizium anisopliae* reveals a plethora of secreted proteins with potential activity in insect hosts. *Microbiology* 149, 239–247.
- Freimoser, F.M., Screen, S., Hu, G., St. Leger, R.J., 2003b. EST analysis of genes expressed by the zygomycete pathogen *Conidiobolus coronatus* during growth on insect cuticle. *Microbiology* 149, 1893–1900.
- Fuhrman, J.A., Lane, W.S., Smith, R.F., Piessens, W.F., Perler, F.B., 1992. Transmission-blocking antibodies recognize microfilarial chitinase in brugian lymphatic filariasis. *Proc. Natl Acad. Sci. USA* 89, 1548–1552.
- Fukamizo, T., 2000. Chitinolytic enzymes: catalysis, substrate binding, and their application. *Curr. Protein Peptide Sci.* 1, 105–124.
- Fukamizo, T., Koga, D., Goto, S., 1995. Comparative biochemistry of chitinases: anomeric form of the reaction products. *Biosci. Biotech. Biochem.* 59, 311–313.
- Fukamizo, T., Kramer, K.J., 1985. Mechanism of chitin hydrolysis by the binary chitinase system in insect moulting fluid. *Insect Biochem.* 15, 141–145.
- Fukamizo, T., Kramer, K.J., 1987. Effect of 20-hydroxyecdysone on chitinase and β -N-acetylglucosaminidase during the larval-pupal transformation in *Manduca sexta* (L.). *Insect Biochem.* 17, 547–550.
- Fukamizo, T., Kramer, K.J., Mueller, D.D., Schaefer, J., Garbow, J., et al., 1986. Analysis of chitin structure by nuclear magnetic resonance spectroscopy and chitinolytic enzyme digestion. *Arch. Biochem. Biophys.* 249, 15–26.
- Furukawa, S., Taniai, K., Yang, J., Shono, T., Yamakawa, M., 1999. Induction of gene expression of antibacterial proteins by chitin oligomers in the silkworm, *Bombyx mori*. *Insect Mol. Biol.* 8, 145–148.
- Fuseti, F., von Moeller, H., Houston, D., Rozeboom, H.J., Dijkstra, B.W., et al., 2002. Structure of human chitotriosidase: implications for specific inhibitor design and function of mammalian chitinase-like lectins. *J. Biol. Chem.* 277, 25537–25544.
- Gagou, M.E., Kapsetaki, M., Turberg, A., Kafetzopoulos, D., 2002. Stage-specific expression of the chitin synthase *DmeChSA* and *DmeChSB* genes during the onset of *Drosophila* metamorphosis. *Insect Biochem. Mol. Biol.* 32, 141–146.
- Gaines, P.J., Brandt, K.S., Eisele, A.M., Wagner, W.P., Bozic, C.M., et al., 2002. Analysis of expressed

- sequence tags from *Ctenocephalides felis* hindgut and Malpighian tubule cDNA libraries. *Insect Mol. Biol.* 11, 299–306.
- Gibbons, B.J., Roach, P.J., Hurley, T.D., 2002. Crystal structure of the autocatalytic initiator of glycogen biosynthesis, glycogenin. *J. Mol. Biol.* 319, 463–477.
- Gilkes, N.R., Henrissat, B., Kilburn, D.G., Miller, R.C., Warren, R.A.J., 1991. Domains in microbial β -1,4-glycanases: sequence conservation, function, and enzyme families. *Microbiol. Rev.* 55, 303–315.
- Gill, J., Rixon, J.E., Bolam, D.N., McQueen-Mason, S., Simpson, P.J., et al., 1999. The type II and X cellulose-binding domains of *Pseudomonas* xylanase A potentiate catalytic activity against complex substrates by a common mechanism. *Biochem. J.* 342, 473–480.
- Girard, C., Jouanin, L., 1999. Molecular cloning of a gut-specific chitinase cDNA from the beetle *Phaedon cochleariae*. *Insect Biochem. Mol. Biol.* 29, 549–556.
- Gongora, C.E., Broadway, R.M., 2002. Plant growth and development influenced by transgenic insertion of bacterial chitinolytic enzymes. *Mol. Breeding* 9, 123–135.
- Gongora, C.E., Wang, S., Barbehenn, R.V., Broadway, R.M., 2001. Chitinolytic enzymes from *Streptomyces albidoflavus* expressed in tomato plants: effects on *Trichoplusia ni*. *Entomol. Experimentia Appl.* 99, 193–204.
- Goo, T.-W., Hwang, J.-S., Sung, G.-B., Yun, E.-Y., Bang, H.-S., et al., 1999. Molecular cloning and characterization of a gene encoding a beta-N-acetylglucosaminidase homologue from *Bombyx mandarina*. *Korean J. Sericult. Sci.* 41, 147–153.
- Gooday, G.W., 1999. Aggressive and defensive roles for chitinases. In: Jolles, P., Muzzarelli, R.A.A. (Eds.), *Chitin and Chitinases*. Birkhäuser Verlag, Basel, pp. 157–169.
- Gopalakrishnan, B., Muthukrishnan, S., Kramer, K.J., 1995. Baculovirus-mediated expression of a *Manduca sexta* chitinase gene: properties of the recombinant protein. *Insect Biochem. Mol. Biol.* 25, 255–265.
- Graack, H., Cinque, U., Kress, H., 2001. Functional regulation of glutamine: fructose-6-phosphate aminotransferase 1 (GFAT1) of *Drosophila melanogaster* in a UDP-N-acetylglucosamine and cAMP-dependent manner. *Biochem. J.* 360, 401–412.
- Gunner, H.P., Met, M.Z., Berger, S., 1985. In: Gamble, D.G., Lewis, F.B. (Eds.), *Microbial Control of Spruce Budworms*. US Forest Service GTR-NE-100, Northeastern Forest Experiment Station, Broomall, PA, p. 102.
- Hamodrakas, S.J., Willis, J.H., Iconomidou, V.A., 2002. A structural model of the chitin-binding domain of cuticle proteins. *Insect Biochem. Mol. Biol.* 32, 1577–1583.
- Harper, M.S., Granados, R.R., 1999. Peritrophic membrane structure and formation of larval *Trichoplusia ni* with an investigation on the secretion patterns of a PM mucin. *Tissue and Cell* 31, 201–211.
- Harper, M.S., Hopkins, T.L., 1997. Peritrophic membrane structure and secretion in European corn borer larvae (*Ostrinia nubilalis*). *Tissue and Cell* 29, 461–475.
- Harper, M.S., Hopkins, T.L., Czaplá, T.H., 1998. Effect of wheat germ agglutinin on formation and structure of the peritrophic membrane in European corn borer (*Ostrinia nubilalis*) larvae. *Tissue and Cell* 30, 166–176.
- Harris, M.T., Fuhrman, J.A., 2002. Structure and expression of chitin synthase in the parasitic nematode *Dirofilaria immitis*. *Mol. Biochem. Parasitol.* 122, 231–234.
- Harris, M.T., Lai, K., Arnold, K., Martinez, H.F., Specht, C.A., et al., 2000. Chitin synthase in the filarial parasite, *Brugia malayi*. *Mol. Biochem. Parasitol.* 111, 351–362.
- Hawtin, R.E., Zarkowska, T., Arnold, K., Thomas, C.J., Gooday, G.W., et al., 1997. Liquefaction of *Autographa californica* nucleopolyhedrosis-infected insects is dependent on the integrity of virus-encoded chitinase and cathepsin genes. *Virology* 238, 243–253.
- Hefner, T., Stockigt, J., 2003. Probing suggested catalytic domains of glycosyltransferases by site-directed mutagenesis. *Eur. J. Biochem.* 270, 533–538.
- Hemmi, H., Ishibashi, J., Tomie, T., Yamakawa, M., 2003. Structural basis for new pattern of conserved amino acid residues related to chitin binding in the antifungal peptide from the coconut rhinoceros beetle *Oryctes rhinoceros*. *J. Biol. Chem.* 278, 22820–22827.
- Henrissat, B., 1999. Classification of chitinase modules. In: Jolles, P., Muzzarelli, R.A.A. (Eds.), *Chitin and Chitinases*. Birkhäuser Verlag, Basel, pp. 137–156.
- Henrissat, B., Bairoch, A., 1996. Updating the sequence-based classification of glycosyl hydrolases. *Biochem. J.* 316, 695–696.
- Honda, Y., Kitaoka, M., Tokuyasu, K., Sasaki, C., Fukamizo, T., et al., 2003. Kinetic studies on the hydrolysis of N-acetylated and N-deacetylated derivatives of 4-methylumbelliferyl chitobioside by the family 18 chitinases ChiA and ChiB from *Serratia marcescens*. *J. Biochem. (Tokyo)* 133, 253–258.
- Hopkins, T.L., Harper, M.S., 2001. Lepidopteran peritrophic membranes and effects on dietary wheat germ agglutinin on their formation and structure. *Arch. Insect Biochem. Physiol.* 47, 100–109.
- Hopkins, T.L., Kramer, K.J., 1992. Insect cuticle sclerotization. *Annu. Rev. Entomol.* 37, 273–302.
- Horst, M.N., 1983. The biosynthesis of crustacean chitin: isolation and characterization of polyprenol-linked intermediates from brine shrimp microsomes. *Arch. Biochem. Biophys.* 223, 254–263.
- Houston, D.R., Reeklies, A.D., Krupa, J.C., van Aalten, D.M.F., 2003. Structure and ligand-induced conformational change of the 39-kDa glycoprotein from human articular chondrocytes. *J. Biol. Chem.* 278, 30206–30212.
- Houston, D.R., Shiomi, K., Arai, N., Omura, S., Peter, M.G., et al., 2002. High-resolution structures of a chitinase complexed with natural product cyclopentapeptide inhibitors: mimicry of carbohydrate substrate. *Proc. Natl Acad. Sci. USA* 99, 9127–9132.

- Hsu, T.A., Takahashi, N., Tsukamoto, Y., Kato, K., Shimada, I., *et al.*, 1997. Differential N-glycan patterns of secreted and intracellular IgG produced in *Trichoplusia ni* cells. *J. Biol. Chem.* 272, 9062–9070.
- Huang, X., Zhang, H., Zen, K.C., Muthukrishnan, S., Kramer, K.J., 2000. Homology modeling of the insect chitinase catalytic domain-oligosaccharide complex and the role of a putative active site tryptophan in catalysis. *Insect Biochem. Mol. Biol.* 30, 107–117.
- Ibrahim, G.H., Smartt, C.T., Kiley, L.M., Christensen, B.M., 2000. Cloning and characterization of a chitin synthase cDNA from the mosquito *Aedes aegypti*. *Insect Biochem. Mol. Biol.* 30, 1213–1222.
- Ishaaya, I., 2001. Biochemical processes related to insecticide action: an overview. In: Ishaaya, I. (Ed.), *Biochemical Sites of Insecticide Action and Resistance*. Springer-Verlag, New York, pp. 1–16.
- Jarvis, D.L., Finn, E.E., 1995. Biochemical analysis of the N-glycosylation pathway in baculovirus-infected lepidopteran insect cells. *Virology* 212, 500–511.
- Johansen, J.S., Christensen, I.J., Riisbro, R., Greenall, M., Han, C., *et al.*, 2003. High serum YKL-40 levels in patients with primary breast cancer is related to short recurrence free survival. *Breast Cancer Res. and Treatment* 80, 15–21.
- Jurgens, G., Wieschaus, E., Nusslein-Volhard, C., Kluding, H., 1984. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. 2. Zygotic loci on the third chromosome. *Roux's Arch. Devel. Biol.* 193, 283–295.
- Kanost, M.R., Zepp, M.K., Ladendorff, N.E., Andersson, L.A., 1994. Isolation and characterization of a hemocyte aggregation inhibitor from hemolymph of *Manduca sexta* larvae. *Arch. Insect Biochem. Physiol.* 27, 123–136.
- Kato, N., Dasgupta, R., Smartt, C.T., Christensen, B.M., 2002. Glucosamine: fructose-6-phosphate aminotransferase: gene characterization, chitin biosynthesis and peritrophic matrix formation in *Aedes aegypti*. *Insect Mol. Biol.* 11, 207–216.
- Khor, E., 2002. Chitin: a biomaterial in waiting. *Curr. Opin. Solid State Materials Sci.* 2, 313–317.
- Kim, M.G., Shin, S.W., Bae, K.S., Kim, S.C., Park, H.Y., 1998. Molecular cloning of chitinase cDNAs from the silkworm, *Bombyx mori*, and the fall webworm, *Hyphantria cunea*. *Insect. Biochem. Mol. Biol.* 28, 163–171.
- Kimura, S., 1976. Insect haemolymph exo- β -N-acetylglucosaminidase from *Bombyx mori*. *Biochim. Biophys. Acta* 446, 399–406.
- Kimura, S., 1977. Exo- β -N-acetylglucosaminidase and chitobiase in *Bombyx mori*. *Insect Biochem.* 7, 237–245.
- Kirkpatrick, R.B., Matico, R.E., McNulty, D.E., Strickler, J.E., Rosenberg, M., 1995. An abundantly secreted glycoprotein from *Drosophila melanogaster* is related to mammalian secretory proteins produced in rheumatoid tissues and by activated macrophages. *Gene* 153, 147–154.
- Kleywegt, G.J., Zou, J.Y., Kjeldgaard, M., Jones, T.A., 2001. In: Around, O., Rossmann, M.G., Arnold, E. (Eds.), *International Tables for Crystallography*. vol. F. Kluwer Academic, Dordrecht, The Netherlands, pp. 353–356.
- Koga, D., Funakoshi, T., Fujimoto, H., Kuwano, E., Eto, M., *et al.*, 1991. Effects of 20-hydroxyecdysone and KK-42 on chitinase and beta-N-acetylglucosaminidase during the larval-pupal transformation of *Bombyx mori*. *Insect Biochem.* 21, 277–284.
- Koga, D., Funakoshi, T., Mizuki, K., Ide, A., Kramer, K.J., *et al.*, 1992. Immunoblot analysis of chitinolytic enzymes in integument and molting fluid of the silkworm, *Bombyx mori* and the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol.* 22, 305–311.
- Koga, D., Isogai, A., Sakuda, S., Matsumoto, S., Suzuki, A., *et al.*, 1987. Specific inhibition of *Bombyx mori* chitinase by allosamidin. *Agric. Biol. Chem.* 51, 471–476.
- Koga, D., Jilka, J., Kramer, K.J., 1983. Insect endochitinases: glycoproteins from moulting fluid, integument and pupal haemolymph. *Insect Biochem.* 13, 295–305.
- Koga, D., Mai, M.S., Dziadik-Turner, C., Kramer, K.J., 1982. Kinetics and mechanism of exochitinase and β -N-acetylglucosaminidase from the tobacco hornworm, *Manduca sexta* L. (*Lepidoptera: Sphingidae*). *Insect Biochem.* 12, 493–499.
- Koga, D., Mitsutomi, M., Kono, M., Matsumiya, M., 1999. Biochemistry of chitinases. In: Jolles, P., Muzzarelli, R.A.A. (Eds.), *Chitin and Chitinases*. Birkhäuser Verlag, Basel, pp. 111–123.
- Koga, D., Sasaki, Y., Uchiumi, Y., Hirai, N., Arakane, Y., *et al.*, 1997. Purification and characterization of *Bombyx mori* chitinase. *Insect Biochem. Mol. Biol.* 27, 757–767.
- Kramer, K.J., Corpuz, L., Choi, H., Muthukrishnan, S., 1993. Sequence of a cDNA and expression of the genes encoding epidermal and gut chitinases of *Manduca sexta*. *Insect Biochem. Mol. Biol.* 23, 691–701.
- Kramer, K.J., Dziadik-Turner, C., Koga, D., 1985. Chitin metabolism in insects. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry, and Pharmacology*, vol. 3. Pergamon Press, Oxford, pp. 75–115.
- Kramer, K.J., Hopkins, T.L., Schaefer, J., 1988. Insect cuticle structure and metabolism. *Adv. Chem.* 379, 160–185.
- Kramer, K.J., Hopkins, T.L., Schaefer, J., 1995. Applications of solids NMR to the analysis of insect sclerotized structures. *Insect Biochem. Mol. Biol.* 25, 1067–1080.
- Kramer, K.J., Koga, D., 1986. Insect chitin: physical state, synthesis, degradation and metabolic regulation. *Insect Biochem.* 16, 851–877.
- Kramer, K.J., Muthukrishnan, S., 1997. Insect chitinases: molecular biology and potential use as biopesticides. *Insect Biochem. Mol. Biol.* 27, 887–900.

- Kramer, K.J., Muthukrishnan, S., Johnson, L., White, F., 1997. Chitinase for insect control. In: Carozzi, N., Koziel, M. (Eds.), *Advances in Insect Control: The Role of Transgenic Plants*. Taylor and Francis, London, pp. 185–193.
- Krieger de Moraes, C., Schrank, A., Vainstein, M.H., 2003. Regulation of extracellular chitinases and proteases in the entomopathogen and acaricide *Metarhizium anisopliae*. *Curr. Microbiol.* 46, 205–210.
- Krishnan, A., Nair, P.N., Jones, D., 1994. Isolation, cloning, and characterization of new chitinase stored in active form in chitin-lined venom reservoir. *J. Biol. Chem.* 269, 20971–20976.
- Kubelka, V., Altmann, F., Marz, L., 1995. The asparagine-linked carbohydrate of honeybee venom hyaluronidase. *Glycoconjugate J.* 12, 77–83.
- Kuranda, M.J., Robbins, P.W., 1991. Chitinase is required for cell separation during growth of *Saccharomyces cerevisiae*. *J. Biol. Chem.* 266, 19758–19767.
- Lanfranco, L., Balsamo, R., Martino, E., Perotto, S., Bonfante, P., 2002. Zinc ions alter morphology and chitin deposition in an ericoid fungus. *Eur. J. Histochem.* 46, 341–350.
- Langsford, M.L., Gilkes, N.R., Singh, B., Moser, B., Miller, R.C., et al., 1987. Glycosylation of bacterial cellulases prevents proteolytic cleavage between functional domains. *FEBS Lett.* 25, 163–167.
- Lehtio, J., Sugiyama, J., Gustavsson, M., Fransson, L., Linder, M., et al., 2003. The binding specificity and affinity determinants of family 1 and family 3 cellulose binding modules. *Proc. Natl Acad. Sci. USA* 100, 484–489.
- Limón, M.C., Margolles-Clark, E., Benítez, T., Penttilä, M., 2001. Addition of substrate-binding domains increases substrate-binding capacity and specific activity of a chitinase from *Trichoderma harzianum*. *FEMS Microbiol. Lett.* 198, 57–63.
- Linder, M., Salovuori, I., Ruohonen, L., Teeri, T.T., 1996. Characterization of a double cellulose-binding domain: synergistic high affinity binding to crystalline cellulose. *J. Biol. Chem.* 271, 21268–21272.
- Linder, M., Teeri, T.T., 1997. The roles and function of cellulose-binding domains. *J. Biotechnol.* 57, 15–28.
- Liu, M., Cai, Q.X., Liu, H.Z., Zhang, B.H., Yan, J.P., et al., 2002. Chitinolytic activities in *Bacillus thuringiensis* and their synergistic effects on larvicidal activity. *J. Appl. Microbiol.* 93, 374–379.
- Locke, M., Huie, P., 1979. Apolysis and the turnover of plasma membrane plaques during cuticle formation in an insect. *Tissue and Cell* 11, 277–291.
- Lu, Y., Zen, K.C., Muthukrishnan, S., Kramer, K.J., 2002. Site-directed mutagenesis and functional analysis of active site acidic amino acid residues D142, D144 and E146 in *Manduca sexta* (tobacco hornworm) chitinase. *Insect Biochem. Mol. Biol.* 32, 1369–1382.
- Lucero, H.A., Kuranda, M.J., Bulik, D.A., 2002. A nonradioactive, high throughput assay for chitin synthase activity. *Anal. Biochem.* 305, 97–105.
- Lysenko, O., 1976. Chitinase of *Serratia marcescens* and its toxicity to insects. *J. Invert. Pathol.* 27, 385–386.
- Macedo, M.L.R., Andrade, L.B.S., Moraes, R.A., Xavier-Filho, J., 1993. Vicilin variants and the resistance of cowpea (*Vigna unguiculata*) seeds to the cowpea weevil (*Callosobruchus maculatus*). *Comp. Biochem. Physiol. C* 105, 89–94.
- Macedo, M.L.R., Damico, D.C.S., Freire, M. D. G. M., Toyama, M.H., Marangoni, S., et al., 2003. Purification and characterization of an N-acetylglucosamine-binding lectin from *Koelreuteria paniculata* seeds and its effect on the larval development of *Callosobruchus maculatus* (Coleoptera: Bruchidae) and *Anagasta kuehniella* (Lepidoptera: Pyralidae). *J. Agric. Food Chem.* 51, 2980–2986.
- McCall, C., Hunter, S., Stedman, K., Weber, E., Hillier, A., et al., 2001. Characterization and cloning of a major high molecular weight house dust mite allergen (Der f 15) for dogs. *Veter. Immunol. Immunopathol.* 78, 231–247.
- McLean, B.W., Boraston, A.B., Brouwer, D., Sanaie, N., Fyfe, C.A., et al., 2002. Carbohydrate-binding modules recognize fine substructures of cellulose. *J. Biol. Chem.* 277, 50245–50254.
- Mikitani, K., Sugasaki, T., Shimada, T., Kobayashi, M., Gustafsson, J.A., 2000. The chitinase gene of the silkworm, *Bombyx mori*, contains a novel Tc-like transposable element. *J. Biol. Chem.* 275, 37725–37732.
- Mikolajczyk, P., Oberlander, H., Silhacek, D., Ishaaya, I., Shaaya, E., 1994. Chitin synthesis in *Spodoptera frugiperda* wing imaginal discs. 1. Chlorfluzuron, diflubenzuron, and teflubenzuron inhibit incorporation but not uptake of [¹⁴C]-N-acetyl-D-glucosamine. *Arch. Insect Biochem. Physiol.* 25, 245–258.
- Morlais, I., Severson, D.W., 2001. Identification of a polymorphic mucin-like gene expressed in the midgut of the mosquito, *Aedes aegypti*, using an integrated bulked segregant and differential display analysis. *Genetics* 158, 1125–1136.
- Morris, O.N., 1976. A 2-year study of the efficacy of *Bacillus thuringiensis*–chitinase combinations in spruce budworm (*Choristoneura fumiferana*) control. *Can. Entomol.* 108, 225.
- Mota, A.C., DaMatta, R.A., Filho, M.L., Silva, C.P., Xavier-Filho, J., 2003. Cowpea (*Vigna unguiculata*) vicilins bind to the peritrophic membrane of larval sugarcane stalk borer (*Diatraea saccharalis*). *J. Insect Physiol.* 49, 873–880.
- Munrow, C.A., Gow, N.A., 2001. Chitin synthesis in human pathogenic fungi. *Med. Mycol.* 39(Suppl. 1), 41–53.
- Muzzarelli, C., Muzzarelli, R.A.A., 2002. Reactivity of quinones towards chitosans. *Trends Glycosci. Glyco-tech.* 14, 223–229.
- Muzzarelli, R.A.A., Littarru, G., Muzzarelli, C., Tosi, G., 2003. Selective reactivity of biochemically relevant quinones towards chitosans. *Carbohydrate Polymers* 53, 109–115.

- Nagahashi, S., Sudoh, M., Ono, N., Sawada, R., Yamaguchi, E., *et al.*, 1995. Characterization of chitin synthase 2 of *Saccharomyces cerevisiae*: implications of two highly conserved domains as possible catalytic sites. *J. Biol. Chem.* 270, 13961–13967.
- Nagamatsu, Y., Yanagisawa, I., Kimoto, M., Okamoto, M., Koga, D., 1995. Purification of a chitoooligosaccharidolytic β -N-acetylglucosaminidase from *Bombyx mori* larvae during metamorphosis and the nucleotide sequence of its cDNA. *Biosci. Biotech. Biochem.* 59, 219–225.
- Nagano, N., Orengo, C.A., Thornton, J.M., 2002. One fold with many functions: the evolutionary relationships between TIM barrel families based on their sequences, structures and functions. *J. Mol. Biol.* 321, 741–765.
- Nagano, N., Porter, C.T., Thornton, J.M., 2001. The ($\beta\alpha$)₈ glycosidases: sequence and structure analyses suggest distant evolutionary relationships. *Protein Eng.* 14, 845–855.
- Neuhaus, J.M., 1999. Plant chitinases (PR-3, PR-4, PR-8, PR-11). In: Datta, S.K., Muthukrishnan, S. (Eds.), Pathogenesis-Related Proteins in Plants. CRC Press, New York, pp. 77–106.
- Nichols, E.J., Beckman, J.M., Hadwiger, L.A., 1980. Glycosidic enzyme activity in pea tissue and pea-*Fusarium solani* interactions. *Plant Physiol.* 66, 199–204.
- Nusslein-Volhard, C., Wieschaus, E., Kluding, H., 1984. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. 1. Zygotic loci on the second chromosome. *Roux's Arch. Devel. Biol.* 193, 267–282.
- Oberlander, H., Silhacek, D., 1998. New perspectives on the mode of action of benzoylphenylurea insecticides. In: Ishaaya, I., Degheele, D. (Eds.), Insecticides with Novel Modes of Action: Mechanism and Application. Springer-Verlag, New York, pp. 92–105.
- Ostrowski, S., Dierick, H.A., Bejsovec, A., 2002. Genetic control of cuticle formation during embryonic development of *Drosophila melanogaster*. *Genetics* 161, 171–182.
- Otsu, Y., Mori, H., Komuta, K., Shimizu, H., Nogawa, S., *et al.*, 2003. Suppression of leaf feeding and oviposition of phytophagous ladybird beetles (Coleoptera: Coccinellidae) by chitinase gene-transformed phylloplane bacteria and their specific bacteriophages entrapped in alginate gel beads. *J. Econ. Entomol.* 96, 555–563.
- Palli, S.R., Retnakaran, A., 1999. Molecular and biochemical aspects of chitin synthesis inhibition. In: Jolles, P., Muzzarelli, R.A.A. (Eds.), Chitin and Chitinases. Birkhäuser Verlag, Basel, pp. 85–98.
- Patil, R.S., Ghormade, V., Deshpande, M.V., 2000. Chitinolytic enzymes: an exploration. *Enzyme Microbiol. Technol.* 26, 473–483.
- Paulson, J.C., 1989. Glycoproteins: what are the sugar chains for? *Trends Biochem. Sci.* 14, 272–276.
- Perchan, T., Cohen, A., Williams, W.P., Luthe, D.S., 2002. Insect feeding mobilizes a unique plant defense protease that disrupts the peritrophic matrix of caterpillars. *Proc. Natl Acad. Sci. USA* 99, 13319–13323.
- Perrakis, A., Tews, I., Dauter, Z., Oppenheim, A.B., Chet, I., *et al.*, 1994. Crystal structure of a bacterial chitinase at 2.3 Å resolution. *Structure* 2, 1169–1180.
- Quentin, M., Ebbelaar, M., Derksen, J., Mariani, C., Van der Valk, H., 2002. Description of a cellulose-binding domain and a linker sequence from *Aspergillus* fungi. *Appl. Microbiol. Biotechnol.* 58, 658–662.
- Quesada-Allue, L.A., 1982. The inhibition of insect chitin biosynthesis by tunicamycin. *Biochem. Biophys. Res. Commun.* 105, 312–319.
- Raikhel, N.V., Lee, H.-I., Broekaert, W.F., 1993. Structure and function of chitin-binding proteins. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 44, 591–615.
- Ramalho-Ortigão, J.M., Traub-Csekö, Y.M., 2003. Molecular characterization of *Llchit1*, a midgut chitinase cDNA from the leishmaniasis vector *Lutzomyia longipalpis*. *Insect Biochem. Mol. Biol.* 33, 279–287.
- Rao, F.V., Houston, D.R., Boot, R.G., Aerts, J. M. F. G., Sakuda, S., van Aalten, D.M.F., 2003. Crystal structures of allosamidin derivatives in complex with human macrophage chitinase. *J. Biol. Chem.* 278, 20110–20116.
- Rayms-Keller, A., McGaw, M., Oray, C., Carlson, J.O., Beaty, B.J., 2000. Molecular cloning and characterization of a metal responsive *Aedes aegypti* mucin cDNA. *Insect Mol. Biol.* 9, 419–426.
- Read, S.M., Bacic, T., 2002. Prime time for cellulose. *Science* 295, 59–60.
- Rebers, J.E., Willis, J.H., 2001. A conserved domain in arthropod cuticular proteins binds chitin. *Insect Biochem. Mol. Biol.* 31, 1083–1093.
- Receveur, V., Czjzek, M., Schülein, M., Panine, P., Heinrissat, B., 2002. Dimension, shape, and conformational flexibility of a two domain fungal cellulase in solution probed by small angle X-ray scattering. *J. Biol. Chem.* 277, 40887–40892.
- Regev, A., Keller, M., Strizhov, N., Sneh, B., Prudovsky, E., *et al.*, 1996. Synergistic activity of a *Bacillus thuringiensis* delta-endotoxin and a bacterial endochitinase against *Spodoptera littoralis* larvae. *Appl. Environ. Microbiol.* 62, 3581–3586.
- Renkema, G.H., Boot, R.G., Au, F.L., Donker-Koopman, W.E., Strijland, A., *et al.*, 1998. Chitotriosidase, a chitinase, and the 39-kDa human cartilage glycoprotein, a chitin-binding lectin, are homologues of family 18 glycosyl hydrolases secreted by human macrophages. *Eur. J. Biochem.* 251, 504–509.
- Richmond, T., 2000. Higher plant cellulose synthases. *Genome Biol.* 1, 3001–3006.
- Rogers, S., Wells, R., Rechsteiner, M., 1986. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* 234, 364–368.
- Roseland, C.R., Grodowitz, M.J., Kramer, K.J., Hopkins, T.L., Broce, A.B., 1985. Stabilization of mineralized and sclerotized puparial cuticle of muscid flies. *Insect Biochem.* 15, 521–528.

- Royer, V., Fraichard, S., Bouhin, H., 2002. A novel putative insect chitinase with multiple catalytic domains: hormonal regulation during metamorphosis. *Biochem. J.* 366, 921–928.
- Saito, A., Fujii, T., Miyashita, K., 2003. Distribution and evolution of chitinase genes in *Streptomyces* species: involvement of gene-duplication and domain-deletion. *Antonie van Leeuwenhoek* 84, 7–16.
- Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425.
- Sales, M.P., Pimenta, P.P., Paes, N.S., Grossi-de-Sá, M.F., Xavier-Filho, J., 2001. Vicilins (7S storage globulins) of cowpea (*Vigna unguiculata*) seeds bind to chitinous structures of the midgut of *Callosobruchus maculatus* (Coleoptera: Bruchidae) larvae. *Brazil. J. Med. Biol. Res.* 34, 27–34.
- Sampson, M.N., Gooday, G.W., 1998. Involvement of chitinases of *Bacillus thuringiensis* during pathogenesis in insects. *Microbiology* 144, 2189–2194.
- Sarauer, B.L., Gillott, C., Hegedus, D., 2003. Characterization of an intestinal mucin from the peritrophic matrix of the diamondback moth, *Plutella xylostella*. *Insect Mol. Biol.* 12, 333–343.
- Schaefer, J., Kramer, K.J., Garbow, J.R., Jacob, G.S., Stejskal, E.O., et al., 1987. Aromatic cross-links in insect cuticle: detection by solid-state ^{13}C and ^{15}N NMR. *Science* 235, 1200–1204.
- Screen, S.E., Hu, G., St. Leger, R.J., 2001. Transformants of *Metarhizium anisopliae* sf. *anisopliae* over expressing chitinase from *Metarhizium anisopliae* sf. *acidum* show early induction of native chitinase but are not altered in pathogenicity to *Manduca sexta*. *J. Invert. Pathol.* 78, 260–266.
- Shapiro, M., Preisler, H.K., Robertson, J.L., 1987. Enhancement of baculovirus activity on gypsy moth (Lepidoptera: Limantidae) by chitinase. *J. Econ. Entomol.* 80, 1113–1115.
- Shen, Z., Jacobs-Lorena, M., 1997. Characterization of a novel gut-specific gene from the human malaria vector *Anopheles gambiae*. *J. Biol. Chem.* 272, 28895–28900.
- Shen, Z., Jacobs-Lorena, M., 1999. Evolution of chitin-binding proteins in invertebrates. *J. Mol. Evol.* 48, 341–347.
- Shinoda, T., Kobayashi, J., Matsui, M., Chinzei, Y., 2001. Cloning and functional expression of a chitinase cDNA from the common cutworm, *Spodoptera litura*, using a baculovirus lacking the virus-encoded chitinase gene. *Insect Biochem. Mol. Biol.* 31, 521–532.
- Shutov, A.D., Kakhovskaya, I.A., Braun, H., Bäumlein, H., Müntz, K., 1995. Legumin-like and vicilin-like seed storage proteins: evidence for a common single-domain ancestral gene. *J. Mol. Evol.* 41, 1057–1069.
- Smirnov, W.A., Valero, R., 1972. Perturbations métaboliques chez *Choristoneura fumiferana* Clemens au cours de l'infection par *Bacillus thuringiensis* seul ou en présence de chitinase. *Rev. Con. Biol.* 31, 163.
- Sneh, B., Schuster, S., Gross, S., 1983. Biological control of *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) by *Bacillus thuringiensis* subsp. *entomocidus* and *Bracon hebetor* Say (Hymenoptera: Braconidae). *Z. Ang. Entomol.* 96, 77–83.
- Spindler, K.D., Spindler-Barth, M., 1999. Inhibitors of chitinases. In: Jolles, P., Muzzarelli, R.A.A. (Eds.), *Chitin and Chitinases*. Birkhäuser Verlag, Basel, pp. 201–209.
- Srisodsuk, M., Reinikainen, T., Penttilä, M., Teeri, T., 1993. Role of the interdomain linker peptide of *Trichoderma reesei* cellobiohydrolase I in its interaction with crystalline cellulose. *J. Biol. Chem.* 268, 20756–20761.
- St. Leger, R.J., Joshi, L., Bidochka, M.L., Rizzo, N.W., Roberts, D.W., 1996. Characterization and ultrastructural localization of chitinases from *Metarhizium anisopliae*, *M. flavoviride*, and *Beauveria bassiana* during fungal invasion of host (*Manduca sexta*) cuticle. *Appl. Environ. Microbiol.* 62, 907–912.
- Suetake, T., Tsuda, S., Kawabata, S., Miura, K., Iwanaga, S., et al., 2000. Chitin-binding proteins in invertebrates and plants comprise a common chitin-binding motif. *J. Biol. Chem.* 275, 17929–17932.
- Sun, Y.J., Chang, N.C., Hung, S.I., Chang, A.C., Chou, C.C., et al., 2001. The crystal structure of a novel mammalian lectin, Ym1, suggests a saccharide-binding site. *J. Biol. Chem.* 276, 17507–17514.
- Sundara Rajulu, G., Aruchami, A., Gowri, N., 1982. Natural deacetylation of chitin to chitosan in the abdominal cuticle of the physogastric queen of *Macrotermes estherae*. In: Proc. 2nd Int. Conf. Chitin and Chitosan, cited in Gooday, G. W., 1985. Chitin deacetylases in invertebrates. In: Muzzerelli, R., Jeuniaux, C., Gooday, G.W. (Eds.), *Chitin in Nature and Technology*. Plenum Press, New York, pp. 263–268.
- Suzuki, K., Taiyoji, M., Sugawara, N., Nikaidou, N., Hernissat, B., et al., 1999. The third chitinase gene (*ChiC*) of *Serratia marcescens* 2170 and the relationship of its product to other bacterial chitinases. *Biochem. J.* 343, 587–596.
- Takahashi, M., Kiuchi, M., Kamimura, M., 2002. A new chitinase-related gene, *BmChiR1*, is induced in the *Bombyx mori* anterior silk gland at molt and metamorphosis by ecdysteroid. *Insect Biochem. Mol. Biol.* 32, 147–151.
- Tantimavanich, S., Pantuwatana, S., Bhumiratana, A., Panbangred, W., 1997. Cloning of a chitinase gene into *Bacillus thuringiensis* subsp. *aizawai* for enhanced insecticidal activity. *J. Gen. Appl. Microbiol.* 43, 341–347.
- Tellam, R.L., 1996. Protein motifs in filarial chitinases: an alternative view. *Parasitol. Today* 12, 291–292.
- Tellam, R.L., Vuocolo, T., Eisemann, C., Briscoe, S., Riding, G., et al., 2003. Identification of an immunoprotective mucin-like protein, peritrophin-55, from the peritrophic matrix of *Lucilia cuprina* larvae. *Insect Biochem. Mol. Biol.* 33, 239–252.
- Tellam, R.L., Vuocolo, T., Johnson, S.E., Jarmey, J., Pearson, R.D., 2000. Insect chitin synthase cDNA sequence, gene organization and expression. *Eur. J. Biochem.* 267, 6025–6043.

- Tellam, R.L., Wijffels, G., Willadsen, P., 1999. Peritrophic matrix proteins. *Insect Biochem. Mol. Biol.* 29, 87–101.
- Tharanathan, R.N., Kittur, F.S., 2003. Chitin: the undisputed biomolecule of great potential. *Crit. Rev. Food Sci. Nutri.* 43, 61–87.
- Thomas, C.J., Gooday, G.W., King, L.A., Possee, R.D., 2000. Mutagenesis of the active site coding region of the *Autographa californica* nucleopolyhedrovirus *chiA* gene. *J. Gen. Virol.* 81, 1403–1411.
- Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680.
- Thummel, C.S., 1996. Flies on steroids: *Drosophila* metamorphosis and the mechanisms of steroid hormone action. *Trends Genet.* 12, 306–310.
- Tjoelker, L.W., Gosting, L., Frey, S., Hunter, C.L., Trong, H.L., et al., 2000. Structural and functional definition of the human chitinase chitin-binding domain. *J. Biol. Chem.* 275, 514–520.
- Tomme, P., Warren, R.A., Miller, R.C., Kilburn, D.G., Gilkes, N.R., 1995. In: Saddler, J.N., Penner, M. (Eds.), *Enzymatic Degradation of Insoluble Polysaccharides*. American Chemical Society, Washington, DC, pp. 142–163.
- Tsigos, I., Martinou, A., Kafetzopoulos, D., Bouriotis, V., 2000. Chitin deacetylases: new, versatile tools in biotechnology. *Trends Biotechnol.* 18, 305–312.
- Turner, C.D., Koga, D., Mai, M.S., Kramer, K.J., 1981. Purification and characterization of two β -N-acetylhexosaminidases from the tobacco hornworm, *Manduca sexta* (L.) (Lepidoptera: Sphingidae). *Arch. Biochem. Biophys.* 212, 546–560.
- Uchiyama, T., Katouno, F., Nikaidou, N., Nonaka, T., Sugiyama, J., et al., 2001. Roles of the exposed aromatic residues in crystalline chitin hydrolysis by chitinase A from *Serratia marcescens* 2170. *J. Biol. Chem.* 276, 41343–41349.
- Väljamäe, P., Pettersson, G., Johansson, G., 2001. Mechanism of substrate inhibition in cellulose synergistic degradation. *Eur. J. Biochem.* 268, 4520–4526.
- van Aalten, D. 2003. Ligand-induced signaling and conformational change of the 39 kD glycoprotein from human chondrocytes. Proc. 9th Int. Chitin Chitosan Conf., Montreal, Abstract Ha. 1, p. 6.
- Venegas, A., Goldstein, J.C., Beauregard, K., Oles, A., Abdulhayoglu, N., et al., 1996. Expression of recombinant microfilarial chitinase and analysis of domain function. *Mol. Biochem. Parasitol.* 78, 149–159.
- Verloop, A., Ferrell, C.D., 1977. Benzoylphenylureas: a new group of insecticides interfering with chitin deposition. *Pesticide Chem. 20th Cent.* 37, 237–270.
- Veronico, P., Gray, L.J., Jones, J.T., Bazzicalupo, P., Arbucci, S., et al., 2001. Nematode chitin synthases: gene structure, expression and function in *Caenorhabditis elegans* and the plant parasitic nematode *Meloidogyne artiellia*. *Mol. Genet. Genomics* 266, 28–34.
- Wang, P., Granados, R.R., 1997. Molecular cloning and sequencing of a novel invertebrate intestinal mucin cDNA. *J. Biol. Chem.* 272, 16663–16669.
- Wang, P., Granados, R.R., 2000a. Molecular structure of the peritrophic membrane (PM): identification of potential PM target sites for insect control. *Arch. Insect Biochem. Physiol.* 47, 110–118.
- Wang, P., Granados, R.R., 2000b. Calcofluor disrupts the midgut defense system in insects. *Insect Biochem. Mol. Biol.* 30, 135–143.
- Wang, X., Ding, X., Gopalakrishnan, B., Morgan, T.D., Johnson, L., et al., 1996. Characterization of a 46 kDa insect chitinase from transgenic tobacco. *Insect Biochem. Mol. Biol.* 26, 1055–1064.
- Watanabe, T., Ariga, Y., Sato, U., Toratani, T., Hashimoto, M., et al., 2003. Aromatic residues within the substrate-binding cleft of *Bacillus circulans* chitinase A1 are essential for crystalline chitin hydrolysis. *Biochem. J.* 376, 237–244. Immediate Publication, doi:10.1042/BJ20030419.
- Watanabe, T., Kobori, K., Miyashita, K., Fujii, T., Sakai, H., et al., 1993. Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity. *J. Biol. Chem.* 268, 18567–18572.
- Watanabe, T., Kono, M., 2002. Molecular studies of family 18 chitinases in animals: structural and functional diversity. *Chitin Chitosan Res.* 8, 95–104.
- Watanabe, T., Oyangi, W., Suzuki, K., Tanaka, H., 1990. Chitinase system of *Bacillus circulans* WL-12 and importance of chitinase A1 in chitin degradation. *J. Bacteriol.* 172, 4017–4022.
- Watanabe, T., Uchida, M., Kobori, K., Tanaka, H., 1994. Site-directed mutagenesis of the Asp-197 and Asp-202 residues in chitinase A1 of *Bacillus circulans* WL-12. *Biosci. Biotechnol. Biochem.* 58, 2283–2285.
- Wiechaus, E., Nusslein-Volhard, C., Jurgens, G., 1984. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. 1 Zygotic loci on the X chromosome and the fourth chromosome. *Roux's Arch. Devel. Biol.* 193, 296–307.
- Wilson, T.G., Cryan, J.R., 1997. Lufenuron, a chitin-synthesis inhibitor, disrupts development of *Drosophila melanogaster*. *J. Exp. Biol.* 278, 37–44.
- Wiwat, C., Lertcanawanichkul, M., Siwayapran, P., Pantuwatana, S., Bhumiratan, A., 1996. Expression of chitinase encoding genes from *Aeromonas hydrophila* and *Pseudomonas maltophilia* in *Bacillus thuringiensis* subsp. *israelensis*. *Gene* 179, 119–126.
- Wiwat, C., Thaithanun, S., Pantuwatana, S., Bhumiratan, A., 2000. Toxicity of chitinase-producing *Bacillus thuringiensis* sp. *kurstaki* HD-1 toward *Plutella xylostella*. *J. Invert. Path.* 76, 270–277.
- Wormleaton, S., Kuzio, J., Winstanley, D., 2003. The complete sequence of the *Adoxophyes orana* granulovirus genome. *Virology* 311, 350–365.
- Yan, J., Cheng, Q., Narashimhan, S., Li, C.B., Aksoy, S., 2002. Cloning and functional expression of a fat

- body-specific chitinase cDNA from the tsetse fly, *Glossina morsitans morsitans*. *Insect Biochem. Mol. Biol.* 32, 979–989.
- Zechel, D.L., Withers, S.G., 2000. Glycosidase mechanisms: anatomy of a finely tuned catalyst. *Acc. Chem. Res.* 33, 11–18.
- Zen, K.C., Choi, H.K., Nandegama, K., Muthukrishnan, S., Kramer, K.J., 1996. Cloning, expression and hormonal regulation of an insect β -N-acetylglucosaminidase gene. *Insect Biochem. Mol. Biol.* 26, 435–444.
- Zhang, H., Huang, X., Fukamizo, T., Muthukrishnan, S., Kramer, K.J., 2002. Site-directed mutagenesis and functional analysis of an active site tryptophan of insect chitinase. *Insect Biochem. Mol. Biol.* 32, 1477–1488.
- Zheng, Y., Zheng, S., Cheng, X., Ladd, T., Lingohr, E.J., *et al.*, 2002. A molt-associated chitinase cDNA from the spruce budworm, *Choristoneura fumiferana*. *Insect Biochem. Mol. Biol.* 32, 1813–1823.
- Zheng, Y.P., Retnakaran, A., Krell, P.J., Arif, B.M., Primavera, M., *et al.*, 2003. Temporal, spatial and induced expression of chitinase in the spruce budworm, *Choristoneura fumiferana*. *J. Insect Physiol.* 49, 241–247.
- Zhou, Y., Wang, F., Xiao, X., 2002. Structures of the cloned chitinase and its truncant from *A. caviae*. *Prog. Nat. Sci.* 12, 587–592.
- Zhu, Y.C., Muthukrishnan, S., Specht, C.A., Dittmer, N., Kanost, M.R., *et al.*, 2002. Sequence of a cDNA and expression of the gene encoding a putative epidermal chitin synthase of *Manduca sexta*. *Insect Biochem. Mol. Biol.* 32, 1497–1506.
- Zimoch, L., Merzendorfer, H., 2002. Immunolocalization of chitin synthase in the tobacco hornworm. *Cell Tissue Res.* 308, 287–297.

12 Cuticular Proteins

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12.1. Introduction

In the previous edition of this series, Silvert (1985) outlined several major areas of uncertainty regarding cuticular proteins. The questions raised were: Were proteins extracted from cuticle authentic cuticular proteins or might some be contaminants of adhering cells and hemolymph? Was the epidermis the sole site of synthesis of cuticular proteins or were some synthesized in other tissues and transported to the cuticle? What was the relation among cuticular proteins of various developmental stages? Did cuticular proteins share common structural features?

That article presented all the cuticular protein sequence data then available – four complete and three partial sequences from *Drosophila melanogaster* and one partial sequence from *Sarcophaga bullata*. The considerable sequence similarity seen with those limited data indicated that cuticular protein genes belonged to multigene families, and

the even more limited genomic information revealed that similar genes were adjacent on a chromosome.

Progress in less than two decades has been spectacular, but not surprising given the advances in relevant techniques. Elegant immunolocalization analyses have solved the problem of the sources of cuticular proteins. Over 300 cuticular protein sequences are now available from six orders and over 20 species of insects. Listed in Tables 1 and 2 are all but those that come exclusively from the annotation of the genomes of *D. melanogaster* and *Anopheles gambiae* or SilkBase, an extensive expressed sequence tag (EST) project in *Bombyx mori* (Mita *et al.*, 1999; SilkBase, 2003). Throughout this chapter, proteins are referred to by the names used in Tables 1 and 2. These tables also provide a gi number or an identifier from SwissProt that provides access to the complete sequence and the relevant references. Recombinant proteins have

Table 1 Characteristics of "structural" cuticular proteins that have been completely sequenced

<i>Order/species</i>	<i>Protein name</i>	<i>Number of amino acids^a</i>	<i>Type</i>	<i>AAP(A/V) Repeats^b</i>	<i>Other features^c</i>	<i>Sequence method^d</i>	<i>Identifier^e</i>
Coleoptera							
<i>Apriona germari</i>	LCP10.7	87	RR-1			CT	16226511
	LCP12.3	114	RR-1			CT	21617523
	LCP12.6	120	RR-1			CT	21617525
<i>Tenebrio molitor</i>	TM-LCP-A1A	174	RR-2	2N 1C	2[AAP(I/L)]	DS	1706191
	TM-LCP-A2B	117	RR-2	1N 1C		DS	1706192
	TM-LCP-A3A	134	RR-2	1N 1C	1[AAP(I/L)]	DS	1706194
	TM-PCP-C1B	161		9	51 aa motif 1[AAP(I/L)]	DS	1706197
	TM-E1A	243		22		DS	913040
	TM-F1A	154		3	5 [AAP(I/L)]	DS	998953
	TM1-F1B	158		4	4 [AAP(I/L)]	DS	998954
	TM-F1C	162		4	5 [AAP(I/L)]	DS	998955
	TM-PCP-G1A	211		5	51 aa motif	DS	1706198
	TM-H1C = TMLPCP22	195		4	51 aa motif 1[AAP(I/L)]	CT DS	3123202*
	PCP5.8	53				DS	7511760
	PCP9.2	92				DS	7511761
	PCP15.6	161		5	1[AAP(I/L)]	DS	7441995
	PCP16.7	166				DS	7511759
	TMLPCP-23	214		2	51 aa motif 1[AAP(I/L)]	CT	3121955*
	TMLPCP29	276	RR-3	11N	1 (18 residue motif) 1[AAP(I/L)]	CT	2275132*
ACP17	167		2	High G	CT	1078986*	
ACP20	191	RR-2		High G	CT	102879	
ACP-22	180	RR-2		2(6G), 1(8G), 1(15G)	CT	113012*	
Dictyoptera							
<i>Blaberus craniifer</i>	BC-NCP1	87			Has 6 Cys, forming 3 potential S-S	DS	3023587
	BC-NCP2	99			8 As; 2 (18 residue motif)	DS	3023589
	BC-NCP4	127			2 (18 residue motif)	DS	3023588
	BC-NCP5	145			2 (18 residue motif)	DS	P82118
	BC-NCP6	139	RR-3		1 (18 residue motif)	DS	P82119
	BC-NCP7	145	RR-3		1 (18 residue motif)	DS	P82120
	BC-NCP8	195	RR-2			DS	P82121
	BC-NCP9	34				DS	P82122
	Diptera						
<i>Anopheles gambiae</i>	AGCP2A	214	RR-2	1N	4 [(S/A)APIAH]	CT	2961109*
	AGCP2B	222	RR-2	1N	5 [(S/A)APIAH]	CT	2961110*
	AGCP2C	214	RR-2	2N	5 [(S/A)APIAH]	CT	2961111*
	AGCP2D	215	RR-2	2N	4 [(S/A)APIAH]	CT	2961113
<i>Drosophila melanogaster</i>	Dacp-1	116		3		CT	7580461*
	LCP-1	114	RR-1			DS CT	17380379*
	LCP-2	110	RR-1			DS CT	117634*
	LCP-3	96	RR-1			DS CT	117635*
	LCP-4	96	RR-1			DS CT	117636*
	PCP = GART INTRON	166	RR-1			CT	157483*
	ACP65A	85	RR-1			CT	1857602*
	LCP65Aa	83	RR-1			CT	1857600*
	LCP65Ab1	86	RR-1			CT pDS	1857597*
	LCP65Ab2	86	RR-1			CT pDS	1857595*
	LCP65Ac	91	RR-1			CT	1857593*
	LCP65Ad	90	RR-1			CT	1857495*
	LCP65Ae	83	RR-1			CT	1857604*
	LCP65Af	84	RR-1			CT	1857606*
LCP65Ag1	87	RR-1			CT pDS	1857608*	
LCP65Ag2	87	RR-1			CT pDS	1857610*	
EDG-78	106	RR-1			CT	117639*	
EDG-84	171	RR-2	1C		CT	117640*	

Table 1 Continued

Order/species	Protein name	Number of amino acids ^a	Type	AAP(A/V) Repeats ^b	Other features ^c	Sequence method ^d	Identifier ^e
	EDG91	138			8 (2G) 6(3G) 3 (4G)	CT	17380419*
	Ccp84Aa	188	RR-2	1N 6C		CT	4389433*
	Ccp84Ab	204	RR-2	1N 7C		CT	4389434*
	Ccp84Ac	199	RR-2	2C		CT	4389435*
	Ccp84Ad	182	RR-2	1N 6C		CT	4389436*
	Ccp84Ae	191	RR-2	1N 3C		CT	4389437*
	Ccp84Af	134	RR-2	1N		CT	4389438*
	Ccp84Ag	173	RR-2	6C		CT	4389439*
	Cry	457	RR-2		7Q, internal M	CT	22946279*
<i>Drosophila miranda</i>	LCP-1	122	RR-1			CT	3023591*
	LCP-2	110	RR-1			CT pDS	3023592
	LCP-3	96	RR-1			CT	231917*
	LCP-4	96	RR-1			CT	231917*
	LCP-3Y	96	RR-1			CT pDS	386246
<i>Drosophila pseudoobscura</i>	CP = GART	177	RR-1			CT	435017*
	INTRON						
<i>Drosophila simulans</i>	DS-06238.4-like	188	RR-2			CT	9966434*
<i>Drosophila yakuba</i>	DS-06238.4-like	194	RR-2			CT	9966436*
<i>Lucilia cuprina</i>	CUT1	102	RR-1			CT	2565392
	CUT12	89	RR-1			CT	2565394
Hemiptera							
<i>Myzus persicae</i>	CP	208	RR-2	1N 4C	[(S/K)APAY] 1N 10C	CT	16798648
<i>Aphis fabae</i>	CP	208	RR-2	3C	[(S/K)APAY] 1N 12C	CT	29124938
<i>Aphis gossypii</i>	CP	203	RR-2	1N 3C	[(S/K)APAY] 10C	CT	29124934
<i>Brevicoryne brassicae</i>	CP	208	RR-2	1N 2C	[(S/K)APAY] 1N 12C	CT	29124932
<i>Lipaphis erysimi</i>	CP	208	RR-2	1N 3C	[(S/K)APAY] 1N 11C	CT	29124930
<i>Rhopalosiphum maidis</i>	CP	210	RR-2	2N 2C	[(S/K)APAY] 13C	CT	29124936
Lepidoptera							
<i>Bombyx mori</i>	PCP	235		3	3 (18 residue motif)	CT	1169137*
	BMWCP1A	205	RR-2	5N		CT	12862579
	BMWCP1B	205	RR-2	5N		CT	12862581
	BMWCP2	231	RR-2	9N		CT	12862583
	BMWCP3	215	RR-2	7N		CT	12862585
	BMWCP4	226	RR-2	3N		CT	12862587
	BMWCP5	274	RR-2	2N 1C		CT	12862589
	BMWCP6	186	RR-2	2N 2C		CT	
	BMWCP7A	157	RR-2	1N		CT	12862593
	BMWCP7B	157	RR-2	1N		CT	12862595
	BMWCP8	221	RR-2			CT	12862597
	BMWCP9	158	RR-1			CT	12862599
	BMWCP10	295	RR-1		Internal M	CT	23096118
	EDG84A	180	RR-2	1N 1C		CT	3608259
	homologue						
	BMCP17	127	RR-1	1N		CT pDS	2204069
	BMCP18	89	RR-1	1N		CT pDS	5360249*
	BMCP22	158	RR-1			CT pDS	2204071
	BMCP30	223	RR-1			CT pDS	6634056*
	GCP1	149			17(GGY)	CT	15146344
<i>Galleria melonella</i>	PCP-52	338		1	Has 2 Cys; 21% Ala	CT	1086154
<i>Helicoverpa armigera</i>	LCP-1	95	RR-1		VVVV	CT	3913261
<i>Hyalophora cecropia</i>	HCCP12	89	RR-1			CT pDS	1169129*

Continued

Table 1 Continued

<i>Order/species</i>	<i>Protein name</i>	<i>Number of amino acids^a</i>	<i>Type</i>	<i>AAP(A/V) Repeats^b</i>	<i>Other features^c</i>	<i>Sequence method^d</i>	<i>Identifier^e</i>	
<i>Manduca sexta</i>	HCCP66	112	RR-2			CT pDS	1169133*	
	LCP-14	109	RR-1			CT	117623	
	CP14.6	90	RR-1			CT	3121956*	
	LCP16/17	123	RR-1			CT	3121953*	
	CP20	182	RR-1			7(GG) 3(GGG); glycosylated	CT DS	19548965
	CP27	165	RR-1			Glycosylated	CT DS	19743772
	CP36	327	RR-1			32(GG) 1(GGG); glycosylated	CT DS	22000820
Orthoptera								
<i>Locusta migratoria</i>	LM-ACP-abd4	116	RR-1		3 glycosylated forms	DS	461860	
	LM-ACP-abd5	82	RR-1		Glycosylated	DS	3913394	
	LM-ACP7	131	RR-2	2N 3C		DS	998751	
	LM-ACP8	148	RR-2	5N		DS	84730	
	LM-ACP19	157	RR-2	6N 1C	GYL motif	DS	1345864	
	LM-ACP21	169	RR-2	6N 3C		DS	3287770	
	LM-ACP38	163		14	GYL motif	DS	72263	
	LM-NCP55	33		2		DS	446069	
	LM-NCP62	88				DS	446070	
	LM-ACP63	157		8	GYL motif	DS	1169130	
	LM-ACP64	152		7	GYL motif	DS	1169131	
	LM-ACP65	145		7	GYL motif	DS	1169132	
	LM-ACP67a	98		6	51 aa motif	DS	416850	
	LM-ACP67b	104		6	51 aa motif	DS	542520	
	LM-ACP70	88		4	GYL motif	DS	416852	
	LM-ACP76	139		6	GYL motif; 51 aa motif	DS	1169134	
	LM-ACP79a	131		3	5 [GGG(L/Y)]	DS	1168721	
	LM-ACP79b	131		3	5 [GGG(L/Y)]	DS	1168722	
	LM-NCP4.9	46		1		DS	P82168	
	LM-NCP5.1	48		1		DS	P82169	
	LM-NCP6.4	62				DS	P82170	
	LM-NCP9.5	90				DS	P82171	
	LM-NCP18.7	193				4 (18 residue motif)	DS	P82165
LM-NCP21.3	200			10		DS	P82167	
LM-NCP19.8	200	RR-2	3N 5C			DS	P82166	
<i>Schistocerca gregaria</i>	SGAbd-1	184	RR-1		Glycosylated; (PPPPPPP)	DS	7511754	
	SGAbd-2	135	RR-1		Glycosylated	DS	7511755	
	SGAbd-3	119	RR-1		Glycosylated	DS	7441999	
	SGAbd-4	116	RR-1		Glycosylated	DS	7441997	
	SGAbd-5	82	RR-1		Glycosylated	DS	3913395	
	SGAbd-6	82	RR-1		Glycosylated	DS	7511756	
	SGAbd-8	139	RR-1		Glycosylated; internal M	DS	7511757	
	SGAb-9	129	RR-1		Glycosylated	DS	7441998	

^aSequence length of mature peptide; signal peptides were deleted using data from authors or SignalP V2.0 (<http://www.cbs.dtu.dk/services/SignalP-2.0/>).

^bIf a protein has an R&R Consensus, location of the AAP(A/V) repeats is given relative to the consensus.

^cSee Section 12.3.2.2 for description of these features.

^dDS, direct sequencing of protein, a p indicates only a partial (generally N-terminal) sequence was obtained; CT, conceptual translation of a cDNA, genomic region, or EST product.

^eProtein sequences and additional annotation can be found at <http://www3.ncbi.nlm.nih.gov/Entrez/index.html>. Sequences that have an identifier that begins with a letter can be found at <http://us.expasy.org>. An asterisk indicates that genomic sequence information is available.

Table 2 Characteristics of some nonstructural proteins that have been found in cuticle

Species	Protein name	Number of amino acids ^a	Function	Sequence method ^b	Identifier ^c
<i>Schistocerca gregaria</i>	Putative carotene binding protein	250	Transfers carotene into cuticle	DS	13959527
<i>Caliphora vicina</i>	ARYLPHORIN A4	743	Found in cuticle	CT	114232
	ARYLPHORIN C223	743		CT	114236
<i>Drosophila melanogaster</i>	YELLOW	520	Positions melanin pigment in cuticle	CT	140623
<i>Bombyx mori</i>	CECROPIN A	41	Defense protein	CT	2493573
	CECROPIN B	41	Defense protein	CT	1705754
	PROPHENOLOXIDASE	675	Melanization enzyme	CT	13591614
<i>Calpodes ethlius</i>	CECP22	169	Cuticle digestion	CT	4104409
<i>Manduca sexta</i>	ARYLPHORIN α	684		CT	114240
	ARYLPHORIN β	687		CT	1168527
	INSECTICYANIN A	189	Blue pigment	CT	102968
	INSECTICYANIN B	189	Blue pigment	CT	124527
	SCOLEXIN A	279	Serine protease immune protein	CT	4262357
	SCOLEXIN B	279	Serine protease immune protein	CT	4262359

^aSequence length of mature peptide; signal peptides were deleted using data from authors or SignalP V2.0 (<http://www.cbs.dtu.dk/services/SignalP-2.0/>).

^bDS, direct sequencing of protein; CT, conceptual translation of a cDNA, genomic region, or EST product.

^cProtein sequences and additional annotation can be found at: <http://www3.ncbi.nlm.nih.gov/Entrez/index.html>.

revealed a function for a highly conserved domain that was present in those first protein sequences discussed by Silvert. Structural predictions have elucidated the basis for this function. It is these developments that will be the focus of this review.

12.2. Cuticle Structure and Synthesis

12.2.1. Cuticle Morphology

12.2.1.1. Terminology The descriptive terms used here to describe the regions of cuticle have been simplified according to Locke's (2001) cogent suggestions for new nomenclature. He proposes the use of the term "envelope" to describe the outermost layer of cuticle, rather than the previous term "cuticulin." At the start of each molt cycle, the smooth apical plasma membrane forms microvilli with plaques at their tips where the new envelope assembles. This discrete layer of 10–30 nm not only serves to protect the underlying epidermis from molting fluid enzymes that begin to digest the old cuticle, but, as Locke points out, affects "resistance to abrasion and infection, penetration of insecticides, permeability, surface reflectivity, and physical colors." The sequences and properties of its constituent proteins remain unknown.

Next formed is the epicuticle, about 1 μ m in thickness. This chitin-free layer (but see Section 12.2.1.3) is stabilized by quinones. It was formerly

referred to as the "inner epicuticle" with cuticulin being the outer.

Former arguments about the precise distinction between *exo*- and *endo*-cuticle are eliminated by Locke's lumping of the inner regions of the cuticle under the term "procuticle," encompassing both preecdysial and postecdysial secretions. The procuticle, then, is the region that combines chitin and cuticular proteins in various combinations and becomes sclerotized and pigmented to varying degrees. This is the region depicted in electron micrographs showing stacks of precisely oriented lamellae. According to Locke (2001), apical microvilli bend in concert across the epithelial sheet and this movement serves to orient the laminae that will form lamellae. While knowledge of the process of secreting and assembling such a highly ordered structure is limited, details about the proteins associated with the lamellae are now voluminous.

12.2.1.2. Growth of the cuticle within an instar Central to the issue of cuticle structure is the important fact that considerable cuticle growth can occur during an intermolt period (Williams, 1980), some of it by a smoothing out of macro- and microscopic folds and pleats (Carter and Locke, 1993). During intrainstar growth, new cuticular proteins are interspersed among the old, necessitating a model of chitin-protein and protein-protein

interactions that will permit such intussusception (Condoulis and Locke, 1966; Wolfgang and Riddiford, 1986).

12.2.1.3. Localization of cuticular proteins within the cuticle Precise localization of cuticular proteins within the cuticle and even within cellular organelles has been made possible with immunogold labeling of electron microscopic sections. Here a specific primary antibody is bound to the sections and visualized with a secondary antibody conjugated to colloidal gold particles.

Antibodies have been raised against extracts of whole cuticle or isolated electrophoretic bands and the specificity of each antibody ascertained with Western blots. While each polyclonal antibody raised against a single band was specific for the immunizing protein, monoclonals raised against cuticular extracts frequently reacted with more than one electrophoretic band.

One concern with immunolocalization is that as cuticular proteins become modified in the cuticle by binding to chitin or by becoming sclerotized, the immunizing epitopes might become masked, a problem that should be more serious with monoclonal than polyclonal antibodies. All groups recognized that while the presence of an antigen is significant, its absence may reflect no more than such masking.

This concern is significant when one considers results of immunolocalization in the assembly zone, the region of cuticle directly above the microvilli. It is here that chitin secreted from the tips of the microvilli interacts with cuticular proteins secreted into the perimicrovillar space. Immunolocalization studies revealed only a few of the cuticular proteins within the perimicrovillar space but the same ones and others were abundant in the assembly zone directly above it (Locke *et al.*, 1994; Locke, 1998). The authors' conclusion was that the assembly zone "is where we should expect proteins to unravel and expose most epitopes in preparation for assuming a new configuration as they stabilize in the maturing cuticle." Wolfgang *et al.* (1986, 1987) found two *D. melanogaster* cuticular proteins exclusively in this zone and suggested they might function in cuticle assembly. Locke *et al.* (1994) point out that it was common for antibodies raised against *Calpodes ethlius* proteins to react more strongly with the assembly zone than with more mature regions of cuticle where sclerotization and chitin binding might mask epitopes. Thus more substantial evidence than the failure to detect a protein in more mature regions is needed to confirm that it belonged exclusively to the assembly zone.

It was known from earlier work on protein and mRNA distribution that cuticles from different metamorphic stages and different anatomical regions had different cuticular proteins and that there may be a change in cuticular proteins synthesized by a single cell within a molt cycle (review: Willis, 1996). Such a transition in proteins synthesized is especially apparent at the time of ecdysis, and, in some insects, late in the instar. Consistent with this, immunolocalization revealed different proteins in morphologically distinct early and late lamellae in *D. melanogaster* pupae, and *Tenebrio molitor* and *Manduca sexta* larvae (Doctor *et al.*, 1985; Fristrom *et al.*, 1986; Wolfgang and Riddiford, 1986; Wolfgang *et al.*, 1986; Lemoine *et al.*, 1989, 1993; Bouhin *et al.*, 1992a, 1992b; Rondot *et al.*, 1996). Only two proteins with known sequence are among this group, TMAP22 and TMLPCP22.

Csikos *et al.* (1999) have used immunohistochemistry to follow some of *Manduca's* cuticular proteins throughout the molt cycle. These proteins are obviously in a dynamic state as they move from epidermis to cuticle to molting fluid to fat body and then apparently back to cuticle via the hemolymph. More detailed studies are needed to learn if the same molecules make the return trip, and whether their initial passage from molting fluid into the hemolymph is solely via uptake and then basal secretion by the epidermis or whether the midgut plays a role, since lepidopteran larvae drink their molting fluid (Cornell and Pan, 1983).

The findings with epicuticle, the first region to be secreted beneath the envelope, were complex. None of the monoclonal antibodies that recognized *Tenebrio* cuticular proteins reacted with epicuticle (Lemoine *et al.*, 1990). On the other hand, arylphorin from *Calpodes* has been localized to epicuticle and no other cuticular region (Leung *et al.*, 1989) and several proteins, of unknown sequence, were found both in the epicuticle and in the lamellar regions of the procuticle in *D. melanogaster* (Fristrom *et al.*, 1986) and *Calpodes* (Locke *et al.*, 1994). This finding of cuticular proteins in both epicuticle and lamellar regions was surprising, since the epicuticle had always been described as lacking chitin (cf. Fristrom *et al.*, 1986; Fristrom and Fristrom, 1993) and thus was expected to have unique proteins.

In addition to temporal differences in the secretion of cuticular proteins by single cells, there may be regional differences in the cuticle secreted by single cells. Individual epidermal cells of the articulating membranes (intersegmental membranes) in *Tenebrio* secrete a cuticle with sclerotized cones

embedded in softer cuticle. Two of the classes of monoclonal antibodies raised against *Tenebrio*'s larval and pupal cuticular proteins recognized proteins in these cones. The same antibodies recognized proteins in cuticles in other regions that were destined to be sclerotized. Different antibodies recognized the proteins in the softer cuticle (Lemoine *et al.*, 1990, 1993).

Locke *et al.* (1994) were able, using carefully reconstructed sections of *Calpodes* larval cuticle, to distinguish one protein (C36) that was found with the same distribution as the chitin microfibrils that had been visualized with wheat germ agglutinin, a lectin that recognizes *N*-acetylglucosamine, while other antigens failed to show this distribution. Notably, only C36 isolated from cuticle reacted with wheat germ agglutinin on lectin blots. Based on this evidence Locke *et al.* (1994) suggest that the isolated protein may have obtained its *N*-acetylglucosamine from chitin.

12.2.1.4. Cuticles formed following disruption of normal metamorphosis Treatment of many insects with juvenile hormone (JH) causes them to resynthesize a cuticle with a morphology characteristic of the current metamorphic stage, rather than the next (see Chapter 8). Thus, in *Tenebrio*, treatment of pupae with JH prior to pupal–adult apolysis causes the formation of a second pupa rather than an adult. Earlier work revealed that these second pupae had proteins with the same electrophoretic mobility as those extracted from normal pupae (Roberts and Willis, 1980b; Lemoine *et al.*, 1989). A combination of Northern analysis and *in situ* hybridization demonstrated that second pupae have the same cuticular protein mRNAs and protein localization as normal pupae (Lemoine *et al.*, 1993; Rondot *et al.*, 1996). Adult cuticular proteins are not deposited in these cuticles and the adult mRNAs do not appear (Lemoine *et al.*, 1989, 1993; Bouhin *et al.*, 1992a, 1992b; Charles *et al.*, 1992). Some JH-treated *Tenebrio* pupae form two cuticles, the first pupalike in morphology and the second with adult features. The adultlike cuticle was shown with immunolocalization to have ACP22 (Bouhin *et al.*, 1992a). If JH is applied too late to form a perfect second pupa, the next cuticle formed will be a composite with morphological features of two metamorphic stages (Willis *et al.*, 1982). Bouhin *et al.* (1992b) found that all the epidermal cells laying down such a composite cuticle had mRNAs for ACP22.

Zhou and Riddiford (2002) used Northern analysis to characterize the somewhat nondescript cuticles made by *D. melanogaster* that had been

manipulated by misexpressing the gene, *broad*, that codes for a transcription factor that appears before the larval–pupal molt in flies and moths. By following mRNAs for the adult cuticular protein ACP65A or the pupal cuticular protein Edg78E, they were able to demonstrate the essential role of *broad* in directing pupal development and thereby helped clarify the perplexing action of juvenoids in the higher Diptera.

12.2.2. The Site of Synthesis of Cuticular Proteins

One of the unresolved issues addressed in Silvert's (1985) review was the site of synthesis of cuticular proteins. This might appear to be a trivial issue, for one would expect that the epidermis that underlies the cuticle would synthesize the cuticular proteins. There are, however, reports in the literature that proteins found in the hemolymph were present in cuticle and even that labeled proteins injected into the hemolymph would appear in cuticle. Silvert discussed the possibility that the injected protein had been broken down and resynthesized so that the cuticular protein was labeled solely because its constituent amino acids had come from a labeled pool.

Five methods have now provided data that address the site of synthesis of cuticular proteins. The most common is to use Northern analysis to learn in what tissues and at which stages mRNA is present for a particular cuticular protein. This method is so common that specific examples will not be given. The second method is to incubate epidermis or integument *in vitro* with radioactive amino acids, separate the proteins, and compare the electrophoretic mobility of the labeled proteins to proteins isolated from cleaned cuticles. A third method is to isolate mRNAs from tissues and translate these *in vitro* with commercially available wheat germ extracts or rabbit reticulocytes and compare the translation products to known cuticular proteins. The fourth method is *in situ* hybridization, and the fifth immunolocalization to visualize proteins within the endoplasmic reticulum and Golgi apparatus.

The first three methods suffer from the possibility that tracheae and adhering tissues, fat body, muscles, hemocytes, contribute to the mRNA pool. Both labeling methods suffer from the problem that cuticular proteins are notoriously sensitive to solubilizing buffer and gel conditions (pH, urea concentration) (Cox and Willis, 1987a) and unless cuticular protein standards and labeled translation products are mixed prior to electrophoresis, they may not show identical electrophoretic mobility even in adjacent lanes. Some workers have precipitated labeled

translation products with antibodies raised against extracts of cuticle or individual cuticular proteins, then solubilized the precipitate, run it on a gel, and detected the labeled product with fluorography. Csikos *et al.* (1999) used Western blots of translation products to identify cuticular proteins. Since cuticular proteins are destined for secretion from cells, they have a signal peptide that is cleaved before the protein is secreted into the cuticle. Hence, translation products made *in vitro* will be larger than the protein extracted from cuticle. There are two methods to circumvent this problem. The translation products can have their signal peptides cleaved by adding a preparation of canine microsomes, or antibodies against cuticular proteins (specific or against an extract) can be used to precipitate the translation products before they are solubilized and run on a gel. Either method allows some certainty in the comparison of these *in vitro* translation products with authentic cuticular proteins. It was also found that some commercial preparations of wheat germ extract have endogenous signal peptide processing activity (Binger and Willis, 1990).

Frequently, ^{35}S -methionine was used for metabolic labeling of integument and for *in vitro* translation. This is an unfortunate choice as almost all mature cuticular proteins lack methionine residues (see Section 12.3.2.1). The initiator methionine will be lost along with the entire signal peptide. Clear differences in labeling patterns with ^{35}S -methionine and ^3H -leucine have been found, with none of the major proteins from pharate adult cuticle of *D. melanogaster* or from larval cuticles of *Hyalophora cecropia* showing methionine labeling (Roter *et al.*, 1985; Willis, 1999). Why then did several studies find all of the known cuticular proteins labeled with methionine? Perhaps the finding that ^{35}S -methionine can donate its label to a variety of amino acids in preformed proteins (Browder *et al.*, 1992; Kalinich and McClain, 1992) explains its appearance and suggests that it needs to be used with caution for such studies with cuticular proteins.

The fourth method is *in situ* hybridization, where specific mRNAs can be identified in the epidermis. Results from several studies are summarized in Table 3. *In situ* hybridization allows one to be somewhat more discerning about the site of synthesis of a cuticular protein because it is possible to monitor the presence or absence of a particular mRNA at the level of an individual cell. With this technique, integument is fixed and sectioned and then probed with a labeled cDNA or cRNA allowing the identification of particular regions of the epidermis by examining the morphology of the overlying cuticle. With

most detection methods, contaminating tissues and precise regions of the epidermis can be identified and the presence of the particular mRNA in them can be assessed. Thus this technique identifies the location of the mRNAs recognized by the specific probe used. It was this technique that revealed the precision with which mRNAs are produced, for abrupt boundaries of expression occur between sclerites and intersegmental membranes (Rebers *et al.*, 1997) or at muscle insertion zones (Horodyski and Riddiford, 1989) or next to specialized epidermal cells (Horodyski and Riddiford, 1989; Rebers *et al.*, 1997). This technique even revealed the presence of mRNA for cuticular proteins in epithelia of imaginal disc from young larvae (Gu and Willis, 2003). A limitation of the technique is that cRNA probes sometimes bind to the cuticle itself, possibly obscuring detection of mRNA in the underlying epidermis (Fechtel *et al.*, 1989; Gu and Willis, 2003). Fechtel *et al.* (1989) found this artifact to be cuticle-type as well as strand- and probe-specific. Results from several species are summarized in Table 3.

The fifth method, immunolocalization, was described earlier in conjunction with localization of specific proteins within the cuticle, but it can also be used to identify the site of synthesis by looking for a particular protein within the endoplasmic reticulum or Golgi apparatus (Sass *et al.*, 1994a, 1994b).

The results from Northern analyses, metabolic tissue labeling, and *in vitro* translations reveal that all cuticular proteins with known sequences or for which specific probes are available are synthesized by the integumental preparations. Different proteins are synthesized at different times in a molt cycle and in different anatomical regions and there are some cuticular proteins whose synthesis is stage-specific. Differences in the presence of mRNA parallel the appearance of labeled proteins indicating that much of the temporal and spatial control of cuticular protein synthesis is at the level of transcription. As mentioned above, however, all three of these methods are limited by the possible contamination of tissues by nonepidermal cells and by their inability to address heterogeneity of cell types within the epidermis.

Studies that have combined tissue labeling or *in vitro* translations with immunolocalization have at last clarified the relationship between hemolymph and cuticular proteins with identical electrophoretic and immunological properties. The most comprehensive studies of protein trafficking, carried out in *Calpodes*, revealed four classes of exported proteins that are handled by the epidermis.

Table 3 Evidence for the association of location or type of cuticle and sequence class of some cuticular proteins

Species	Protein	Sequence class	Localization ^a	Nature of evidence ^b	When deposited	Reference
<i>Bombyx mori</i>	BMLCP18	RR-1	Imaginal discs	EST		Gu and Willis (2003)
<i>Drosophila melanogaster</i>	EDG-78	RR-1	Larval and imaginal cells of prepupa	ISH		Fechtel <i>et al.</i> (1989)
<i>Drosophila melanogaster</i>	EDG-84	RR-2	Imaginal disc cells	ISH		Fechtel <i>et al.</i> (1989)
<i>Drosophila melanogaster</i>	PCP	RR-1	Prepupal thorax and abdomen	ISH		Henikoff <i>et al.</i> (1986)
<i>Hyalophora cecropia</i>	HCCP12	RR-1	Soft cuticle; imaginal discs	CD and ISH		Cox and Willis (1985), Gu and Willis (2003)
<i>Hyalophora cecropia</i>	HCCP66	RR-2	Hard cuticle	CD and ISH		Cox and Willis (1985), Gu and Willis (2003)
<i>Locusta migratoria</i>	LM-ACP7	RR-2	Hard cuticle	CD		Andersen <i>et al.</i> (1995a)
<i>Locusta migratoria</i>	LM-ACP8	RR-2	Hard cuticle	CD		Andersen <i>et al.</i> (1995a)
<i>Locusta migratoria</i>	LM-ACP19	RR-2	Hard cuticle	CD		Andersen <i>et al.</i> (1995a)
<i>Manduca sexta</i>	CP14.6	RR-1	Soft cuticle	ISH		Rebers <i>et al.</i> (1997)
<i>Manduca sexta</i>	LCP16/17	RR-1	Soft cuticle	ISH		Horodyski and Riddiford (1989)
<i>Tenebrio molitor</i>	ACP17	Glycine-rich	Hard cuticle	ISH	Strongest post-ecdysis	Mathelin <i>et al.</i> (1995, 1998)
<i>Tenebrio molitor</i>	ACP20	RR-2	Hard cuticle	ISH	Primarily pre-ecdysis	Charles <i>et al.</i> (1992)
<i>Tenebrio molitor</i>	ACP-22	RR-2	Hard cuticle	ISH, mAB	Pre-ecdysis	Bouhin <i>et al.</i> (1992a, 1992b)
<i>Tenebrio molitor</i>	TMLPCP22	51 aa motif	Hard and soft cuticle pre-ecdysis, then only soft cuticle	ISH, mAB	Primarily pre-ecdysis	Rondot <i>et al.</i> (1998)
<i>Tenebrio molitor</i>	TMLPCP23	51 aa motif	Hard and soft cuticle	ISH	Only pre-ecdysis	Rondot <i>et al.</i> (1998)
<i>Tenebrio molitor</i>	TMLPCP29	RR-3 and 18-residue motif	Hard and soft cuticle, except not posterior borders of sclerites	ISH	Post-ecdysis	Mathelin <i>et al.</i> (1998)

^aFor *in situ* hybridization, cuticle type was determined by nature of cuticle overlying the epidermis.

^bCD, careful dissection prior to extraction of proteins; ISH, *in situ* hybridization used to localize mRNA; mAB, monoclonal antibody immunolocalization; EST, from *Bombyx* EST project (Mita *et al.* 1999).

These findings are so important that the experimental methodology is worth discussing. The first approach used was to seal sheets of final instar integument into a bathing chamber so there could be no leakage from the cut edges of the tissue and then find what proteins were made in a 2 h exposure to ³⁵S-methionine. Three classes of proteins were identified with this procedure. One was secreted exclusively into the cuticle (C class), a second appeared in the bathing fluid, hence has been secreted basally (B class) while the third was secreted in both directions (BD class) (Palli and Locke, 1987). Immunolocalization of numerous other *Calpodes* proteins (of unknown sequence) confirmed the

existence of these three routing classes of epidermal proteins. A fourth, T class, for proteins transported into cuticle, but not synthesized by the epidermis, was identified. Its presence eliminated any concerns that the classes might be artifacts from labeling with ³⁵S-methionine (Sass *et al.*, 1993).

One member of the T class (T66) was studied in more detail. It was localized by immunogold throughout the cuticle, and although found in epidermal cells was not found in association with the Golgi apparatus, confirming its transcellular transport, rather than synthesis by the epidermis. A subsequent study identified the exclusive site of its synthesis as spherulocytes (Sass *et al.*, 1994a).

Whether the BD proteins are secreted from both apical and basal borders of epidermal cells is still not clear. Locke (1998, 2003) now favors the possibility that all secretion is apical, where the Golgi are concentrated, and that the secreted proteins are subsequently taken back into the cell from the perimicrovillar space and transported in vesicles to the basal surface where the contents are released into the hemolymph.

In conclusion, it is now clear that the epidermis can synthesize both cuticular and hemolymph proteins. It can also transport proteins made in tissues other than epidermis from hemolymph to cuticle.

12.2.3. Tracheal Cuticular Proteins

An often-neglected source of cuticle in insects is the tracheal system. Since tracheae are associated with all insect tissues, caution is needed in interpreting the significance of the presence of mRNAs or cuticular proteins from nonintegumental tissues. Cox and Willis (1985) recognized that some of the proteins from tracheae had the same isoelectric points as proteins isolated from integumentary cuticle. A further study was carried out a decade later by Sass *et al.* (1994b), combining electrophoretic analysis with immunogold labeling. Chitin was localized with wheat germ agglutinin and found in all regions of tracheae and tracheoles except the taenidial cushion. Antibodies that had been raised against individual electrophoretic bands from integumentary extracts represented proteins from all four classes of integumentary peptides. Some C proteins, those from the surface cuticle, were found associated with chitin but only in taenidia, other C proteins were in the general matrix with and without chitin. The B and BD peptides were only found in the taenidial cushion, the region lacking chitin. It appears that hemolymph peptides that are synthesized by the epidermis may be tracheal cuticle precursors. The one T protein studied (T66, made in spherulocytes) was also found in the general matrix. An important insight from this study was the conclusion that: "The extremely thin tracheal epithelium suggests that transepithelial transport might supply proteins to the tracheal cuticle more evenly than Golgi complex secretions" (Sass *et al.*, 1994b).

12.3. Classes of Proteins Found in Cuticles

12.3.1. Nonstructural Proteins

Nonstructural proteins that have been identified in cuticle are listed in Table 2.

12.3.1.1. Pigments Proteins from three classes of pigments used in cuticle – insecticyanins and two different yellow proteins – have been sequenced. The insecticyanins are blue pigments made by the epidermis and secreted into both hemolymph and cuticle. They are easily extracted from cuticle with aqueous buffers. Members of the lipocalin family, they are present as tetramers with the gamma isomer of biliverdin IX situated in a hydrophobic pocket. In the cuticle, in cooperation with carotenes, they confer green coloration. Their structure has been determined to 2.6 Å by X-ray diffraction (Holden *et al.*, 1987), making them structurally the best characterized cuticular proteins. Two genes code for insecticyanins in *Manduca* (Li and Riddiford, 1992).

The yellow protein in *D. melanogaster* has been localized with immunocytochemistry in cuticles destined to become melanized (Kornezos and Chia, 1992). Thus it was found in association with larval mouth hooks, denticle belts, and Keilin's organs. Mutants of the gene *yellow* lack black pigment in the affected cuticular region. Mutant analysis revealed two classes of mutants, those that affect all types of cuticle at all stages, and those affecting only particular areas of specific stages. At least 40 different adult cuticular structures could express their color independently (Nash, 1976), and the regulatory regions responsible for some of the stage and regional specificity have been identified (Geyer and Corces, 1987). The yellow protein has been described as a structural component of the cuticle that interacts with products from the gene *ebony*, a β -alanyl-dopamine synthase, to allow melanin to be deposited. Flybase (2003) reports that 740 different alleles of *yellow* have been described, in 542 references beginning in 1916. The complete sequence of *yellow* has been determined for 13 species of *Drosophila* in addition to *D. melanogaster*. An examination of *yellow* expression revealed that both *cis*- and *trans*-regulation are responsible for differences in pigmentation patterns among different species (Wittkopp *et al.*, 2002). There is no evidence for a known chitin-binding domain in the yellow protein; the only domain recognized is pfam03022 (major royal jelly protein). Although the sequence for yellow is 37% identical and 56% similar to a dopachrome conversion enzyme from *Aedes aegypti* that is involved in the melanotic encapsulation immune response, yellow itself evidently is devoid of enzyme activity (Han *et al.*, 2002).

Another cuticular protein implicated in pigmentation, putatively β -carotene binding, has been isolated from extracts of cuticle from mature adult

Schistocerca gregaria using column chromatography to isolate a protein that was yellow in color. It bears significant sequence similarity to various insect JH-binding proteins (see **Chapter 8**), as well as odorant-binding proteins. Wybrandt and Andersen (2001) suggest that it is involved in the transport of carotenes into epidermis and then the cuticle.

12.3.1.2. Enzymes Some of the enzymes involved in sclerotization have been identified in cuticle. Since they are discussed by Andersen they will not be considered here.

Some enzymes that belong to the molting fluid become evident as the electrophoretic banding pattern of cuticular proteins changes as *Calpodes* initiates molting at the end of the fifth instar, with the most conspicuous change being the appearance of a band of 19 kDa. Antibodies raised against this protein were used to isolate a cDNA from a library cloned in an expression vector. The conceptual translation revealed a protein (CECP22). Its sequence suggested it might have amidase activity. Further analysis revealed that the protein was present in the cuticle before each molt, and was also found in molting fluid. Marcu and Locke (1998, 1999) present evidence that this protein may be activated by proteolysis and speculate that it may function to cleave an amidic bond between *N*-acetylglucosamine from chitin and amino acids in cuticular proteins.

Enzymes involved in digesting the old cuticle are temporary residents in cuticle. These include proteases and chitinases. Their interaction is discussed by Marcu and Locke (1998).

12.3.1.3. Defense proteins Also found in the cuticle are components of the insect defense system. In one study, cuticle was removed from *Bombyx* larvae 24 h after they had been abraded with emery paper and exposed to bacteria. The antibacterial peptide cecropin was purified from the cuticles (Lee and Brey, 1994). Both prophenoloxidase and a zymogen form of a serine protease capable of activating it have been extracted from *Bombyx* larval cuticle. Colloidal gold secondary antibodies revealed that the prophenoloxidase was localized throughout the epicuticle and procuticle, and in a conspicuous orderly array on the basal side of the helicoidal chitin lamellae. An extraepidermal source is likely for this enzyme since no labeling was found in the epidermis, nor was mRNA detected in the epidermal cells. It is assumed to function in the melanization that occurs in response to injury (Ashida and Brey, 1995).

Molnar *et al.* (2001) presented immunological evidence for a protein related to the defense protein scolexin in the cuticle of *Manduca*. This protein exists in two forms in *Manduca*, but the antibody used did not distinguish between them.

12.3.1.4. Arylphorins The final class of nonstructural proteins is the arylphorins, proteins with high content of aromatic amino acids and some lipid. These proteins, assumed to be hemolymph proteins, have been of special interest since the discovery by Scheller *et al.* (1980) that although calliphorin (the arylphorin from *Calliphora*) was found in cuticle, it seemed to come from the hemolymph, because radioactively labeled calliphorin injected into the hemolymph appeared in cuticle. But there is also evidence that the epidermis is capable of synthesizing arylphorins, for Riddiford and Hice (1985) had detected arylphorin mRNA in the epidermis of *Manduca*.

Palli and Locke (1987) used an anti-arylphorin antibody to identify an 82 kDa protein made in *Calpodes* integumental sheets *in vitro* that appeared in both cuticle and media. Thus arylphorin appeared to be a bidirectionally secreted integumentary protein. Next, colloidal gold secondary antibodies were used to visualize the location of anti-arylphorin in ultrathin sections of various tissues (Leung *et al.*, 1989). The resolution afforded by this method made it possible to recognize arylphorin in epicuticle (but not lamellar cuticle) and in the Golgi complexes of the fat body, and to show by quantifying gold particles that it was also found in Golgi complexes of epidermis, midgut, pericardial cells, and hemocytes as well as the meshwork of fibrous cuticle in tracheae. Thus, while the possibility remains that some arylphorin is transported from hemolymph to cuticle, it need not be, for the epidermis itself is capable of synthesizing and secreting this protein. These studies further demonstrated that a given protein can be synthesized by multiple tissues. Whether it is the same gene that functions in all tissues remains to be determined.

The role of arylphorin remains unknown. It is generally assumed to be participating in sclerotization because of its high tyrosine content. Is it degraded in the cuticle so that its constituent amino acids are released or does it remain an integral part of the cuticle? The latter is favored by the available evidence because calliphorin has been shown to bind strongly to chitin *in vitro* (Agrawal and Scheller, 1986) and no breakdown products were detected after injection of labeled calliphorin (Konig *et al.*, 1986).

12.3.2. Structural Proteins

12.3.2.1. Overview Slightly less than a decade ago, a comprehensive and insightful review of cuticular proteins presented the complete sequence and full citation for all 40 cuticular proteins known at that time and identified features that remain their hallmarks (Andersen *et al.*, 1995a). As of June 2003, in addition to the nonstructural cuticular proteins discussed above, there are now 139 sequences available for what are postulated to be structural proteins. These numbers do not include almost 200 more that have been identified by protein prediction programs used to annotate the *D. melanogaster* and *Anopheles gambiae* genomes. These have been omitted because their annotation is still in a state of flux. All 139 sequences and some of their key features are listed in **Table 1**. Marcu and Locke (1998) have also published tabular summaries of a smaller number of cuticular proteins.

Unfortunately, cuticular protein terminology is not uniform. Most workers have included the initials of the genus and species. Some have named their proteins based on their molecular mass, others on the order in which they obtained them. Many *D. melanogaster* proteins have been designated by the chromosomal band to which a gene-specific probe hybridized. Some have been named after their sequence similarity to a particular cuticular protein from another species. Capitalization and the use of hyphens are erratic. Two different groups have worked on proteins from *Tenebrio*, and given two different names to one protein. Some of the names have included a designator for genus, species, and metamorphic stage (e.g., TMLCP-A1A). Although this designation is an accurate indication of the stage from which the protein was purified, it can inadvertently support the misconception that a particular cuticular protein is stage-specific. In *Tenebrio*, larval and pupal cuticular proteins are indistinguishable electrophoretically (Andersen, 1975; Roberts and Willis, 1980a; Lemoine and Delachambre, 1986; Andersen *et al.*, 1995b), and molecular analyses of several *Tenebrio* cDNAs found that all expressed in pupae are also expressed in larvae (Mathelin *et al.*, 1998; Rondot *et al.*, 1998). Furthermore, proteins that are a major component of the cuticle of one stage can be a minor component of another (Cox and Willis, 1985; Willis, 1986). A final complication is whether two almost identical proteins are allelic variants or distinct proteins. In some cases an “isoform” has been described. Genomic sequences, however, have revealed that stretches coding for proteins with very similar or indeed identical sequence may be linked on a chromosome (Charles *et al.*, 1997;

Dotson *et al.*, 1998) (see Section 12.4.2). Thus the finding of “isoforms” may reflect distinct genes and hence distinct proteins.

Table 1 includes proteins from discrete genes even when two or more may have the same amino acid sequence. In **Table 1**, proteins isolated from cleaned cuticles were counted as cuticular proteins, as were proteins whose nucleic acid sequences were obtained using partial protein sequences or antibodies raised against cuticular protein to select corresponding cDNAs. In addition, **Table 1** contains numerous proteins that had been classified as cuticular proteins by their “discoverers” because their sequence, or a part thereof, was similar to a cuticular protein already in the databases. For many of those in the latter category, the source of the cDNA that led to the sequence came from integumental epidermis or imaginal discs or a cDNA hybridized to epidermal RNA in a Northern analysis. For some, especially those from *Tenebrio* studied by Delachambre’s group, confirmation came from *in situ* hybridization of specific probes (see Section 12.2.1.3 and **Table 3**). But for many, sequence similarity served as the sole criterion.

Most of the structural cuticular proteins whose sequences were known in 1995 came from the efforts of Svend Andersen and his group, and a significant fraction (42%) still does. All of their sequences come from direct sequencing of purified cuticular proteins. Most of the other protein sequences come from sequencing cDNA or genomic DNA. For these, the length of the mature proteins can only be deduced by subtracting the amino acids of the signal peptide. In a few cases, N-terminal sequence data is available to assure that the signal peptide has been correctly identified. In cases where this information was not available, or when the original submissions did not provide this information, it was determined using the program SignalP (Nielsen *et al.*, 1997; Nielsen and Krogh, 1998). All lengths in **Table 1** represent the mature, processed protein.

One notable feature of the structural cuticular proteins is that almost all lack cysteine and methionine residues in the mature protein; the five exceptions to this are indicated in **Table 1** and **Figure 4**. Andersen suggests that the reactivity of cystine and cysteine with *ortho*-quinones could interfere with sclerotization.

Most of the cuticular proteins identified to date are quite short. Those less than 100 amino acids account for 27% of the 139 sequences in **Table 1**, while those between 100 and 199 account for an additional 52%. Only three proteins have more than 300 amino acids. The largest is the gene for

a *D. melanogaster* corneal lens protein (Cry, drosocrystallin) with 457 amino acids, and a perfect RR-2 consensus (Janssens and Gehring, 1999). (See Section 12.3.2.3 for discussion of this consensus.) A cDNA of the appropriate size has been described (gi:2143072). The next largest is for the only cuticular protein characterized from *Galleria* (Kollberg *et al.*, 1995). This protein is unusual in that its only resemblance to known cuticular proteins is an abundance of alanine residues, and it is unique in having two cysteine residues. Yet its cDNA was selected with a polyclonal antibody raised against pupal cuticular proteins. The third largest protein is MSCP36; this is a high glycine protein that has a RR-1 consensus.

12.3.2.2. Motifs found in cuticular proteins

The review by Andersen *et al.* (1995a) was the first to assemble a variety of motifs found in cuticular proteins. The occurrence of such motifs is given in Table 1 and summarized in Table 4. Most common of these is a 28-residue region, first recognized by Rebers and Riddiford (1988) in seven cuticular proteins that is commonly referred to as the R&R Consensus. The original R&R Consensus is part of a longer conserved sequence – pfam00379 – and it is now apparent that there are three distinct forms of the extended R&R Consensus. These matters are discussed in detail below (see Section 12.3.2.3).

After the R&R Consensus, the next most common motifs were repeats of A-A-P-(A/V). These repeats were found in cuticular proteins both with and lacking the R&R Consensus; in sequences with the R&R Consensus they may occur N- or C-terminal to the extended Consensus. They are found in 46% of the sequences in Table 1. Thus, while abundant in cuticular proteins, they certainly are not diagnostic for this class of protein.

Andersen *et al.* (1995a) recognized several sequences with stretches of glycine, leucine, and tyrosine, beginning G-Y-G-L- or G-L-L-G. In Table 1, they are combined under the designation, G(Y/L) motifs. Other cuticular proteins are also high in glycine, but with less regular motifs; these are designated by the number of consecutive Gs. Proteins enriched in glycine residues are found in a

variety of structures such as plant cell walls, cockroach ootheca, and silk (see Bouhin *et al.* (1992a) for discussion). Subsequent to their 1995 review, Andersen and his colleagues recognized two additional motifs. There is an 18-residue motif found in seven cuticular proteins from four orders of insects (and two crustaceans), and its consensus has been described (Andersen, 2000). It occurs in proteins with and without the R&R Consensus. Also reported was a 51-residue motif so far identified only in cuticular proteins from *Locusta* and *Tenebrio*. It has not been found in proteins with the R&R Consensus (Andersen *et al.*, 1997). Other short repeats have been found in a limited number of proteins, from a single species. Proteins with the various motifs are identified in Table 1.

The basic sequences of the three long repeats are:

Original R&R Consensus: [G-x(8)-G-x(6)-Y-x(2)-A-x-E-x-G-F-x(7)-P-x-P.]

18 amino acid repeat: [(PV)-x-D-T-P-E-V-A-A-A-(KR)-A-A-(HF)-x-A-A-(HY).]

51 amino acid repeat: [x(6)-A-x(9)-R-S-x-G-x(4)-V-S-x-Y-x-K-x(2)-D-x(3)-S-S-V-x-K-x-D-x-R-x(2)-N-x(3).]

With this nomenclature, x is any amino acid, the number in parentheses represents the number of amino acids, and multiple letters in parentheses indicate that either of two amino acids may be present. This is the format used by MOTIF (2003), a resource that lets you search a given motif against various databases.

Andersen (2000) presented a model where proteins with the R&R Consensus bind to chitin and the other structural proteins remain free in the interfilament space.

12.3.2.3. Proteins with the R&R consensus The R&R Consensus is a common feature of cuticular proteins from all six orders of insects examined to date and it has also been recognized in cuticular proteins from arachnids and crustaceans (review: Willis, 1999).

Three distinct forms of the consensus have been recognized and named by Andersen (1998, 2000) RR-1, RR-2, and RR-3 (Figures 1–3). RR-1 is present in 51 (37%) of the proteins in Table 1.

Table 4 Summary characteristics of the 139 “structural” cuticular proteins that have been completely sequenced

	Number with AAP(AV)	Number lacking AAP(AV)	Number 18-residue motif	Number 51-residue motif	Mean number H + K in extended R&R region (range)	Number with Met/Cys residues	Total proteins in class
RR-1	2	49	0	0	3.7 (0–9)	3/0	51
RR-2	36	8	0	0	7.4 (2–19)	0/0	44
RR-3	1	2	3	0	6.3 (5–8)	0/0	3
Not RR	26	15	4	7		0/2	41

RR-1-bearing proteins have been isolated from flexible cuticles, while RR-2 proteins have been associated with hard cuticle. This generalization, based on relatively few cases (Table 3), has been used to link proteins to cuticle types in the absence of any other evidence.

The RR-1 proteins have the essential features of the original consensus (Figure 1). The vast majority have the short sequence -Y-x-A-x-E-x-G-(FY)-x(7)-P. N-terminal to this region the sequences diverge, but most have a series of three aromatic residues, such as Y-x-F-x-Y, that begins about 32 amino acids N-terminal to the start of the R&R Consensus (Figure 1). Another distinguishing feature defined by Andersen (1998) is a glutamic acid residue found in a conserved position [Y-x-A-x-E-G-(FY)] in 90% of the sequences in Figure 1.

The RR-2 proteins have a considerably extended consensus, first recognized by Bouhin *et al.* (1992a) and Charles *et al.* (1992). What is extraordinary about the RR-2 consensus is its conservation across six orders of insects. Only two single amino acid gaps are required to accommodate all 44 RR-2 sequences with this variant listed in Table 1. Twenty-two of the 70 residues in the extended consensus (31%) are virtually invariant and an additional 21 are represented by a single amino acid in over half of the proteins (Figure 2). The first few RR-2 sequences identified suggested that the residues G-F-N-A-V-V would be diagnostic (Andersen, 1998). The identification of more RR-2 sequences revealed that that region of the consensus is not perfectly conserved. Rather, all of the sequences have G-F-x-A-x-V, a configuration found in none of the RR-1 sequences.

There are other differences between RR-1- and RR-2-bearing proteins. Most of the RR-2 (82 %) have at least one A-A-P-(AV) motif, while only 2 (4%) of the RR-1 type have this motif (Table 1). Histidine and lysine residues can be more abundant in the extended consensus region of RR-2 proteins (Table 4, Figures 1 and 2). Only one RR-1 protein has been found with more than six histidine plus lysines in this region, while seven or more were present in 20 of the 44 RR-2 sequences. There is an invariant lysine in all RR-2 sequences and several other positions appear to be favorable for either of these amino acids. Over half of the RR-2 proteins, but only a quarter of the RR-1 proteins, have histidine as their final or penultimate C-terminal amino acid. Histidines and lysines are known to be reactive sites for sclerotizing agents, so it is possibly significant that proteins from "hard" sclerotized cuticles would have these amino acids in abundance. The number of potential sclerotization sites may also be related to whether a cuticle can grow by

intussusception, something that would be impossible if the proteins were extensively cross-linked. The six aphid cuticular proteins, while all of the RR-2 type, have relatively few histidines plus lysines (only four or five); this paucity may reflect the need for cuticular expansion with the type of feeding and brooding of progeny that occurs in these animals.

An RR-3 form of the consensus has been based on three sequences from insects and two from other arthropods (Andersen, 2000). A tentative consensus (Figure 3) was constructed from the sequence alignment in Andersen (2000).

Whole genome sequencing has led to the need to classify annotated sequences. A valuable website, Pfam (2003a) has used hidden Markov modeling to define motifs characteristic of particular classes of proteins (Bateman *et al.*, 2002). When a protein sequence is searched against all known (and predicted) proteins using the BLAST server (Blast, 2003), the first information that is presented is an indication of matches to Pfam entries. The Pfam sequence that allows annotators to classify a protein as a cuticular protein is Pfam00379, a 68 amino acid sequence that includes the extended R&R Consensus. It also goes under the name "chitin_bind_4," for reasons that will become apparent later (see Section 12.5.4). The pfam00379 was obviously based on proteins of both RR-1 and RR-2 classes, for it matches neither particularly well (Figure 3). This makes it particularly useful for a preliminary classification of a putative cuticular protein sequence.

The pfam00379 is found in 70% of the 139 cuticular proteins in Table 1, i.e., all the RR-1, RR-2, and RR-3 sequences. A complete listing of all sequences with pfam00379 can be found at Pfam (2003b) or at ENTREZ (2003), where you search Domains for pfam00379. There are no nonarthropod sequences with this consensus. Now that two species of insects (*D. melanogaster* and *A. gambiae*) have had their genomes completely sequenced and pfam00379 is being used to recognize cuticular proteins, the representation of proteins bearing this motif will be disproportionate. As of August 2003, 90 *D. melanogaster* sequences beyond those listed in Table 1 have been found to have pfam00379, and using the ENTREZ site, *A. gambiae* had over 100 beyond those in Table 1.

Pfam00379 so far has been found to occur only once in a given protein, with the notable exception of a protein from the tailfin of the prawn *Penaeus japonicus*. The entire sequence of this protein is made up of 14 consecutive pfam00379 motifs (Ikeya *et al.*, 2001). In the insect proteins, this motif has been found near the N- or C-terminus, or within the protein.

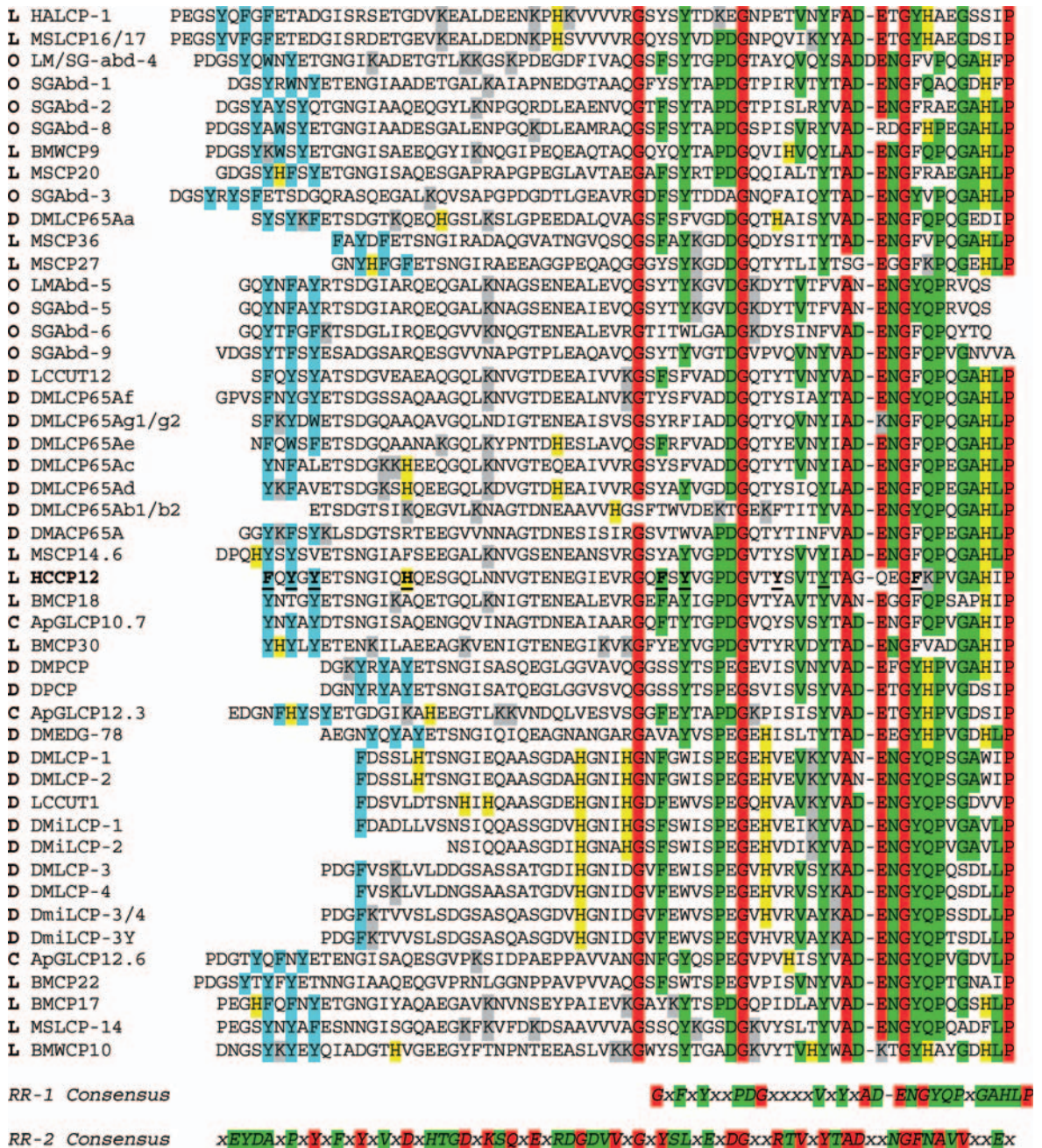


Figure 1 Alignment of the pfam00379 regions of 51 proteins with the RR-1 consensus. The pfam 00379 regions for RR-1 proteins were aligned with ClustalW (<http://clustalw.genome.ad.jp/>); only one internal gap was used to allow direct comparison with the RR-2 consensus. Orders of insects are: (C) Coleoptera, (D) Diptera, (L) Lepidoptera, (O) Orthoptera. Abbreviations for proteins as in **Table 1**. Four pairs of identical sequences are each presented on a single line. Red represents amino acids present in at least 95% of the proteins, green in the majority. Histidines are shown in yellow, lysines in light gray. A common triad of aromatic residues is shown in light blue. Bolded and underlined are several residues from HCCP12 that are shown in **Figure 5a-c**. An RR-1 consensus based on these sequences is given. The bottom line gives the consensus for RR-2 proteins from **Figure 2**, except that two single amino acid gaps needed to accommodate three atypical sequences were eliminated.

The wealth of information on cuticular protein sequences and the unraveling of how the structure of some contributes to the interaction of chitin and protein (see Section 12.5) is only a beginning. Essential properties of cuticle remain to be explained, and important questions raised in the older literature about various means of achieving cuticle plasticity and the importance of hydration in cuticle stabilization must not be forgotten (Vincent, 2002 and references therein).

D DS/DY	DHHD ^H SHA ^H EYD ^F FEYGVKDHKTGDVKSQ ^S ES ^R RHGH-TVTGHYE ^L LIDADG-HKRTV ^H HYTAD ^K HK ^H GF ^E AHVHREK
Dy BC-NCP8	PQYD ^N PNPQY ^T TF ^S YNVDDPETGDSK ^S Q ^E ETRNGD-NVQGRYS ^V IESD ^G -SRRVVEYSADAVSGFN ^A VVHREA
D DM ^C ry	EDYD ^T TRPQYS ^F AYDVRDSL ^T GGDDK ^R Q ^E EKRDGD-LVKGQYS ^L IE ^F PDG-TRRIVEY ^T AD ^D VSGFN ^A IVSKQR
O LM-ACP21	AEYD ^N PNPQYSY ^A Y ^N VQDAL ^T GD ^S KAQ ^Q ETRDGD-VVQGSYS ^L VE ^F PDG-SIRTVDY ^T AD ^P VNGFN ^A VVHKEA
O LM-NCP19.8	AEYD ^P HPQYSY ^G YSVNDAL ^T GD ^S SKS ^Q Q ^E SRDGD-VVQGSYS ^L VE ^F PDG-SVRTVDY ^T AD ^P VNGFN ^A VVHKEP
C TM-LCP-A1A	DEYD ^N PNPQYS ^F GYD ^V QDGL ^T GD ^S KNQ ^V ESRSGD-VVQGSYS ^L V ^D PDG-TRRTVEY ^T AD ^P INGFN ^A VVHREP
C TM-LCP-A2B	DEYD ^P HPQYQY ^G YD ^V QDGL ^T GD ^S KS ^Q IESRSGD-VVQGSYS ^L V ^D PDG-TRRTVEY ^T AD ^P INGFN ^A VVHREP
C TM-LCP-A3A	DEYD ^P HPQYSY ^G YDI ^Q DGL ^T GD ^S KNQ ^Q ETRDGD-VVQGSYS ^L V ^D PDG-TRRTVEY ^T AD ^P INGFN ^A VVHREP
L BMWCP6	EEYD ^A HPQYS ^F AYD ^V QDSL ^T GD ^S KTQ ^H ETRDGD-VVQGSYS ^V V ^D PDG-TKRTVDY ^T AD ^P HNGFN ^A VVHKEP
D DM ^C Cp84Aa	EEYD ^P HPQYR ^F SYGVDD ^K L ^T GD ^N KG ^V Q ^V EERDGD-VVRGEYS ^L LI ^D ADG-YKRIVQY ^T AD ^P INGFN ^A VVNREP
D DM ^C Cp84Ab	EEYD ^P HPQYR ^F SYGVDD ^K L ^T GD ^N KG ^V Q ^V EERDGD-VVRGEYS ^L LI ^D ADG-YKRIVQY ^T AD ^P INGFN ^A VVNREP
D DM ^C Cp84Ae	EEYD ^P HPQY ^T YSYD ^V QD ^T LS ^G DNKG ^H VEERDGD-VVRGEYS ^L LI ^D ADG-FKRTVTY ^T AD ^S INGFN ^A VVRRP
D DM ^C Cp84Ad	EEYD ^P HPQYKY ^A YD ^V QD ^S LS ^G DKS ^Q VEERDGD-VVRGEYS ^L LI ^D ADG-YKRIVQY ^T AD ^P INGFN ^A VVNREP
D DM ^C Cp84Af	EEYD ^P HPQYKY ^F AYD ^V QD ^S LS ^G SKS ^Q VEERDGD-VVHGEYS ^L LI ^D SDG-YKRIVQY ^T SD ^P INGFN ^A VVNRP
D DM ^C Cp84Ag	EEYD ^P HPQYTY ^G YD ^V KD ^A IS ^G DKS ^Q ETRDGD-VVQGSYS ^L ND ^A DG-YRRTV ^D Y ^T AD ^P INGFN ^A VVRRP
D DM ^C Cp84Ac	PDD ^D PHPKY ^N FAYD ^V QD ^A LS ^G DKS ^Q VEERDGD-VVQGEYS ^L DD ^A DG-FRRTV ^K Y ^T AD ^S VNGFN ^A VVHREP
D DMEDG84	DTYD ^S HPQYS ^F NYD ^V QD ^P ETG ^D VKS ^Q ES ^R RDGD-VVHGQYS ^V ND ^A DG-YRRTVDY ^T AD ^D V ^R GFN ^A VVRRP
D AnGCP2b, c, d	VEHHAPAN ^Y E ^F SYSV ^H DEH ^T GD ^I KNQ ^H ETRH ^G D-EVHGQYS ^L LD ^S DG-HQRI ^V DY ^H AD ^H H ^T GFN ^A VVRRP
D AnGCP2a	VEHHAPAN ^Y E ^F SYSV ^H DEH ^T GD ^I KNQ ^H ETRH ^G D-EVHGQYS ^L LD ^S DG-HQRI ^V DY ^H AD ^H H ^T GFN ^A VVRRP
L HCCP66	SDFSS ^F SYGVAD ^P STG ^D FKS ^Q IES ^R LGD-NVQGSYS ^L LES ^D G-TQRTVDY ^A AGSE-GFN ^A VVKDP
L BMWCP1B, 2	EEEY ^A HPKYD ^F AYSVAD ^G HSGDNKS ^Q ESR ^D GD-AVHGEY ^T L ^V EADG-SVRKVEY ^T AD ^D H ^H GFN ^A VVSNSA
L BMWCP1A	EEEY ^A HPKYD ^F AYSVAD ^G HSGDNKS ^Q ESR ^D GD-AVHGEY ^T L ^V EADG-SVRKVEY ^T AD ^D H ^H GFN ^A IVSNTA
L BMEDG84A	HD ^T Y ^A HPKNDY ^A Y ^S VAD ^P H ^T GGHKS ^Q HENR ^D DG-AVHGSYS ^L VE ^F PDG-SVRKVDN ^T AD ^D H ^H GFN ^A VVHKTP
L BMWCP3	AEEI ^A YPKY ^E F ^N YSVAD ^G HSGV ^N KS ^Q Q ^E VRDGD-AVKGSYS ^F HE ^A DG-SIRTVEY ^T AD ^A HNGFN ^A VVHNTA
L BMWCP4	VDEY ^A HPKYGY ^S SVED ^P H ^T GD ^H KS ^Q HE ^T RDGD-VVKGEYS ^L LQ ^P PDG-SFRKV ^T Y ^T AD ^H HNGFN ^A VVHNTP
L BMWCP5	VEDHAPAKY ^E F ^S YSVED ^P H ^T GD ^H KS ^Q HE ^T RDGD-VVKGEYS ^L LQ ^P PDG-SIRKVEY ^T AD ^H HNGFN ^A IVHNSE
L BMWCP7A, B	EDYD ^A HPKYA ^F EYK ^I ED ^P H ^T GD ^L KS ^Q HE ^T RDGD-VVKGY ^S L ^H EADG-SIRVVEY ^S AD ^K HNGFN ^A VVKHTA
L BMWCP8	EDHY ^A YPKYA ^F EYK ^I ED ^P H ^T GD ^N KY ^Q HE ^I RDGD-VVKGEYS ^L H ^E ADG-SIRTV ^K Y ^T AD ^K KS ^G FN ^A EV ^I NSG
O LM-ACP19	VDY ^S YYPKYA ^F EYGVND ^P H ^T GD ^V KRQ ^W EERDGD-VVRGEYS ^L LE ^F PDG-TT ^R T ^V Y ^T AD ^A HNGFN ^A VVHRSG
O LM-ACP7	IEYD ^N PNPHYS ^F EYSVSDA ^H TGD ^Q KAQ ^H ETRDGD-VVQGSYS ^L VE ^F PDG-SVRTVEY ^T AD ^P HNGFN ^A VVHRQA
O LM-ACP8	AEPV ^A YPKY ^E F ^N YGVHDA ^H TGD ^I KQ ^S EARDGD-VVKGSYS ^L VE ^F PDG-STRTVEY ^Q AD ^D HNGFN ^A VVHRTP
H 5 sp.	ESYDAPAPY ^N F ^E YSVND ^P H ^T YD ^V KS ^Q EYAD ^G NGY ^V KGSYS ^L VE ^F PDG-STRTVEY ^T AD ^D HNGFN ^A VVKKEG
H AGCP	ESYDAPAPY ^N F ^E YSVND ^P H ^T YD ^V KS ^Q EYAD ^G NGY ^V KGSYS ^L VE ^F PDG-STRTVEY ^T AD ^E DYNGFN ^A VVKKEG
C TMACP20	VDLHTPAHY ^Q F ^K YGVED ^H RTG ^D RKQ ^Q AEV ^R VDG-VVKGEYS ^L AE ^F PDG-TVRV ^V KY ^T AD ^D HNGFN ^A VVSRVG
C TMACP22	IHLKAH ^F EYHS ^D YHVAD ^H KT ^K DF ^K SKHE ^V RDGY-KVKGTYS ^L LE ^P DK ^T V ^R V ^D Y ^S V ^D KK ^R GF ^I ARV ^S YR ^K

RR-2 Consensus xEYDAxPxYx^FxYxVxDxHTGDxK^SQxExRDGD-VVxGxYSLxExDG-xxRTVxYTADxxNGFNxVxEx

Figure 2 Alignment of the pfam00379 region of 44 cuticular proteins with the RR-2 consensus. Abbreviations as in **Figure 1** plus Dictyoptera (**Dy**) and Hemiptera (**H**). All hemipteran proteins except the one from *Aphis gossypii* are indicated by [**H**5 sp]. The order of the proteins was based on alignment by ClustalW. Red represents amino acids present in at least 95% of the proteins, green in the majority. Histidines are shown in yellow, lysines in light gray. The seven histidines in AGCP2b are also discussed in the text (see Section 12.5.4) and modeled in **Figure 5d** are bolded and underlined, beginning with residue 99. An RR-2 consensus is given.

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pfam00379:
  PDGSNNYAAETSNGIADQETGDLKSQGEERDGVVQGSYSYVDPDGQTRTVTYTAD-ENGFQPVGAHLP
RR-1 Consensus:
  GxFxYxxxPDGxxxxVxYxAD-ENGYQPxGAHLP
RR-2 Consensus:
  xEYDAxPxYxFxYxVxDxHTGDxKSQxExRDGDVVxGxYSLxExDGxxRTVxYTADxxNGFNxVxEx
RR-3 Consensus:
  V-xVxFxYHAQDxLGQxSFGHxxxxQRxExxDAAGNKxGSYxYVDExGKVxxxxYVAD-AxGFRVAxx-NLPVxF

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Figure 3 Consensus regions from the three types of RR cuticular proteins plus pfam00379. For pfam00379, the three aromatic residues found in RR-1 and RR-2 sequences (**Figures 1** and **2**) are shown in light blue. Indicated in red is the original R&R Consensus; the dash (-) was inserted to facilitate alignment with the other sequences. The green F was a Y in the original. RR-1 and RR-2 consensus are from **Figures 1** and **2**, respectively. Red residues were found in at least 95% of the sequences, green in at least 50%. RR-3 was taken from Andersen (2000) using the sequences from the three insects and two arthropods arachnid and crustacean, that was identified as RR-3. Red residues were found in all five RR-3 sequences, green in three or four. The first and third dashes were inserted to allow alignment among all RR-3 sequences; the second was necessary to allow alignment with the RR-2 sequence.

12.3.2.4. Resilin The name resilin has been given to the rubberlike proteins responsible for the elasticity of jumping fleas and vibrating wings. Resilins are characterized by a high percentage of glycine (35–40%) and proline (7–10%). They are cross-linked with di- and tri-tyrosine residues (Andersen and Weis-Fogh, 1964). An intact protein corresponding to resilin has never been isolated from cuticle, presumably reflecting its insolubility after cross-linking. Ardell and Andersen (2001) used short peptide sequences that they had obtained from resilin-bearing regions of *Schistocerca* cuticle to probe the annotated *D. melanogaster* genome. Two candidate proteins had good matches to the locust peptides and to some that Lombardi and Kaplan (1993) had obtained from resilin in *Periplaneta americana*.

Ardell and Andersen concluded that predicted protein CG15920 (gi:24654243) of 620 amino acids was most likely to be a true proresilin (the non-cross-linked version), for in addition to the peptide matches, it had 35% glycine and 11% proline. Its 18 N-terminal copies of a 15-residue repeat and 13 C-terminal copies of a 13-residue repeat were predicted to contribute to a β -spiral, a common form for proteins with elastic properties (Ardell and Andersen, 2001). A cDNA (gi:27820115) is available that corresponds to most of the genomic sequence, but it lacks 45 internal amino acids. These correspond to the predicted second exon in the genomic sequence and contain almost the entire match to the locust proresilin peptides as well as most of the match to pfam00379. All of the repeat regions, however, are present. Thus, in order for CG15920 to match the locust resilin, it would have to be coded by an alternatively spliced form.

The second *D. melanogaster* gene that had “resilin peptides” was CG9036. Ardell and Andersen considered it to be a less likely candidate because it lacked both a predicted signal peptide and features expected for elastic properties. The original version they described has been replaced (gi:19922620) and a cDNA identical to this new sequence has been obtained. The new version has a predicted signal peptide of 19 amino acids. The mature protein of 198 amino acids has a pfam00379 region (occupying one-third of the mature protein) and is 20% glycine and 10% proline; it has neither of the repeats found in the other candidate protein.

Proof that either of these sequences is proresilin will require localization of the mRNA or protein to the tendons shown to have resilin in Diptera (Andersen and Weis-Fogh, 1964). The special properties of resilin justify further work to establish its sequence.

12.3.2.5. Glycosylation of cuticular proteins Glycosylation of cuticular proteins was first reported

by Trim (1941) and in limited subsequent reports (review: Cox and Willis, 1987b). In recent years, posttranslational modifications of cuticular proteins have been determined by staining gels with periodic acid Schiff (PAS), by using labeled lectins to probe blots of electrophoretically separated proteins or by discovering discrepancies in masses of peptide fragments experimentally determined by matrix-assisted laser desorption ionization – mass spectrometry (MALDI-MS) analysis and calculated from Edman sequencing.

Most of the major cuticular proteins seen on gels stained with Coomassie Blue are not recognized by PAS or lectins, while some minor ones are glycosylated. This was true for *H. cecropia* where PAS staining revealed glycosylated proteins in extracts of flexible cuticles and a screen with eight lectins revealed the presence of mannose and *N*-acetylgalactosamine, with more limited binding to *N*-acetylglucosamine, galactose, and fucose, in a few of the proteins from all stages (Cox and Willis, 1987b). A comparable study in *Tenebrio* revealed one major band of water-soluble larval and pupal cuticular proteins that had *N*-acetylglucosamine; a few other bands were weakly visualized with lectins; none of the proteins from adult cuticle reacted with the lectins (Lemoine *et al.*, 1990). In another coleopteran, *Anthonomus grandis*, glycosylation was found in cuticular proteins extracted from all three metamorphic stages (Stiles, 1991). In *Calpodes*, all the BD peptides (see Section 12.2.2) extracted from the cuticle were associated with α -D-glucose and α -D-mannose, just like most of the hemolymph proteins but very few of the C class proteins. Some of each class appeared to be modified with *N*-acetylglucosamine. T66, a protein synthesized in spherulocytes, transported to epidermis, and then secreted into the cuticle, however, was not glycosylated. In none of these species is the amino acid sequence of a glycosylated protein known.

Sequence-related information about glycosylation is available for cuticular proteins isolated from locusts and *Manduca* (see Table 1) where the direct analysis of residues had been used. In *Locusta migratoria*, one to three threonine residues were modified in the protein LM-ACP-abd4. In each case, the modification was with a moiety with a mass of 203, identified as *N*-acetylglucosamine (Talbo *et al.*, 1991). Each of the three threonine residues occurred in association with proline (FPTPPP, LATLPPTPE). All eight of the cuticular proteins that have been sequenced from *Schistocerca gregaria* nymphs had evidence for glycosylation with a moiety with a mass of 203, all at a threonine residue found in a cluster of prolines (Andersen, 1998). Three proteins recently isolated from *Manduca* were similarly shown to be

glycosylated on threonines also in proline-rich regions. Surprisingly in these cases, masses of the adducts were varied (184, 188, and 189) and their nature was not determined (Suderman *et al.*, 2003). In all of these cases, the available evidence indicates that the threonine residues had been O-glycosylated. The significance of such glycosylation awaits further elucidation.

12.4. Genomic Information

12.4.1. Introduction

The first four cuticular proteins whose complete sequences were determined were also the first to have their genes described (Snyder *et al.*, 1982). The wealth of experimental detail and thoughtful discussion in that paper make it a classic in the cuticular protein literature. These four genes were for *D. melanogaster* cuticular proteins LCP-1, -2, -3, and -4, and were found to occupy 7.9 kb of DNA, along with what appeared to be a pseudogene. Each gene had a single intron and that intron interrupted the protein-coding region between the third and fourth amino acid. LCP-1 and -2 were in the opposite orientation of LCP-3 and -4. The nucleic acid sequences in the protein coding regions for LCP-1 and -2 were 91% identical, for LCP-3 and -4, 85%, with similarity between the two groups about 60%. For the noncoding regions of the mRNAs, the 5' upstream regions had more sequence similarity than the 3' downstream. A consensus poly(A) addition site, AATAAA, was found for two of the genes, 110 bp from the stop codon, while similar but not identical sequences (AATACA, AGTAAA), were found for the other two. The four genes were all expressed in the third instar and several short, shared elements were found in their 5' regions upstream from the transcription start site. Snyder *et al.* (1982) also speculated on the origin of the cluster through gene duplication and inversion. These

features of those four genes (coding for RR-1 proteins) have turned out to be the common elements of most of the cuticular protein genes that are known – hence linkage, shared and divergent orientation, an unusually placed intron that interrupts the signal peptide, presence of a pseudogene in the cluster, atypical poly(A) addition sites, and divergence of 3'-untranslated regions have been found for cuticular protein genes in Diptera, Lepidoptera, and Coleoptera.

12.4.2. Chromosomal Linkage of Cuticular Proteins Genes

In addition to the four *D. melanogaster* genes discussed in the previous section, several more instances of linked cuticular proteins genes were described prior to sequencing entire genomes. In some cases, the evidence for these genes was restricted to cross-hybridization of the genomic fragment, and complete sequences are not known for all the members. A summary of such linked genes is presented in Table 5. Many more instances will become known as annotation of the *A. gambiae* and *D. melanogaster* genomes is completed.

A detailed analysis of the cluster of genes at 65A allowed Charles *et al.* (1997, 1998) to describe important features that most likely contributed to the multiplication and diversification of cuticular protein genes. Twelve genes (Table 5) were identified in a stretch of 22 kb with the direction of transcription, or more accurately the strand used, was

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The third gene in the cluster appeared to be a pseudogene. Several important features were found. First, the number of *Lcp-b* genes within the cluster was variable among different strains of *D. melanogaster*, two in the original line (with identical coding sequences), one in another, and three in a third. On the other hand, three copies of the *Lcp-g*

Table 5 Linked cuticular protein genes

Species	Length of DNA examined	Number of genes found	Protein names	Reference
<i>Anopheles gambiae</i>	17.4 kb	3	AGCP2a, 2b, 2c	Dotson <i>et al.</i> (1998)
<i>Drosophila melanogaster</i>	9 kb	6	DM-LCP1, 1ψ, 2, 3, 4 and one other	Snyder <i>et al.</i> (1982)
<i>Drosophila melanogaster</i>	20.5 kb	8	EDG-84, 84Aa, Ab, Ac, Ad, Ae, Af, Ag	Apple and Fristrom (1991), Kaufman <i>et al.</i> (1990), genome annotation
<i>Drosophila melanogaster</i>	22 kb	12	ACP65A, LCP65Aa, aψ, b1, b2, c, d, e, f, g1, g2, g3	Charles <i>et al.</i> (1997, 1998)
<i>Manduca sexta</i>	20 kb	3	LCP16/17 + 2 not named	Horodyski and Riddiford (1989)
<i>Tenebrio molitor</i>	3.9 kb	2	TMLPCP 22, 23	Rondot <i>et al.</i> (1998)

genes (also with 100% sequence identity) were found in all three strains. Comparison of cDNA sequences and genes revealed that *Lcp-b1* and *-b2* lacked introns. Both *Lcp-b1* and *-b2* had tracts of As at the 3' end of the genes, as well as short flanking direct repeats. These features are consistent with the *Lcp-b* genes arising in this cluster by retrotransposition. The sequence data also indicated that *Acp* and *Lcp-a* lacked introns. The rest of the genes had introns but not the common one interrupting the signal peptide (see Section 12.4.3).

Evidence for gene conversion between the *Lcp-c* gene and those on the right side of the cluster was also found after a careful analysis of the sequences (Charles *et al.*, 1997).

The consequences of gene duplication in terms of gene expression are an important issue. It could be that duplicated genes were preserved to boost the amount of product made in the short period that the single-layer epidermis is secreting cuticle. Alternatively, duplication may allow for precise regulation of expression of genes both spatially and temporally. Subtle difference in protein sequence may be advantageous for particular structures. A detailed analysis of mRNA levels with Northern blot analysis demonstrated that some members of the 65A cuticular protein cluster have quite different patterns of expression. *Acp* was expressed only in adults. Expression was not detected for *Lcp-a*; all other *Lcp* genes were expressed in all larval stages, and all but *Lcp-b* and *-f* also contributed to pupal cuticle (Charles *et al.*, 1998).

12.4.3. Intron Structure of Cuticular Protein Genes

Genomic sequence data is available for 45 cuticular proteins in Table 1. None of these has more than two introns; most have only one, and these introns are in a very conserved positions. Thirty-five of these proteins have an intron that interrupts the signal peptide. In 25 of these sequences, interruption occurs after four amino acids (12 bp); in the remainder, two to eight amino acids are coded for before the intron begins. The PSORT tool (Psort, 2003) calculates the location of discrete regions of a potential signal peptide using modifications of McGeoch's method (McGeoch, 1985) and reports this information as "PSG" data. These short stretches, confined to the first exon, were shown to be identical to the N-terminal positively charged region in 20 cases; all but four of the rest were but one amino acid longer. Whether this correlation of the coding region of the first exon with the N-region of the signal peptide is because it is so short, or

because it reflects something more fundamental awaits further exploration. Most of the putative cuticular protein genes in the annotated *A. gambiae* database are missing their initiator methionine, probably because it resides in a short exon, coding for these few amino acids of the signal plus 5' untranslated nucleotides. The programs, unfortunately, are not yet trained to recognize a configuration with such a short open reading frame.

Genes for four of the proteins listed in Table 1 (HCCP12 and MSLCP 16/17, MSCP14.6, and TMAPC22) have two introns, one interrupting the signal peptide, the other occurring shortly after the beginning of the pfam00379 region. The intron-bearing *D. melanogaster* genes in the cluster at 65A (see above) have their sole intron at the internal position. This led Charles *et al.* (1997) to postulate that the primitive condition for introns in insect cuticular proteins would be two; over time, some genes lost one, some the other, and some lost both or arrived in the genome by retrotransposition.

There is also a *Drosophila* cuticular protein whose gene is located within the region corresponding to the first intron of *Gart*, a gene that encodes three proteins involved in the purine pathway. The gene for this RR-1 protein (*Gart Intron*) is read off the opposite strand and has its own intron, conventionally placed interrupting the signal peptide (Henikoff *et al.*, 1986). A comparably placed gene with 70% amino acid sequence identity is found in *D. pseudoobscura* (Henikoff and Eghtedarzadeh, 1987).

12.4.4. Regulatory Elements

One of the attractions of studying cuticular proteins is that they are secreted at precise times in the molt cycle and are thus candidates for genes under hormonal control (Riddiford, 1994). It would be expected, therefore, that some might have hormone response elements (see Chapter 7). Imperfect matches to ecdysteroid response elements (EcREs) from *D. melanogaster* have been found on two of its cuticular protein genes: EDG78 and EDG84 (Apple and Fristrom, 1991). These genes are activated in imaginal discs exposed to a pulse of ecdysteroids, but if exposed to continuous hormone, no message appears. The two cuticular protein genes that have been studied in *H. cecropia* have regions close to their transcription start sites that resemble EcREs (Binger and Willis, 1994; Lampe and Willis, 1994) and upstream from MSCP14.6 are also two regions that match (Rebers *et al.*, 1997).

Both *Bombyx* PCP and *H. cecropia* HCCP66 have response elements for members of the POU

family of receptors (Nakato *et al.*, 1992; Lampe and Willis, 1994). POU proteins are transcription factors used for tissue-specific regulation in mammals (Scholer, 1991). Gel mobility shift experiments established that there was a protein in epidermal cells that could bind to this element (Lampe and Willis, 1994).

As more genomic sequence information becomes available, identification of regulatory elements and verification of their action is certain to be productive.

12.5. Interaction of Cuticular Proteins with Chitin

Ever since the R&R Consensus was recognized in 1988, it has been predicted that it must be playing an important role in cuticle. As more and more sequences were discovered with the Consensus, and as it was learned that it also is present in cuticles formed by arachnids and crustaceans, this prediction became more likely. Several workers suggested that the role of the R&R Consensus might be to bind to chitin (Bouhin *et al.*, 1992a; Charles *et al.*, 1992; Andersen *et al.*, 1995a).

Four complementary routes have been followed to learn more about the function of this consensus region. The first was to analyze it with appropriate programs to generate predictions of secondary structure. The second approach was to use spectroscopic techniques on cuticular components to gain information about the conformation of their protein constituents *in situ*. Third, the tertiary structure of the extended Consensus has been modeled, and the fourth route was a direct experimental approach to test whether the extended Consensus could bind to chitin.

12.5.1. Secondary Structure Predictions

Prediction of secondary structure was carried out on the extended R&R Consensus region (67–68 amino acids, the pfam00379 region) of cuticular proteins representing different metamorphic stages and four different orders (Iconomidou *et al.*, 1999). For each protein, individual predictions of α -helix, β -sheet, and β -turn/coil/loops were carried out using several different prediction programs. These predictions on individual proteins were combined to produce joint prediction histograms for the two classes of proteins. (See Iconomidou *et al.* (1999) for details of proteins analyzed, programs used, and pictorial representation of results.)

The results indicated that the extended R&R domain of cuticular proteins has a considerable

proportion of β -pleated sheet structure and a total absence of α -helix. There appeared to be four β -strands in the RR-2 proteins and only three in the RR-1. Three other features were immediately apparent:

1. The three invariant glycines of the original R&R Consensus correspond exactly at the maxima of β -turn/loop predictions, and it is well known that glycines are good turn/loop formers (Chou and Fasman, 1974a, 1974b).
2. With both classes of cuticular proteins, the sheets showed an amphipathic character, i.e., one face is polar, the other nonpolar. Alternating residues along a strand point in the opposite direction on the two faces of a β -sheet. With these proteins, it is the aromatic or hydrophobic amino acids that alternate with other, sometimes hydrophilic, residues. The aromatic rings are thus positioned to stack against faces of the saccharide rings of chitin. This type of interaction is fairly common in protein–saccharide complexes (Vyas, 1991; Hamodrakas *et al.*, 1997; Tews *et al.*, 1997).
3. The turn/loop regions frequently contained histidines. This would place them “exposed” at the “edges” of a β -pleated sheet. Histidines are involved in cuticular sclerotization and are involved in the variations of the water-binding capacity of cuticle and the interactions of its constituent proteins. This occurs because small changes of pH can affect the ionization of their imidazole group (Andersen *et al.*, 1995a).

The suggestion that cuticular proteins adopt a β -sheet configuration is not new. Fraenkel and Rudall (1947) provided evidence from X-ray diffraction that the protein associated with chitin in insect cuticle has a β -type of structure.

12.5.2. Experimental Studies of Cuticular Protein Secondary Structure

The next step in probing the structure of cuticular proteins involved direct measurements on intact cuticles, on proteins extracted from them with a strong denaturing buffer with 8 M guanidine hydrochloride, and on the extracted cuticle. The cuticles came from the flexible abdominal cuticle of larvae of *H. cecropia*, and extracts have HCCP12, a RR-1 protein, as a major constituent (Cox and Willis, 1985). The same prediction programs described above were used on the sequence for HCCP12, and it indicated that the entire protein had a considerable proportion of β -pleated sheet and total absence of α -helix. Fourier-transform Raman spectroscopy

(FT-Raman), attenuated total reflectance infrared spectroscopy (ATR-FT-IR), and circular dichroism spectroscopy (CD) were carried out on these preparations (Iconomidou *et al.*, 2001). These techniques eliminated problems that had been found previously with more conventional laser-Raman spectra due to the high fluorescent background associated with cuticle.

The FT-Raman spectra of both the intact and extracted cuticle were dominated by the contribution of bands due to chitin. Certain features of the Raman spectrum of the intact cuticle signified the presence of proteins. The protein contribution to the spectrum of intact cuticle was revealed by subtracting the spectrum of the extracted cuticle, after scaling the discrete chitin bands of both preparations. The comparison of this difference spectra to that from the isolated proteins revealed striking similarities suggesting that the former gave a reliable physical picture of the cuticle protein vibrations in the native state. While Iconomidou *et al.* (2001) presented a detailed analysis of the spectra and the basis for each assignment, only a few features will be reviewed here. Several of the bands could be attributed to side-chain vibrations of amino acids with aromatic rings, tyrosine, phenylalanine, and tryptophan. Bands in the amide I region ($1600\text{--}1700\text{ cm}^{-1}$) of the Raman spectra of the extracted cuticle proteins and of the difference spectrum exhibited a well-defined maximum at 1669 cm^{-1} , typical of β -sheet structure. The absence of bands at $\sim 1650\text{ cm}^{-1}$ indicates that α -helical structures are not favored. The amide III range ($1230\text{--}1320\text{ cm}^{-1}$) is relatively free from side group vibrations and, thus, highly diagnostic of secondary structure. The extracted proteins had a doublet at 1241 and 1268 cm^{-1} ; the former can be assigned to β -sheet and the latter to β -turns or coil.

Results from ATR-FT-IR spectra from the extracted proteins were in good agreement with their FT-Raman spectra. These spectra had been obtained on lyophilized samples: the CD spectrum, on the other hand, was obtained with proteins solubilized in water. Detailed analysis of the CD spectrum indicated a high percentage (54%) of β -sheet conformation with a small contribution of α -helix ($\sim 13\%$). The contributions of β -turns/loops and random coil were estimated as 24% and 9% respectively (Iconomidou *et al.*, 2001). These results demonstrated that the main structural element of cuticle protein is the antiparallel β -pleated sheet. Comparable results were obtained from lyophilized proteins and intact cuticles and from proteins in solutions, thus negating the concern that lyophilization might

increase the β -sheet content of proteins as discussed by Griebenow *et al.* (1999). These direct measurements confirm the results from secondary structure prediction discussed above (see Section 12.5.1).

These findings are in accord with the prediction of Atkins (1985) that the antiparallel β -pleated sheet part of cuticular proteins would bind to α -chitin. His proposal was based mainly on a two-dimensional lattice matching between the surface of α -chitin and the antiparallel β -pleated sheet structure of cuticular proteins.

There seem to have been several independent solutions in nature whereby chitin binds to protein; in all surface aromatic residues appear to be significant (Shen and Jacobs-Lorena, 1999). In several cases β -sheets have been implicated. The chitin-binding motifs of two lectins studied at atomic resolution contain a two-stranded β -sheet (Suetake *et al.*, 2000). In bacterial chitinases, an antiparallel β -sheet barrel has also been postulated to play an important role in “holding” the chitin chain in place to facilitate catalysis. Four conserved tryptophans on the surface of the β -sheet are assumed to interact firmly with chitin, “guiding” the long chitin chains towards the catalytic “groove” (Perrakis *et al.*, 1997; Uchiyama *et al.*, 2001).

12.5.3. Modeling of Chitin-Binding Domains of Cuticular Proteins

Secondary structure prediction and experimental data summarized above (see Sections 12.5.1 and 12.5.2) indicated that β -pleated sheet is most probably the underlying molecular conformation of a large part of the extended R&R Consensus, especially the part which contains the R&R Consensus itself, and that this conformation is most probably involved in β -sheet/chitin-chain interactions of the cuticular proteins with the chitin filaments (Iconomidou *et al.*, 1999, 2001). Can this information be translated into a three-dimensional model?

Unexpectedly, a distant (20%) sequence similarity was found between RR-1-bearing cuticular proteins and the crystallographically determined C-terminal, β -sheet barrel portion, of bovine plasma retinol-binding protein (RBP). When, following alignment, both conservative substitutions and identities were combined, the similarity rises to 60% of the total HCCP12 sequence (Hamodrakas *et al.*, 2002). This similarity allowed the construction, by “homology” modeling, of a structural model of the “extended R&R consensus” (Hamodrakas *et al.*, 2002). This modeling was successful even though it is clear that RBP and the R&R Consensus-bearing cuticular

proteins are not strictly homologous, for HCCP12 lacks the N-terminal region that is conserved in members of the lipocalin superfamily to which RBP belongs. The original model (Figure 5a) comprises the C-terminal 66 residues (out of 89 in total) of HCCP12 and corresponds to the “extended R&R consensus” (see Section 12.3.2.3).

Does this model fit both major classes of RR proteins? Stereo plots of this model of HCCP12 (Figure 4a) can be compared to comparable models of two RR-2 proteins (HCCP66 and AGCP2b) (Figure 4b and c). These models demonstrated that the extended R&R Consensus of both “soft” and “hard” cuticle proteins may easily adopt the proposed conformation.

How would this proposed structure interact with chitin? A low-resolution docking experiment of an extended *N*-acetylglucosamine tetramer to the model of HCCP12, utilizing the docking program GRAMM (Vakser, 1996) revealed that the proposed model for cuticle proteins accommodates, rather comfortably, at least one extended chitin chain (Figure 4d) (Hamodrakas *et al.*, 2002). The features revealed by secondary structure predictions (see Section 12.5.1) and by experimental spectroscopic analysis (see Section 12.5.2) work exceedingly well with this model. It is an antiparallel β -sheet structure with a “cleft” full of conserved aromatic residues that

form “flat” hydrophobic surfaces on one “face,” perfectly positioned to stack against faces of the saccharide rings of chitin. One unpredicted feature in the model is a short (seven-residue) two-turn α -helix at the C-terminus of the extended R&R Consensus of HCCP12, starting and ending with two proline residues, present in 60% of the “soft” cuticle proteins in Figure 1. This C-terminal part of the model is reminiscent in some respects of the chitin-binding domain of an invertebrate chitin-binding lectin, a two-stranded β -sheet followed by a helical turn (Suetake *et al.*, 2000). The structures of these two different chitin-binding proteins cannot be superimposed, however, and show no sequence similarity.

For this review, the proposed half-barrel model has been used as a basis for more detailed docking experiments. A “high-resolution” docking experiment with the same tetramer was performed and the results are displayed and discussed in Figure 5. A new possibility emerges from this “high-resolution” experiment: the chitin chains can run either parallel to the β -strands of the half β -barrel model (Figure 5b and c), in good agreement with the observations of Atkins (1985), or perpendicular to the β -strands (Figure 5a). Figure 5b and c also provide an instructive view as to how a twisted helicoidal structure might arise from a close packing interaction of half- β -barrel models of cuticle proteins with chitin chains. It can

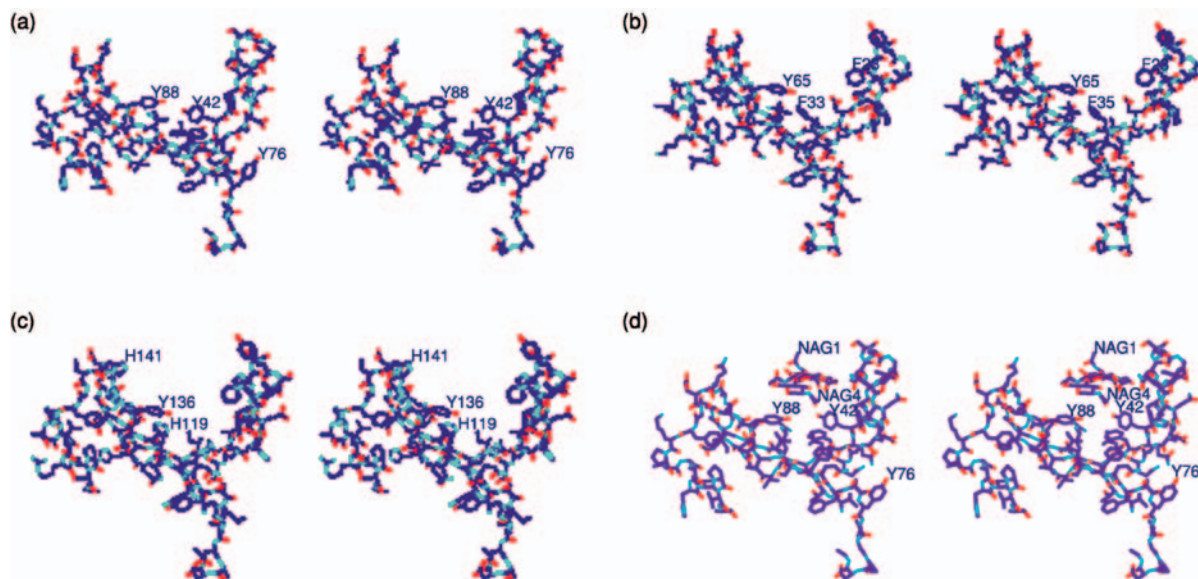


Figure 4 Stereo pairs of cuticular proteins and their interaction with chitin. Stereo pairs of cuticular proteins drawn with the program O (Jones *et al.*, 1991). The numbering scheme used is that of the unprocessed proteins. (a) View of the “soft” cuticle protein HCCP12. (b) View of the “hard” cuticle protein HCCP66. The terminal Ile83 residue could not be modeled and is not shown. (c) View of “hard” cuticle protein AGCP2b. His140 and the terminal Val155 residues could not be modeled and are not shown. (d) HCCP12 shown with an *N*-acetyl glucosamine (NAG) tetramer in an extended conformation. The complex was derived from a “low-resolution” docking experiment of a NAG tetramer, in an extended conformation, with the model of HCCP12, utilizing the docking program GRAMM (Vakser, 1996) and the default parameters of the program. (Reprinted with permission from Hamodrakas, S.J., Willis, J.H., Iconomidou, V.A., 2002. A structural model of the chitin-binding domain of cuticle proteins. *Insect Biochem. Mol. Biol.* 32, 1577–1583, © Elsevier.)

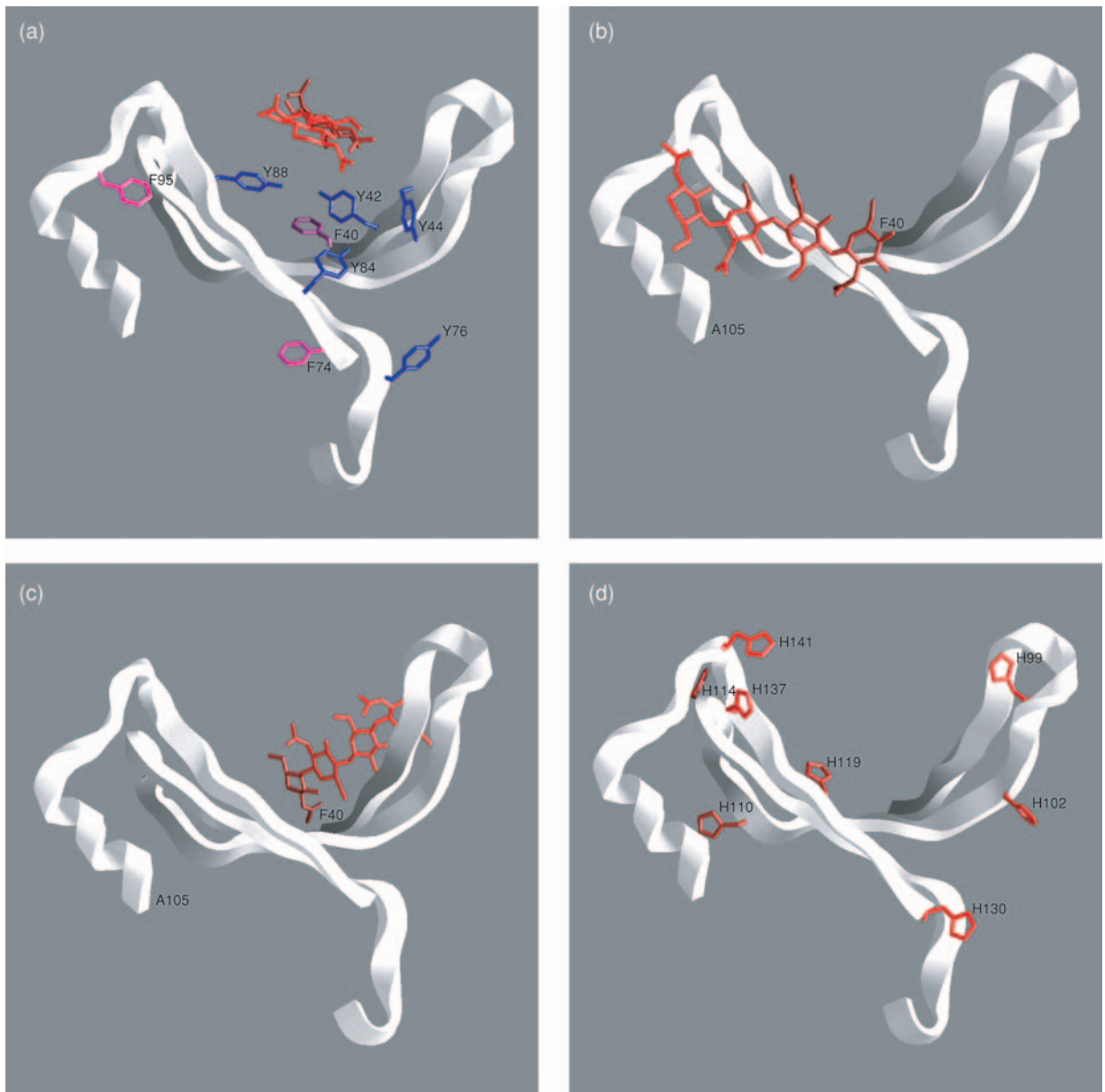


Figure 5 Ribbon models of cuticular proteins derived from homology modeling. (a) A ribbon model of cuticle protein structure, displayed using GRASP (Nicholls *et al.*, 1991). The structure of the representative “soft” cuticle protein HCCP12 was modeled on that of bovine retinol binding protein (RBP; PDB code 1FEN) (Zanotti *et al.*, 1994) utilizing the program WHAT IF (Vriend, 1990). Further details are in Hamodrakas *et al.* (2002). The side chains of several aromatic residues are shown and numbered, following the numbering scheme of the unprocessed HCCP12 sequence. These are: F40, Y42, Y44, H52, F74, Y76, Y84, Y88, and F95, underlined and bolded in **Figure 1**. The model structure has a “cleft” full of aromatic residues, which form “flat” surfaces of aromatic rings (upper side), ideally suited for cuticle protein–chitin chain interactions, and an outer surface (lower side) which should be important for protein–protein interactions in cuticle. The model is a complex of HCCP12 with an *N*-acetyl glucosamine (NAG) tetramer in an extended conformation. The complex was derived from a “low-resolution” docking experiment of a NAG tetramer, in an extended conformation, with the model of HCCP12, utilizing the docking program GRAMM (Vakser, 1996) and the default parameters of the program (a view similar to **Figure 4d**). (b) and (c) Two more possible complexes of HCCP12 with an NAG tetramer in an extended conformation derived from a “high-resolution” docking experiment, utilizing the program GRAMM (Vakser, 1996) and the default parameters of the program for “high resolution.” The two models presented in (b) and (c) are the two “top on the list,” most favorable complexes, whereas third on the list is a structure similar to that of (a). The one in (b) has the NAG tetramer more or less parallel to the last β -strand of the HCCP12 half β -barrel model, whereas that in (c) has the NAG tetramer more or less parallel to the first β -strand of the HCCP12 half β -barrel model. Note that, both in (b) and (c) the chitin chain runs parallel to the β -strands, whereas in (a) the chain is arranged perpendicular to the β -strands. (d) A display of a model of the “hard cuticle” protein AGCP2b. The numbering is that of the unprocessed protein. Histidine (H) side chains are shown as “ball and sticks,” in red, with their corresponding numbering. The corresponding residues are underlined in the sequence for AGCP2b in **Figure 2**.

be seen that the chitin chains, although more or less parallel to the β -strands, are forming an angle of the order of 10–15 degrees with the β -strands, and this, together with the inherent twist of the β -strands in the β -barrel, could provide the basis for the twisted helicoidal structure of the cuticle in general. Thus, both the inherent twist of the half-barrel β -sheet of the cuticle proteins and its packing arrangement at an angle with the chitin chains may provide a molecular basis for the morphological observation of a helicoidal twist in cuticle.

The model proposed by Hamodrakas *et al.* (2002) was subjected to a further test, namely that it should provide for the right positioning of histidine residues in the “hard cuticle” proteins, so that these histidines might play a significant role in cuticle sclerotization (Neville, 1975; Andersen *et al.*, 1995a). Histidines are a common feature of the extended R&R Consensus of many RR-2 proteins and many of their positions are conserved (Figure 2) (see Section 12.3.2.3). If they are to function in protein cross-linking by sclerotizing agents, they must reside on opposite faces to the aromatic residues that were postulated to interact with chitin (Iconomidou *et al.*, 1999; Hamodrakas *et al.*, 2002). In Figure 5d, a model of the “hard cuticle” protein AGCP2b is shown, similar in orientation to Figure 5a, indicating the positions of the histidine side chains. The relevant histidines are underlined and in bold in the sequence for AGCP2b in Figure 2. Three histidines, H102, H110, and H141 (the second, third, and last) are at sites where histidines are common. Such an interspecies conservation of these histidines, most probably signifies their very important structural and functional role (see below).

All the bolded and underlined histidines occupy “exposed” positions either in turns (like H99, H114, H137, H141), or at the “edges” of the half- β -barrel or its periphery (like H102, H110, H130), in excellent positions to be involved in cuticular sclerotization, readily reacting with activated *N*-acetyldopamine residues. Alternatively, they could be involved in the variations of the water-binding capacity of cuticle and the interactions of its constituent proteins, because small changes of pH can affect the ionization of their imidazole groups (Andersen *et al.*, 1995a).

These observations are in excellent agreement with the predictions made several years ago for the

role of histidines from secondary structure predictions (Iconomidou *et al.*, 1999) and strengthen further the value of the model previously proposed both for “soft” and “hard” cuticle proteins (Hamodrakas *et al.*, 2002).

12.5.4. Fusion Proteins Establish a Role for the Extended R&R Consensus

Predictions of secondary and tertiary structure and experimental evidence supporting them (discussed above in Sections 12.5.1–12.5.3) established that the extended R&R Consensus has the properties to serve as a chitin-binding motif. In particular, the planar surfaces of the predicted β -sheets will expose aromatic residues positioned for protein–chitin interaction. The ultimate test of these predictions would be to show that the extended consensus region is sufficient to confer chitin binding on a protein.

Rebers and Willis (2001) investigated this possibility by creating fusion proteins using the extended R&R Consensus from the *A. gambiae* putative cuticular protein, AGCP2b. First they expressed this protein in *Escherichia coli* and isolated it from cell lysates. The construct used coded for the complete protein minus the predicted signal peptide and had a histidine-tag added to the N-terminus to facilitate purification (Dotson *et al.*, 1998). AGCP2b is a protein of 222 amino acids, with a RR-2 type of consensus. The purified protein bound to chitin beads and could be eluted from these beads with 8 M urea or boiling SDS. This established unequivocally that AGCP2b was a chitin-binding protein. Chitin binding previously had been obtained with mixtures of protein extracted from cuticles of two beetles and *D. melanogaster* (Hackman, 1955; Fristrom *et al.*, 1978; Hackman and Goldberg, 1978).

The next, and essential, step was to create a fusion protein uniting a protein that did not bind to chitin with the extended R&R Consensus region. Such a fusion was created between glutathione-S-transferase (GST) and 65 amino acids for AGCP2b, covering the region of pfam00379, as shown in Figure 6.

The GST and the fusion protein were each affinity purified using a glutathione–sepharose column. GST alone did not bind to chitin but the fusion protein did, requiring denaturing agents for release.

Other experiments defined in more detail the requirements for converting GST into a chitin-binding protein. A shorter fragment of AGCP2b,

APANYEFSYSVHDEHT**IGD**IKSQHETRHGDEVHGOYSLLDSDGHQRIVD**VHADHHTG**INAVVRREP

Figure 6 The pfam00379 region of AGCP2b used to construct fusion proteins. For details, see text.

40 amino acids (underlined in **Figure 6**) with the strict R&R Consensus (shown in italics) did not bind chitin. Nor did the full construct when either the Y and F (bolded and highlighted) of the strict R&R Consensus or the T and D (highlighted) of the extended consensus were “mutated” to alanine (Rebers and Willis, 2001).

These experiments established, at last, that the extended R&R Consensus is sufficient to confer chitin-binding properties on a protein and thereby resolved years of speculation on the importance of this region.

Chitinase, some lectins, and proteins from peritrophic membranes all bind chitin (review: Shen and Jacobs-Lorena, 1999). What is unique about the extended R&R Consensus is that it lacks cysteine residues. These residues serve essential roles in the other types of chitin-binding proteins, forming disulfide bonds that hold the protein in the proper configuration for binding. While these other chitin-binding proteins have weak sequence similarities to one another, they do not approach the sequence conservation seen in the R&R Consensus throughout the arthropods. Rebers and Willis (2001) suggested that this conservation (see **Figures 1** and **2**) could well be due to the need to preserve a precise conformation of the chitin-binding domain in the absence of stabilizing disulfide bonds.

In addition to establishing a function of the extended R&R Consensus, these experiments also provided confirmation of key elements in the models discussed above (see Section 12.5.4). Substitution of the two conserved aromatic residues abolished chitin binding. With the TD “mutations,” alanines were substituted for two other conserved residues. These flank a glycine that is conserved in position in the “extended consensus” of all hard and many soft cuticles (Iconomidou *et al.*, 1999). According to the proposed model (**Figure 5a**), these two polar residues would point away from the hydrophobic “cleft” and thus should not participate in chitin binding. It should be noted, however, that this glycine is located at a sharp turn, at the end of the second β -strand (in the vicinity of H102 of **Figure 5d**). The substitution of two polar residues by two alanines may result in destruction of this turn and to improper folding, thus leading to a structure not capable of binding chitin.

12.5.5. Summary

Four different types of data have been presented above (see Section 12.5) analyzing the extended R&R Consensus: secondary structure predictions of antiparallel β -sheets (see Section 12.5.1),

experimental spectroscopic evidence from cuticles and cuticle extracts for the predominance of such β -sheets in cuticular protein conformation (see Section 12.5.2), models showing organization of the consensus into a half β -barrel with a groove that can accommodate chitin (see Section 12.5.3), and direct demonstration that the extended consensus is sufficient to confer chitin binding on a protein (see Section 12.5.4). These four types of data are all in agreement that the highly conserved amino acid sequence of the extended R&R Consensus forms a novel chitin-binding domain, albeit one that displays an essential feature of other proteins that interact with chitin, namely the presentation of aromatic residues in a planar surface. Crystal structures of the cuticular protein–chitin complex are needed to assure that these inferences are correct.

12.6. Comparison of Cuticle and Chorion: Structure and Proteins

Silkmoth and fish chorions (eggshells) and cuticle are known to have a helicoidal architecture (Neville, 1975; Hamodrakas, 1992). Excellent reviews on helicoidal architecture and its appearance in biological systems have been made by Bouligand (1972, 1978a, 1978b) and Neville (1975, 1981, 1986). These works describe, in a beautiful and most comprehensive way, how helicoids are identified, how widespread they are, and the basic molecular principles of their formation as well as their geometrical, physical, and biological properties. The close analogy between the helicoidal structures of (usually extracellular) biological materials and the structure of cholesteric liquid crystals suggests that these structures self-assemble according to a mechanism that is very similar to the process allowing materials to form liquid crystals. Apparently, helicoids should pass through a liquid crystalline phase before solidifying. It is assumed that this occurs in the assembly zone during cuticle formation. Self-assembling systems are important in biology, as they are economical in energy terms, requiring neither enzymatic control nor the expenditure of energy-rich bonds. They are particularly appropriate for building extracellular skeletal structures outside of the cells that secrete the components (Bouligand, 1978a, 1978b; Neville, 1986).

Silkmoth chorion is produced by the follicular cells that surround the oocyte (Regier and Kafatos, 1985 and references therein). Fish eggshell is mainly produced by the oocyte, with minor contributions from the follicular cells (Hamodrakas, 1992 and references therein) and cuticle is produced by the epidermis.

Natural helicoidal composites occur in several combinations such as polysaccharide fibers in a polysaccharide matrix (plant cell walls), polysaccharide fibers in a protein matrix (arthropod cuticle), and protein fibers in a protein matrix (insect and fish eggshells). In all cases, principles of molecular recognition and weak intermolecular interactions should govern the self-assembly mechanisms (Neville, 1986).

In silkmoth chorion, disulfide bonds, and in fish eggshell, isopeptide bonds between the side chains of R-K and D-E, are major contributors to stabilization. These covalent bonds, however, are totally absent in cuticle, where stabilization occurs via protein–chitin interaction and by cross-linking by sclerotization compounds.

It is clear that the main characteristic of chorion proteins is the presence of exact, tandemly repeating hexapeptide motifs that adopt a characteristic antiparallel β -pleated sheet structure. This is the main structural unit of silkmoth chorion fibrils and, apparently, the molecular denominator, which dictates formation of the helicoidal architecture (Hamodrakas, 1992). The ellipsoidal shape of silkmoth chorions is, most probably, due to the fact that the basic building-blocks, chorion protein fibrils, are so uniform in shape. By contrast, in cuticle, despite the fact that there are regions of the molecules rich in tandem repeats of certain motifs (see Section 12.3.2.2), the sequences are mainly characteristic of globular proteins, and cuticle may adopt all sorts of shapes depending on the local needs of the arthropods producing it. The majority of cuticular proteins contain a conserved domain, rich in a characteristic antiparallel β -pleated sheet structure, a half β -barrel (see Section 12.5.3) which again should serve as the molecular denominator determining the helicoidal structure of cuticle, interacting with chitin crystalline chains and giving rise to a plethora of architectural plans as needed locally.

Apparently, an antiparallel β -pleated sheet type of structure is the common molecular denominator, that dictates the helicoidal architecture adopted by the chorion of Lepidoptera and fish and also by the arthropod cuticle.

12.7. Summary and Future Challenges

This review has summarized the wealth of information about cuticular proteins amassed since Silvert's review in 1985. Most striking is that the 35-fold increase in sequences for structural cuticular proteins has revealed that the majority has a conserved

domain (pfam00379) that is an extended version of the R&R Consensus. A group of proteins that appears to contribute to hard cuticles have a highly conserved extended consensus (RR-2). It is now known that RR-2 proteins interact with chitin and we can predict in some detail the features of their sequence that confer this property. It is not known whether the RR-1 proteins are as effective in binding chitin. We have not yet begun to analyze how the regions outside the consensus contribute to cuticular properties, nor have we learned how the proteins lacking the consensus but with other conserved features contribute to cuticle structure.

Cuticular proteins with pfam00379 are one of the largest multigene families found in *Drosophila* (Lespinet *et al.*, 2002). We need to learn whether this multiplicity serves to allow rapid synthesis of cuticle or whether different genes are used to construct cuticles in different regions. If the latter, the question becomes whether subtle differences in sequence are important for different cuticular properties, or if gene multiplication has been exploited to allow precise temporal and spatial control. The elegant immunolocalization studies that have been carried out were done with antibodies against proteins whose sequences for the most part are unknown. Now that we recognize that several genes may have almost identical sequences, we have to be very careful in designing specific probes for use in Northern analyses, for *in situ* hybridization, and for immunolocalization, if our goal is to learn the use to which each individual gene is put.

Cuticular protein sequences are certain to be described in ever-increasing numbers as more insect genomes are analyzed. Describers need to be careful to submit to databases an indication of whether assignment as a cuticular protein is based on sequence alone or on some type of corroborating evidence. It would be helpful if there were a more consistent system for naming cuticular proteins. At the very least, each protein should have a designation of genus and species and a unique number.

A wealth of information is available already but many challenges lie ahead for those who wish to continue to further our understanding of how the diverse forms and properties of cuticle are constructed extracellularly as these proteins self-assemble in proximity to chitin.

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References

- Agrawal, O.P., Scheller, K., 1986. The formation of the chitin-arylphorin complex *in vitro*. In: Muzzarelli, R., Jeuniaux, C., Gooday, G.W. (Eds.), *Chitin in Nature and Technology*. Plenum Press, New York, pp. 316–320.
- Andersen, S.O., 1975. Cuticular sclerotization in the beetles *Pachynoda epphiata* and *Tenebrio molitor*. *J. Insect Physiol.* 21, 1225–1232.
- Andersen, S.O., 1998. Amino acid sequence studies on endocuticular proteins from the desert locust, *Schistocerca gregaria*. *Insect Biochem. Mol. Biol.* 28, 421–434.
- Andersen, S.O., 2000. Studies on proteins in post-ecdysial nymphal cuticle of locust, *Locusta migratoria*, and cockroach, *Blaberus cranifer*. *Insect Biochem. Mol. Biol.* 30, 569–577.
- Andersen, S.O., Hojrup, P., Roepstorff, P., 1995a. Insect cuticular proteins. *Insect Biochem. Mol. Biol.* 25, 153–176.
- Andersen, S.O., Rafn, K., Krogh, T.N., Hojrup, P., Roepstorff, P., 1995b. Comparison of larval and pupal cuticular proteins in *Tenebrio molitor*. *Insect Biochem. Mol. Biol.* 25, 177–187.
- Andersen, S.O., Rafn, K., Roepstorff, P., 1997. Sequence studies of proteins from larval and pupal cuticle of the yellow meal worm, *Tenebrio molitor*. *Insect Biochem. Mol. Biol.* 27, 121–131.
- Andersen, S.O., Weis-Fogh, T., 1964. Resilin: a rubber-like protein in arthropod cuticle. *Adv. Insect Physiol.* 2, 1–65.
- Apple, R.T., Fristrom, J.W., 1991. 20-Hydroxyecdysone is required for, and negatively regulates, transcription of *Drosophila* pupal cuticle protein genes. *Devel. Biol.* 146, 569–582.
- Ardell, D.H., Andersen, S.O., 2001. Tentative identification of a resilin gene in *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* 31, 965–970.
- Ashida, M., Brey, P.T., 1995. Role of the integument in insect defense: pro-phenol oxidase cascade in the cuticular matrix. *Proc. Natl Acad. Sci. USA* 92, 10698–10702.
- Atkins, E.D.T., 1985. Conformations in polysaccharides and complex carbohydrates. *Proc. Int. Symp. Biomol. Struct. Interactions, Suppl. J. Biosci.* 8, 375–387.
- Bateman, A., Birney, E., Cerruti, L., Durbin, R., Etwiller, L., et al., 2002. The Pfam protein families database. *Nucleic Acids Res.* 30, 276–280.
- Binger, L.C., Willis, J.H., 1990. *In vitro* translation of epidermal RNAs from different anatomical regions and metamorphic stages of *Hyalophora cecropia*. *Insect Biochem.* 20, 573–583.
- Binger, L.C., Willis, J.H., 1994. Identification of the cDNA, gene and promoter for a major protein from flexible cuticles of the giant silkworm *Hyalophora cecropia*. *Insect Biochem. Mol. Biol.* 24, 989–1000.
- BLAST, 2003. <http://www.ncbi.nlm.nih.gov/BLAST/>
- Bouhin, H., Charles, J.-P., Quenedey, B., Courrent, A., Delachambre, J., 1992a. Characterization of a cDNA clone encoding a glycine-rich cuticular protein of *Tenebrio molitor*: developmental expression and effect of a juvenile hormone analogue. *Insect Mol. Biol.* 1, 53–62.
- Bouhin, H., Charles, J.-P., Quenedey, B., Delachambre, J., 1992b. Developmental profiles of epidermal mRNAs during the pupal–adult molt of *Tenebrio molitor* and isolation of a cDNA encoding an adult cuticular protein: effects of a juvenile hormone analogue. *Devel. Biol.* 149, 112–122.
- Bouligand, Y., 1972. Twisted fibrous arrangements in biological materials and cholesteric mesophases. *Tissue and Cell* 4, 189–217.
- Bouligand, Y., 1978a. Cholesteric order in biopolymers. *ACS Symposium Series* 74, 237–247.
- Bouligand, Y., 1978b. Liquid crystalline order in biological materials. In: Blumstein, A. (Ed.), *Liquid Crystalline Order in Polymers*. Academic Press, London, pp. 261–297.
- Browder, L.W., Wilkes, J., Rodenhiser, D.I., 1992. Preparative labeling of proteins with [³⁵S]methionine. *Anal. Biochem.* 204, 85–89.
- Carter, D., Locke, M., 1993. Why caterpillars do not grow short and fat. *Int. J. Insect Morphol. Embryol.* 22, 81–102.
- Charles, J.-P., Bouhin, H., Quenedey, B., Courrent, A., Delachambre, J., 1992. cDNA cloning and deduced amino acid sequence of a major, glycine-rich cuticular protein from the coleopteran *Tenebrio molitor*: temporal and spatial distribution of the transcript during metamorphosis. *Eur. J. Biochem.* 206, 813–819.
- Charles, J.-P., Chihara, C., Nejad, S., Riddiford, L.M., 1997. A cluster of cuticle protein genes of *Drosophila melanogaster* at 65A: sequence, structure and evolution. *Genetics* 147, 1213–1226.
- Charles, J.-P., Chihara, C., Nejad, S., Riddiford, L.M., 1998. Identification of proteins and developmental expression of RNAs encoded by the 65A cuticle protein gene cluster in *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* 28, 131–138.
- Chou, P., Fasman, G.D., 1974a. Conformational parameters for amino acids in helical, β -sheet and random coil regions calculated from proteins. *Biochemistry* 13, 211–221.
- Chou, P., Fasman, G.D., 1974b. Prediction of protein conformation. *Biochemistry* 13, 222–245.
- Condoulis, W.V., Locke, M., 1966. The deposition of endocuticle in an insect, *Calpodus ethlius* Stoll (Lepidoptera, Hesperidae). *J. Insect Physiol.* 12, 311–323.
- Cornell, J.C., Pan, M.L., 1983. The disappearance of moulting fluid in the tobacco hornworm, *Manduca sexta*. *J. Exp. Biol.* 107, 501–504.
- Cox, D.L., Willis, J.H., 1985. The cuticular proteins of *Hyalophora cecropia* from different anatomical regions and metamorphic stages. *Insect Biochem.* 15, 349–362.
- Cox, D.L., Willis, J.H., 1987a. Analysis of the cuticular proteins of *Hyalophora cecropia* with two dimensional electrophoresis. *Insect Biochem.* 17, 457–468.
- Cox, D.L., Willis, J.H., 1987b. Post-translational modifications of the cuticular proteins of *Hyalophora cecropia*

- from different anatomical regions and metamorphic stages. *Insect Biochem.* 17, 469–484.
- Csikos, G., Molnar, K., Borhegyi, N.H., Talian, G.C., Sass, M., 1999. Insect cuticle, an *in vivo* model of protein trafficking. *J. Cell Sci.* 112, 2113–2124.
- Doctor, J., Fristrom, D., Fristrom, J.W., 1985. The pupal cuticle of *Drosophila*: biphasic synthesis of pupal cuticle proteins *in vivo* and *in vitro* in response to 20-hydroxyecdysone. *J. Cell Biol.* 101, 189–200.
- Dotson, E.M., Cornel, A.J., Willis, J.H., Collins, F.H., 1998. A family of pupal-specific cuticular protein genes in the mosquito *Anopheles gambiae*. *Insect Biochem. Mol. Biol.* 28, 459–472.
- ENTREZ, 2003. <http://www3.ncbi.nlm.nih.gov/Entrez/index.html>
- Fechtel, K., Fristrom, D.K., Fristrom, J.W., 1989. Pre-pupal differentiation in *Drosophila*: distinct cell types elaborate a shared structure, the pupal cuticle, but accumulate transcripts in unique patterns. *Development* 106, 649–656.
- Flybase, 2003. Genetic data on *Drosophila*. <http://flybase.bio.indiana.edu/>
- Fraenkel, G., Rudall, K.M., 1947. The structure of insect cuticles. *Proc. Roy. Soc. B* 134, 111–143.
- Fristrom, D., Doctor, J., Fristrom, J.W., 1986. Procuticle proteins and chitin-like material in the inner epicuticle of the *Drosophila* pupal cuticle. *Tissue and Cell* 18, 531–543.
- Fristrom, D., Fristrom, J.W., 1993. The metamorphic development of the adult epidermis. In: Bate, M., Martinez Arias, A. (Eds.), *The Development of Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 843–897.
- Fristrom, J.W., Hill, R.J., Watt, F., 1978. The procuticle of *Drosophila*: heterogeneity of urea-soluble proteins. *Biochemistry* 17, 3917–3924.
- Geyer, P.G., Corces, V.G., 1987. Separate regulatory elements are responsible for the complex pattern of tissue-specific and developmental transcription of the *yellow* locus in *Drosophila melanogaster*. *Genes and Devel.* 1, 996–1004.
- Griebenow, K., Santos, A.M., Carrasquillo, K.G., 1999. Secondary structure of proteins in the amorphous dehydrated state probed by FTIR spectroscopy: dehydration-induced structural changes and their prevention. *Int. J. Vibr. Spectrosc.* 3, 1–34.
- Gu, S., Willis, J.H., 2003. Distribution of cuticular protein mRNAs in silk moth integument and imaginal discs. *Insect Biochem. Mol. Biol.* 33, 1177–1188.
- Hackman, R.H., 1955. Studies on chitin. 3. Absorption of proteins to chitin. *Austral. J. Biol. Sci.* 8, 530–536.
- Hackman, R.H., Goldberg, M., 1978. The non-covalent binding of two insect cuticular proteins by a chitin. *Insect Biochem.* 8, 353–357.
- Hamodrakas, S.J., 1992. Molecular architecture of helical proteinaceous eggshells. In: Case, S.T. (Ed.), *Results and Problems in Cell Differentiation*, vol. 19. Springer-Verlag, Berlin, pp. 116–186.
- Hamodrakas, S.J., Kanellopoulos, P.N., Pavlou, K., Tucker, P.A., 1997. The crystal structure of the complex of concanavalin A with 4-methylumbelliferyl- α -D-glucopyranoside. *J. Struct. Biol.* 118, 23–30.
- Hamodrakas, S.J., Willis, J.H., Iconomidou, V.A., 2002. A structural model of the chitin-binding domain of cuticle proteins. *Insect Biochem. Mol. Biol.* 32, 1577–1583.
- Han, Q., Fang, J., Ding, H., Johnson, J.K., Christensen, B.M., *et al.*, 2002. Identification of *Drosophila melo-gaster* yellow-f and yellow-f2 proteins as dopachrome-conversion enzymes. *Biochem. J.* 368, 333–340.
- Henikoff, S., Eghtedarzadeh, M.K., 1987. Conserved arrangement of nested genes at the *Drosophila Gart* locus. *Genetics* 117, 711–725.
- Henikoff, S., Keene, M.A., Fechtel, K., Fristrom, J.W., 1986. Gene within a gene: nested *Drosophila* genes encode unrelated proteins on opposite DNA strands. *Cell* 44, 33–42.
- Holden, H.M., Rypniewski, W.R., Law, J.H., Rayment, I., 1987. The molecular structure of insecticyanin from the tobacco hornworm *Manduca sexta* L. at 2.6 Å resolution. *EMBO J.* 6, 1565–1570.
- Horodyski, F.M., Riddiford, L.M., 1989. Expression and hormonal control of a new larval cuticular multigene family at the onset of metamorphosis of the tobacco hornworm. *Devel. Biol.* 132, 292–303.
- Iconomidou, V.A., Chryssikos, G.D., Gionis, V., Willis, J.H., Hamodrakas, S.J., 2001. “Soft”-cuticle protein secondary structure as revealed by FT-Raman, ATR-FT-IR and CD spectroscopy. *Insect Biochem. Mol. Biol.* 31, 877–885.
- Iconomidou, V.A., Willis, J.H., Hamodrakas, S.J., 1999. Is β -pleated sheet the molecular conformation which dictates formation of helicoidal cuticle? *Insect Biochem. Mol. Biol.* 29, 285–292.
- Ikeya, T., Persson, P., Kono, M., Watanabe, T., 2001. The *DD5* gene of the decapod crustacean *Penaeus japonicus* encodes a putative exoskeletal protein with a novel tandem repeat structure. *Comp. Biochem. Physiol. B* 128, 379–388.
- Janssens, H., Gehring, W.J., 1999. Isolation and characterization of *drosocrystallin*, a lens crystallin gene of *Drosophila melanogaster*. *Devel. Biol.* 207, 204–214.
- Jones, T.A., Zou, J.Y., Cowan, S.W., Kjeldgaard, M., 1991. Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr. A* 47, 110–119.
- Kalinich, J.F., McClain, D.E., 1992. An *in vitro* method for radiolabeling proteins with ^{35}S . *Anal. Biochem.* 205, 208–212.
- Kaufman, T.C., Seeger, M.A., Olsen, G., 1990. Molecular and genetic organization of the Antennapedia gene complex of *Drosophila melanogaster*. *Adv. Genet.* 27, 309–362.
- Kollberg, U., Obermaier, B., Hirsch, H., Kelber, G., Wobert, P., 1995. Expression cloning and characterization of a pupal cuticle protein cDNA of *Galleria mellonella* L. *Insect Biochem. Mol. Biol.* 25, 355–363.

- Konig, M., Agrawal, O.P., Schenkel, H., Scheller, K., 1986. Incorporation of calliphorin into the cuticle of the developing blowfly, *Calliphora vicina*. *Roux's Arch. Devel. Biol.* 195, 296–301.
- Kornezos, A., Chia, W., 1992. Apical secretion and association of the *Drosophila yellow* gene product with developing larval cuticle structures during embryogenesis. *Mol. Gen. Genet.* 235, 397–405.
- Lampe, D.J., Willis, J.H., 1994. Characterization of a cDNA and gene encoding a cuticular protein from rigid cuticles of the giant silkworm, *Hyalophora cecropia*. *Insect Biochem. Mol. Biol.* 24, 419–435.
- Lee, W.-J., Brey, P.T., 1994. Isolation and identification of cecropin antibacterial peptides from the extracellular matrix of insect integument. *Anal. Biochem.* 217, 231–235.
- Lemoine, A., Delachambre, J., 1986. A water-soluble protein specific to the adult cuticle in *Tenebrio*: its use as a marker of a new programme expressed by epidermal cells. *Insect Biochem.* 16, 483–489.
- Lemoine, A., Millot, C., Curie, G., Delachambre, J., 1989. A monoclonal antibody against an adult-specific cuticular protein of *Tenebrio molitor* (Insecta, Coleoptera). *Devel. Biol.* 136, 546–554.
- Lemoine, A., Millot, C., Curie, G., Delachambre, J., 1990. Spatial and temporal variations in cuticle proteins as revealed by monoclonal antibodies, immunoblotting analysis and ultrastructural immunolocalization in a beetle, *Tenebrio molitor*. *Tissue and Cell* 22, 177–189.
- Lemoine, A., Millot, C., Curie, G., Massonneau, V., Delachambre, J., 1993. Monoclonal antibodies recognizing larval- and pupal-specific cuticular proteins of *Tenebrio molitor* (Insecta, Coleoptera). *Roux's Arch. Devel. Biol.* 203, 92–99.
- Lespinet, O., Wolf, Y.I., Koonin, E.V., Aravind, L., 2002. The role of lineage-specific gene family expansion in the evolution of eukaryotes. *Genome Res.* 12, 1048–1059.
- Leung, H., Palli, S.R., Locke, M., 1989. The localization of arylphorin in an insect, *Calpodes ethlius*. *J. Insect Physiol.* 35, 223–231.
- Li, W., Riddiford, L.M., 1992. Two distinct genes encode two major isoelectric forms of insecticyanin in the tobacco hornworm, *Manduca sexta*. *Eur. J. Biochem.* 205, 491–499.
- Locke, M., 1998. Epidermis. In: Harrison, F.W., Locke, M. (Eds.), *Microscopic Anatomy of Invertebrates*, vol. 11A. Wiley-Liss, New York, pp. 75–138.
- Locke, M., 2001. The Wigglesworth Lecture: Insects for studying fundamental problems in biology. *J. Insect Physiol.* 47, 495–507.
- Locke, M., 2003. Surface membranes, golgi complexes and vacuolar systems. *Annu. Rev. Entomol.* 48, 1–27.
- Locke, M., Kiss, A., Sass, M., 1994. The cuticular localization of integument peptides from particular routing categories. *Tissue and Cell* 26, 707–734.
- Lombardi, E.C., Kaplan, D.L., 1993. Preliminary characterization of resilin isolated from the cockroach, *Periplaneta americana*. *Mat. Res. Soc. Proc.* 292, 3–7.
- Marcu, O., Locke, M., 1998. A cuticular protein from the moulting stages of an insect. *Insect Biochem. Mol. Biol.* 28, 659–669.
- Marcu, O., Locke, M., 1999. The origin, transport and cleavage of the molt-associated cuticular protein CECP22 from *Calpodes ethlius* (Lepidoptera, Hesperidae). *J. Insect Physiol.* 45, 861–870.
- Mathelin, J., Bouhin, H., Quennedey, B., Courrent, A., Delachambre, J., 1995. Identification, sequence and mRNA expression pattern during metamorphosis of a cDNA encoding a glycine-rich cuticular protein in *Tenebrio molitor*. *Gene* 156, 259–264.
- Mathelin, J., Quennedey, B., Bouhin, H., Delachambre, J., 1998. Characterization of two new cuticular genes specifically expressed during the post-ecdysial molting period in *Tenebrio molitor*. *Gene* 211, 351–359.
- McGeoch, D.J., 1985. On the predictive recognition of signal peptide sequences. *Virus Res.* 3, 271–286.
- Mita, K., Morimyo, M., Okano, K., Shimada, T., Maeda, S., 1999. The construction of EST database for genome analysis of *Bombyx mori*. *RIKEN Rev.* 22, 63–67.
- Molnar, K., Borhegyi, N.H., Csikos, G., Sass, M., 2001. The immunoprotein scolexin and its synthesizing sites: the midgut epithelium and the epidermis. *Acta Biol. Hungarica* 52, 473–484.
- MOTIF, 2003. <http://motif.genome.ad.jp/MOTIF2.html>
- Nakato, H., Izumi, S., Tomino, S., 1992. Structure and expression of gene coding for a pupal cuticle protein of *Bombyx mori*. *Biochim. Biophys. Acta* 1132, 161–167.
- Nash, W.G., 1976. Patterns of pigmentation color states regulated by the γ locus in *Drosophila melanogaster*. *Devel. Biol.* 48, 336–343.
- Neville, A.C., 1975. *Biology of the Arthropod Cuticle*. Springer-Verlag, Berlin.
- Neville, A.C., 1981. Cholesteric proteins. *Mol. Cryst. Liq. Cryst.* 76, 279–286.
- Neville, A.C., 1986. The physics of helicoids: multidirectional “plywood” structures in biological systems. *Phys. Bull.* 37, 74–76.
- Nicholls, A., Sharp, K.A., Honig, B., 1991. Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. *Proteins: Struct. Funct. Genet.* 11, 281–296.
- Nielsen, H., Engelbrecht, J., Brunak, S., von Heijne, G., 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* 10, 1–6.
- Nielsen, H., Krogh, A., 1998. Prediction of signal peptides and signal anchors by a hidden Markov model. *Proc. Int. Conf. Intell. Syst. Mol. Biol.* 6, 122–130.
- Palli, S.R., Locke, M., 1987. The synthesis of hemolymph proteins by the larval epidermis of an insect *Calpodes ethlius* (Lepidoptera: Hesperidae). *Insect Biochem.* 17, 711–722.
- Perrakis, A., Ouzounis, C., Wilson, K.S., 1997. Evolution of immunoglobulin-like modules in chitinases: their structural flexibility and functional implications. *Folding and Design* 2, 291–294.

- Pfam, 2003a. <http://www.sanger.ac.uk/Software/Pfam/>
- Pfam, 2003b. <http://pfam.wustl.edu/cgi-bin/getdesc?acc=PF00379>
- Psort, 2003. <http://psort.ims.u-tokyo.ac.jp/>
- Rebers, J.E., Niu, J., Riddiford, L.M., 1997. Structure and spatial expression of the *Manduca sexta* MSCP14.6 cuticle gene. *Insect Biochem. Mol. Biol.* 27, 229–240.
- Rebers, J.F., Riddiford, L.M., 1988. Structure and expression of a *Manduca sexta* larval cuticle gene homologous to *Drosophila* cuticle genes. *J. Mol. Biol.* 203, 411–423.
- Rebers, J.E., Willis, J.H., 2001. A conserved domain in arthropod cuticular proteins binds chitin. *Insect Biochem. Mol. Biol.* 31, 1083–1093.
- Regier, J.C., Kafatos, F.C., 1985. Molecular aspects of chorion formation. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry, and Pharmacology*, vol. 1. Pergamon Press, Oxford, pp. 113–151.
- Riddiford, L.M., 1994. Cellular and molecular actions of juvenile hormone I: general considerations and premetamorphic actions. *Adv. Insect Physiol.* 24, 213–274.
- Riddiford, L.M., Hice, R.H., 1985. Developmental profiles of the mRNAs for *Manduca* arylphorin and two other storage proteins during the final larval instar of *Manduca sexta*. *Insect Biochem.* 15, 489–502.
- Roberts, P.E., Willis, J.H., 1980a. The cuticular proteins of *Tenebrio molitor*. 1. Electrophoretic banding patterns during postembryonic development. *Devel. Biol.* 75, 59–69.
- Roberts, P.E., Willis, J.H., 1980b. Effects of juvenile hormone, ecdysterone, actinomycin D, and mitomycin C on the cuticular proteins of *Tenebrio molitor*. *J. Embryol. Exp. Morph.* 56, 107–123.
- Rondot, I., Quennedey, B., Courrent, A., Lemoine, A., Delachambre, J., 1996. Cloning and sequencing of a cDNA encoding a larval–pupal-specific cuticular protein in *Tenebrio molitor* (Insecta, Coleoptera): developmental expression and effect of a juvenile hormone analogue. *Eur. J. Biochem.* 235, 138–143.
- Rondot, I., Quennedey, B., Delachambre, J., 1998. Structure, organization and expression of two clustered cuticle protein genes during the metamorphosis of an insect *Tenebrio molitor*. *Eur. J. Biochem.* 254, 304–312.
- Roter, A.H., Spofford, J.B., Swift, H., 1985. Synthesis of the major adult cuticle proteins of *Drosophila melanogaster* during hypoderm differentiation. *Devel. Biol.* 107, 420–431.
- Sass, M., Kiss, A., Locke, M., 1993. Classes of integument peptides. *Insect Biochem. Mol. Biol.* 23, 845–857.
- Sass, M., Kiss, A., Locke, M., 1994a. Integument and hemocyte peptides. *J. Insect Physiol.* 40, 407–421.
- Sass, M., Kiss, A., Locke, M., 1994b. The localization of surface integument peptides in tracheae and tracheoles. *J. Insect Physiol.* 40, 561–575.
- Scheller, K., Zimmermann, H.-P., Sekeris, C.E., 1980. Calliphorin, a protein involved in the cuticle formation of the blowfly, *Calliphora vicina*. *Z. Naturforsch.* 35c, 387–389.
- Scholer, H.R., 1991. Octamania: the POU factors in murine development. *Trends Genet.* 7, 323–328.
- Shen, Z., Jacobs-Lorena, M., 1999. Evolution of chitin-binding proteins in invertebrates. *J. Mol. Evol.* 48, 341–347.
- SilkBase, 2003. Expressed sequence tags in *Bombyx mori*. <http://www.ab.a.u-tokyo.ac.jp/silkbase>
- Silvert, D.J., 1985. Cuticular proteins during postembryonic development. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry, and Pharmacology*, vol. 2. Pergamon Press, Oxford, pp. 239–254.
- Snyder, M., Hunkapiller, M., Yuen, D., Silvert, D., Fristrom, J., et al., 1982. Cuticle protein genes of *Drosophila*: structure, organization and evolution of four clustered genes. *Cell* 29, 1027–1040.
- Stiles, B., 1991. Cuticle proteins of the boll weevil, *Anthonomus grandis*, abdomen: structural similarities and glycosylation. *Insect Biochem.* 21, 249–258.
- Suderman, R.J., Andersen, S.O., Hopkins, T.L., Kanost, M.R., Kramer, K.J., 2003. Characterization and cDNA cloning of three major proteins from pharate pupal cuticle of *Manduca sexta*. *Insect Biochem. Mol. Biol.* 33, 331–343.
- Suetake, T., Tsuda, S., Kawabata, S., Miura, K., Iwanaga, S., et al., 2000. Chitin-binding proteins in invertebrates and plants comprise a common chitin-binding structural motif. *J. Biol. Chem.* 275, 17929–17932.
- Talbo, G., Hojrup, P., Rahbek-Nielsen, H., Andersen, S.O., Roepstorff, P., 1991. Determination of the covalent structure of an N- and C-terminally blocked glycoprotein from endocuticle of *Locusta migratoria*: combined use of plasma desorption mass spectrometry and Edman degradation to study post-translationally modified proteins. *Eur. J. Biochem.* 195, 495–504.
- Tews, I., Scheltiga, T., Perrakis, A., Wilson, K.S., Dijkstra, B.W., 1997. Substrate-assisted catalysis unifies 2 families of chitinolytic enzymes. *J. Am. Chem. Soc.* 119, 7954–7959.
- Trim, A.R.H., 1941. Studies in the chemistry of insect cuticle. 1. Some general observations on certain arthropod cuticles with special reference to the characterization of the proteins. *Biochem. J.* 35, 1088–1098.
- Uchiyama, T., Katouno, F., Nikaidou, N., Nonaka, T., Sugiyama, J., et al., 2001. Roles of the exposed aromatic residues in crystalline chitin hydrolysis by chitinase A from *Serratia marcescens* 2170. *J. Biol. Chem.* 276, 41343–41349.
- Vakser, I.A., 1996. Low-resolution docking: prediction of complexes for undetermined structures. *Biopolymers* 39, 455–464.
- Vincent, J.F.V., 2002. Arthropod cuticle: a natural composite shell system. *Composites: Part A* 33, 1311–1315.
- Vriend, G., 1990. WHAT IF: A molecular modeling and drug design package. *J. Mol. Graph.* 8, 52–56.
- Vyas, N.K., 1991. Atomic features of protein–carbohydrate interactions. *Curr. Opin. Struct. Biol.* 1, 723–740.

- Williams, C.M., 1980. Growth in insects. In: Locke, M., Smith, D.S. (Eds.), *Insect Biology in the Future*. Academic Press, London, pp. 369–383.
- Willis, J.H., 1986. The paradigm of stage-specific gene sets in insect metamorphosis: time for revision! *Arch. Insect Biochem. Physiol.* (Suppl. 1), 47–57.
- Willis, J.H., 1996. Metamorphosis of the cuticle, its proteins, and their genes. In: Gilbert, L.I., Atkinson, B.G., Tata, J. (Eds.), *Metamorphosis/Post-Embryonic Reprogramming of Gene Expression in Amphibian and Insect Cells*. Academic Press, London, pp. 253–282.
- Willis, J.H., 1999. Cuticular proteins in insects and crustaceans. *Amr. Zool.* 39, 600–609.
- Willis, J.H., Rezaur, R., Sehnal, F., 1982. Juvenoids cause some insects to form composite cuticles. *J. Embryol. Exp. Morph.* 71, 25–40.
- Wittkopp, P.J., Vaccaro, K., Carroll, S.B., 2002. Evolution of *yellow* gene regulation and pigmentation in *Drosophila*. *Curr. Biol.* 12, 1547–1556.
- Wolfgang, W.J., Fristrom, D., Fristrom, J.W., 1986. The pupal cuticle of *Drosophila*: differential ultrastructural immunolocalization of cuticle proteins. *J. Cell Biol.* 102, 306–311.
- Wolfgang, W.J., Fristrom, D., Fristrom, J.W., 1987. An assembly zone antigen of the insect cuticle. *Tissue and Cell* 19, 827–838.
- Wolfgang, W.J., Riddiford, L.M., 1986. Larval cuticular morphogenesis in the tobacco hornworm, *Manduca sexta*, and its hormonal regulation. *Devel. Biol.* 113, 305–316.
- Wybrandt, G.B., Andersen, S.O., 2001. Purification and sequence determination of a yellow protein from sexually mature males of the desert locust, *Schistocerca gregaria*. *Insect Biochem. Mol. Biol.* 31, 1183–1189.
- Zanotti, G., Marcello, M., Malpeli, G., Folli, C., Sartori, G., *et al.*, 1994. Crystallographic studies on complexes between retinoids and plasma retinol-binding protein. *J. Biol. Chem.* 269, 29613–29620.
- Zhou, X., Riddiford, L.M., 2002. Broad specifies pupal development and mediates the “status quo” action of juvenile hormone on the pupal–adult transformation in *Drosophila* and *Manduca*. *Development* 129, 2259–2269.

Relevant Website

<http://bioinformatics2.biol.uoa.gr> – A relational database of arthropod cuticular proteins established by C.K. Magkrioti, I.C. Spyropoulos, V.A. Iconomidou, J.H. Willis, and S.J. Hamodrakas.

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13 Insect Cytochrome P450

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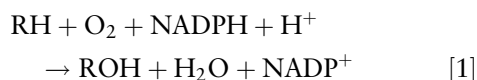
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13.1. Introduction

13.1.1. Overview

Cytochromes P450 or *CYP* genes constitute one of the largest family of genes with representatives in virtually all living organisms, from bacteria to protists, plants, fungi, and animals (Werck-Reichhart and Feyereisen, 2000). An ever-growing number of P450 sequences are available, and the role of P450 enzymes is being documented in an ever-growing number of physiological processes. The human genome carries about 57 P450 genes and insect genomes carry about a hundred. Each P450 protein is the product of a distinct *CYP* gene and P450 diversity is the result of successive gene (or genome) duplications followed by sequence divergence. The typically 45–55 kDa P450 proteins are heme-thiolate enzymes that are known collectively to catalyze at least 60 chemically distinct reactions. Their essential common feature is the absorbance peak

near 450 nm of their Fe^{II}-CO complex for which they are named. P450 enzymes are best known for their monooxygenase role, catalyzing the transfer of one atom of molecular oxygen to a substrate and reducing the other to water. The simple stoichiometry (eqn [1]) commonly describes the monooxygenase or mixed-function oxidase reaction of P450:



However, oxygen atom transfer is not the only catalytic function of P450 enzymes. They also show activity as oxidases, reductases, desaturases, isomerases, etc. (Mansuy, 1998). There are soluble forms of P450 (in bacteria) and membrane-bound forms (in microsomes and mitochondria of eukaryotes). P450s are dependent on redox partners for their supply of reducing equivalents from NADH or NADPH (NADPH cyt P450 reductase and cyt *b*₅ in

microsomes; a ferredoxin and a ferredoxin reductase in mitochondrial and bacterial systems). Some bacterial P450s are fusion proteins with their redox partners, either P450 reductase (see below) or a phthalate family oxygenase reductase (PFOR) (De Mot and Parret, 2002). Because of their complex catalytic mechanism, P450 enzymes often, perhaps always, generate superoxide or hydrogen peroxide from “unsuccessful,” or uncoupled reactions, leading to oxidative stress in cells.

Many P450 proteins are specialized in the metabolism of endogenous substrates (steroid hormones, lipids, etc.) but much of their notoriety has been associated with the metabolism or detoxification of xenobiotics (natural products, drugs, pesticides, etc.). In insects, they are involved in many cases of insecticide resistance. In well-studied cases, P450 enzymes generate more toxic compounds, e.g., carcinogenic metabolites of aryl hydrocarbons. Many foreign compounds, as well as endogenous metabolites, can induce the transcription of P450 genes through complex interactions with nuclear receptors and bHLH-PAS proteins, such as the *Ah* (aryl hydrocarbon) receptor of vertebrates. There are many excellent reviews and books clearly presenting the state of knowledge on P450s, generally with an emphasis on mammalian and bacterial enzymes (Omura *et al.*, 1993; Schenkman and Greim, 1993; Ortiz de Montellano, 1995b). Reliance on the advances made in noninsect systems is both necessary and risky. Necessary because the structural and functional homology of P450 enzymes allows general principles to emerge. Risky because such principles may not always apply to peculiar aspects of insect P450 evolution. The chapter will certainly fail to be comprehensive in view of the enormous literature pertaining to P450 but it will try to convey the state of our current understanding as a basis for future research. Many aspects of P450 biology are intertwined and complex (structure, activity, induction) so the linear and compartmentalized treatment of the subject as done here will probably fail to pass muster with the P450 novice and specialist alike. Indeed, the interpretations and choices of examples cited are mine, and the reader is invited to browse through text and references repeatedly. To help fill the enormous gaps in our knowledge on insect P450, the reader is also invited to join the field of P450 research.

13.1.2. Historical Framework

This chapter humbly tries to update the exhaustive reviews of Hodgson on microsomal monooxygenases (Hodgson, 1985) and of Agosin on the role

of microsomal oxidations in insecticide degradation (Agosin, 1985) published in the first edition of this series. The present chapter will focus on advances since 1985, and the earlier literature will be cited when needed for clarity or historical import. Both 1985 chapters had insect cytochromes P450 as their main focus and both revealed the enticing complexity of P450 catalysis and regulation. These two chapters, as well as insightful reviews that preceded them (Wilkinson and Brattsten, 1972; Brattsten, 1979b; Brooks, 1979), give a good account of the spirit of the times.

The great variety of reactions catalyzed by P450s was well recognized, but it was not really known how the multiplicity of enzymes and the suspected loose substrate specificity were blended to achieve this variety. The existence of multiple P450 forms was accepted but the true measure of this multiplicity was not foreseen. This multiplicity, once understood at the molecular genetic level, led to the early adoption of a common nomenclature in 1987 – the CYP genes. The substrate specificity of individual P450 enzymes was a matter of conjecture, because enzyme purification from insect sources and reconstitution of significant activity *in vitro* proved to be extremely difficult. Many cases of insecticide resistance were clearly attributable to alterations in the P450 system – a quantitative increase and a qualitative change. The molecular basis for this change was unknown, only a few resistance genes had been formally recognized at the genetic level, and the nature of the mutations leading to resistance was barely discussed (Terriere and Yu, 1974).

The role of insect P450 enzymes in the biosynthesis and degradation of endogenous compounds was becoming increasingly clear, but their relation to P450s involved in xenobiotic metabolism was unclear – were they truly the same class of enzymes? In contrast, an evolutionary link between the capacity to metabolize, and resist, synthetic insecticides and the ability of insects to thrive on chemically well-defended plants was recognized early on (Gordon, 1961). The metabolism of plant secondary compounds by P450s and the role these compounds played in regulating P450 activities brought insects forward as exquisite models to study the ecological role of P450 enzymes. Insects have now become equally exquisite models to study the evolution of P450s.

The first cloning and sequencing of a P450 cDNA (rat CYP2B1) was in 1982 (Fuji-Kuriyama *et al.*, 1982) and that of an insect P450 (housefly CYP6A1) followed in 1989 (Feyereisen *et al.*, 1989). The first crystal structure of a soluble bacterial P450, P450cam, was published in 1985 (Poulos

et al., 1985). The first structure of a mammalian microsomal P450 engineered for solubility followed in 2000 (Williams *et al.*, 2000a). After several heroic attempts at wresting activities from purified insect enzymes reconstituted *in vitro*, it was the heterologous expression of cloned insect P450 cDNAs in 1994 (Andersen *et al.*, 1994; Ma *et al.*, 1994) that allowed the rational study of individual P450 enzymology. Genome sequencing projects finally revealed the cast of P450 characters: insects have twice as many CYP genes as mammals, but only a third that of plants (David Nelson's cytochrome P450 homepage (web link)).

13.1.3. Vocabulary

Although P450 enzymes and CYP genes are displacing mixed-function oxidases (MFOs), microsomal oxidases, polysubstrate monooxygenases (PSMO) or simply monooxygenases in the vocabulary, words of caution are needed at the outset. The word "cytochrome" was initially given to the liver P450 pigment (Omura, 1993; Estabrook, 1996) and it is still associated with P450 enzymes. However, P450s are formally not cytochromes but rather heme-thiolate proteins (Mansuy, 1998). Not all P450-dependent reactions are monooxygenations, and not all monooxygenases are P450 enzymes (Mansuy and Renaud, 1995). In particular, flavin monooxygenases or FMOs are NADPH-dependent enzymes that catalyze some reactions similar or identical to those catalyzed by P450 enzymes. In mammalian species, FMOs are microsomal enzymes best known for their *N*- and *S*-oxidation activities (Ziegler, 2002). Until recently, FMOs had not been identified in insects. In the cinnabar moth, *Tyria jacobaea*, a soluble FMO specifically *N*-oxidizes pyrolizidine alkaloids such as senecionine, seneciophylline, monocrotaline, and axillarine (Lindigkeit *et al.*, 1997; Naumann *et al.*, 2002). Only two FMO genes of as yet unknown function have been recognized in the *Drosophila* genome, but there may well be many more in other species. Whether insect FMOs are involved in xenobiotic *N*- or *S*-oxidations is still unknown.

A word of caution, too, about the "microsomal" oxidases. This chapter will show that insect P450s can be found in microsomal membranes as well as in mitochondria, and that subcellular localization does not portend a particular physiological role or catalytic competence.

13.1.4. Nomenclature

A nomenclature of P450 genes and proteins was introduced when only 65 sequences were known

(Nebert *et al.*, 1987). Gene families were initially designated by Roman numerals, but the proliferation of diverse sequences rapidly became discouraging, even to those versed in classics. The rules of nomenclature were then revised to their current form (Nebert *et al.*, 1991; Nelson *et al.*, 1993, 1996) where a CYP prefix, followed by an arabic numeral designates the family (all members nominally >40% identical), a capital letter designates the subfamily (all members nominally >55% identical) and an arabic numeral designates the individual gene (all italics) or message and protein (no italics) (**Figure 1**). Different P450 enzymes are generally products of different genes; they are not isozymes or isoforms. The identity (%) rules for family and subfamily designations are not strictly adhered to, but names once adopted are rarely changed. Initially, many insect P450s were arbitrarily lumped into the CYP6 and the CYP4 families even though they had less than 40% amino acid identity with CYP6A1 or with vertebrate CYP4 proteins. Naming genes in the lump mode made the CYP6 and CYP4 families the largest ones in insects by a cascade effect. CYP6B1 is only 32% identical to CYP6A1 (Cohen *et al.*, 1992), so placing it in the CYP6 family "forced" many subsequent sequences into that family even if they did not meet the 40% criterion. The splitter mode prevailed at the completion of genome projects, which led to a new proliferation of CYP families in insects, the CYP300 series. A termite P450 claimed the welcoming designation of CYP4U2 (GenBank AF046011). Gotoh (1993) has introduced a useful nomenclature of higher order than CYP families: the E (for eukaryotic type) and B (for bacterial type) "classes" and subclasses (I, II, III, etc.) that regroup CYP families on the basis of phylogeny. Nelson (1998) has similarly introduced the notion of "clans," but the precise criteria for naming Gotoh's "classes" and Nelson's "clans" have not been defined.

Alleles of a gene are named as subscripts v_1 , v_2 (e.g., CYP6B1 v_2 , Cohen *et al.*, 1992). The human P450 polymorphisms are named according to a clear nomenclature. Pseudogenes are noted by the

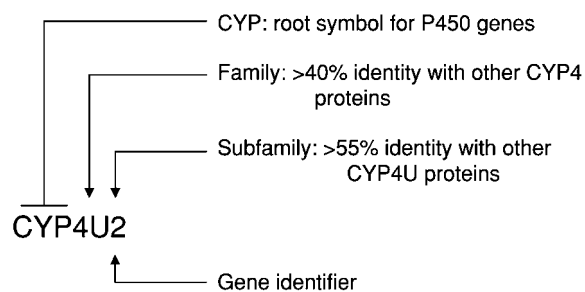


Figure 1 Scheme of the P450 nomenclature.

suffix P. This suffix ought to be added to the closest paralog that is an active gene, e.g., *CYP9E2* and *CYP9E2P1* in *Blattella germanica* (Wen *et al.*, 2001). However this is not always done, as the closest paralog is sometimes not easily recognized. In following the tradition that predates the CYP nomenclature, P450 enzymes can be named with a small suffix, such as P450cam, the camphor hydroxylase of *Pseudomonas putida* later named CYP101; P450BM3 the fatty acid hydroxylase of *Bacillus megaterium* (CYP102); or P450scc, the cholesterol side-chain cleavage enzyme (CYP11A1). In insects, few P450 enzymes have been named in this way. P450Lpr is the predominant P450 in the pyrethroid-resistant strain Learn-Pyr of the housefly, later identified as CYP6D1 (Tomita and Scott, 1995). P450hyd (Reed *et al.*, 1994) is the P450 forming hydrocarbons in the housefly. P450MA is a P450 purified from the Munsyana strain of the German cockroach (Scharf *et al.*, 1998). In the *Drosophila* gene nomenclature (Lindsley and Zimm, 1992), only the initial letter is capitalized, hence *CYP6A1* in the housefly and *Cyp6a2* in *Drosophila*.

The CYP nomenclature of Nebert *et al.* was clearly designed to reflect the evolutionary relationships between the genes as evidenced by the degree of sequence identity of the proteins they encoded. As such it is a Darwinian nomenclature. P450 proteins have been categorized into classes (Ravichandran *et al.*, 1993) that reflect the types of electron delivery to the active site. Class I proteins require both an FAD-flavoprotein reductase and a ferredoxin-type protein, class II P450s require only an FAD and FMN diflavin reductase, class III enzymes are self-sufficient, and class IV P450s receive electrons directly from NADPH. The utility of this nonevolutionary classification is debatable.

13.2. Diversity and Evolution of Insect P450 Genes

13.2.1. Sequence Diversity

13.2.1.1. P450 sequences from classical cloning techniques The first insect P450s cloned and sequenced were CYP6A1 from *Musca domestica* in 1989 (Feyereisen *et al.*, 1989), CYP4C1 from *Blaberus discoidalis* in 1991 (Bradfield *et al.*, 1991), CYP6A2 and CYP4D1 from *Drosophila* (Gandhi *et al.*, 1992; Waters *et al.*, 1992) as well as CYP6B1 from *Papilio polyxenes* in 1992 (Cohen *et al.*, 1992), and CYP4D2 from *Drosophila* in 1994 (Frolov and Alatortsev, 1994). The methods used in these early studies were screening of cDNA expression libraries with polyclonal (CYP6A1) or monoclonal

(CYP6A2) antibodies to (partially) purified P450 proteins of insecticide-resistant flies (CYP6A1, CYP6A2). For CYP6B1, microsequencing of N-terminal and internal sequences of a P450-sized band on a SDS-PAGE gel of *P. polyxenes* larval midgut proteins led to the design of degenerate oligonucleotide probes for RT-PCR on midgut poly(A)⁺ RNA. The cloned PCR product was used in turn as a probe to screen a xanthotoxin-induced midgut cDNA library. Significantly, two cDNAs representing alleles of the *CYP6B1* gene were thus isolated. Classical molecular approaches of this type have continued to yield new P450 sequences (Tomita and Scott, 1995; Wang and Hobbs, 1995; Winter *et al.*, 1999) and the initial P450 sequences have served as probes to isolate related sequences in the same species (Cohen and Feyereisen, 1995; Hung *et al.*, 1995a, 1996; Maitra *et al.*, 1996), or in phylogenetically close species (Li *et al.*, 2000a, 2001). Interestingly, CYP9A1 of *Heliothis virescens* was cloned by screening an expression library with monoclonal antibodies that also served to clone CYP6A2 of *Drosophila*, despite the fact that the two sequences are only 32.4% identical (Rose *et al.*, 1997).

13.2.1.2. Serendipity and P450 discovery In contrast to the targeted approaches, CYP4C1 was obtained in 1991 by differential screening of a cockroach fat body cDNA library (Bradfield *et al.*, 1991). The probes consisted of cDNA obtained from fat bodies of hypertrehalosemic hormone-treated roaches, or decapitated controls. The cloning and identification of *Drosophila Cyp4d1* and *Cyp4d2* were equally serendipitous, as investigators were interested in transcripts in the *prune* region at the tip of the X chromosome (Gandhi *et al.*, 1992; Frolov and Alatortsev, 1994). *Drosophila Cyp18* was initially cloned in a screen for ecdysone-inducible genes (Hurban and Thummel, 1993) and subsequently obtained as full-length cDNA (Bassett *et al.*, 1997). *Cyp4e1* deserves a special historical mention. Its sequence was discovered independently in a 9 kb genomic sequence of the 44D region of *Drosophila* encompassing a cluster of cuticle protein genes (GenBank Acc. K00045) by T. Holton and D. Nero in 1991, each searching databases with P450 sequences (personal communications). This partial sequence of insect *Cyp4e1* is thus the first (1983), though not annotated, record of an insect P450 in GenBank.

13.2.1.3. Exponential amplification of new P450 sequences by PCR By 1994, sufficient information about vertebrate and insect P450s allowed the

isolation of fragments of P450 cDNAs and genes by PCR methods with degenerate oligonucleotides corresponding to consensus sequences. A variety of approaches were taken, two of which being particularly successful: in the first, sequences in the I helix and surrounding the conserved cysteine (see Section 13.3.3.1) were used to isolate PCR products of about 450–500 bp that coded for about 130 amino acids or almost a third of the full-length P450. In the second, the sequence around the conserved cysteine was used to design a first primer, with oligo(dT) serving as anchor on the poly(A)⁺ message. Fragments of varying length were obtained by this 3'-RACE strategy, the C-terminal 30–50 amino acids sequence of the P450 and a variable 3' UTR sequence (review: Snyder *et al.*, 1996). The PCR approaches led to the description of 17 new *CYP4* genes in *A. albimanus* (Scott *et al.*, 1994); 5 *CYP4* genes from *Manduca sexta* (Snyder *et al.*, 1995); 8 *CYP* genes from *H. armigera* (Pittendrigh *et al.*, 1997); 4 genes of the new *CYP28* family from *Drosophila* species (Danielson *et al.*, 1997); 14 P450 fragments from *Ceratitidis capitata* (Danielson *et al.*, 1999), 95 new sequences from 16 drosophilid species (Fogleman *et al.*, 1998), etc. (Amichot *et al.*, 1994; Stevens *et al.*, 2000; Tares *et al.*, 2000; Scharf *et al.*, 2001; Wen *et al.*, 2001; Ranson *et al.*, 2002b). The PCR method has often been used as a first step in the isolation of full-length P450 clones in insects (Snyder *et al.*, 1995; Danielson and Fogleman, 1997; Hung *et al.*, 1997; Danielson *et al.*, 1998, 1999; Guzov *et al.*, 1998; Kasai *et al.*, 1998a, 2000; Ranasinghe and Hobbs, 1998; Sutherland *et al.*, 1998; Stevens *et al.*, 2000; Wen *et al.*, 2001; Wen and Scott, 2001b; Liu and Zhang, 2002) and ticks (Crampton *et al.*, 1999; He *et al.*, 2002).

The large number of partial P450 sequences obtained by cloning and sequencing an even larger number of PCR products has led to two related problems. The first is that small sequence differences can be found between very closely related PCR products. Are these artifactual, or do they represent allelic variation? In the study on *A. albimanus* for instance, 64 clones were sequenced of which 47 encoded P450 fragments, describing 17 genes (Scott *et al.*, 1994). In some clones, up to seven nucleotide differences from the closest sequence were seen. A few nucleotides may not just differentiate two alleles of the same gene, but may be sufficient to differentiate two genes, as the complete sequence of the *Drosophila* and *A. gambiae* genomes subsequently showed. This causes a nomenclature problem, when two distinct genes are prematurely described as allelic variants of a single gene. The second problem is that the P450 fragments obtained

by PCR, one third of the full sequence or less, make the calculation of percentage identity (the base of the nomenclature rules) difficult. In the *A. albimanus* study (Scott *et al.*, 1994) this problem was resolved by establishing a function that derives identity over the full length P450 from the percentage identity of the PCR product. Further validations of this approach have been presented (Danielson *et al.*, 1999; Fogleman and Danielson, 2000). Nonetheless, the practice of bestowing an official CYP designation to a short, partial P450 sequence should be abandoned.

13.2.1.4. Genome sequences: diversity revealed

The *D. melanogaster* complete genome sequence was published in 2000 (Adams *et al.*, 2000). Annotation of the P450 sequences (Tijet *et al.*, 2001) was done by multiple Basic Local Alignment Search Tool (BLAST) searches, taking into account intron/exon structure, pairwise and multiple alignments, EST sequences, and known features of well-characterized P450. This task gave 90 sequences, of which 83 appeared to code for potentially functional P450s. Seven sequences were either partial sequences or obvious pseudogenes. Forty genomic sequences were not represented by ESTs. With release 3 of the *Drosophila* genome (Celniker *et al.*, 2002), the “official count” of potentially functional P450 genes is 85 with five pseudogenes. A similar annotation of the P450 genes from the complete *A. gambiae* genome published in 2002 (Holt *et al.*, 2002) gave 111 genes (Ranson *et al.*, 2002a) of which five are thought to represent pseudogenes. The need for “manual” annotation remains critical because of the error-prone gene- and transcript-calling programs, as noted for P450s by Gotoh (1998). These difficulties remain a major challenge to the “completion” of a genome program (Misra *et al.*, 2002). Potentially confusing is the proliferation of GenBank accessions for transcripts identified *in silico*, with only scant notice that they represent nothing but software-digested genomic sequences. Some of the sources of errors noted for the *Drosophila* annotation (Misra *et al.*, 2002) were plainly evident with the *A. gambiae* genome sequence as well. They typically include the fusion of two neighboring P450 genes into one, or the truncation of a gene. Comparison of the intron/exon structure of closely related genes and alignments with EST sequences when these are available can facilitate P450 gene annotation in a majority of cases. Independent evidence, such as the cloning of full-length cDNAs, or functional expression is rarely available to resolve annotation problems, so that the complete description of the P450 gene complement of any

species remains an ongoing task. A website presents information on the P450 sequences from completely sequenced genomes (*D. melanogaster*, *A. gambiae*) as well as links to other P450 websites. The complete sequence of an increasing number of insect genomes (*Drosophila pseudoobscura*, *Apis mellifera*, *Bombyx mori*, *Tribolium castaneum*) and the continuous addition of P450 sequences in EST projects from various species is redefining the approach to P450 research.

13.2.2. Genomic Organization: Clusters

The presence of P450 genes in clusters was revealed in early studies with *Drosophila* and the housefly (Frolov and Alatorsev, 1994; Cohen and Feyereisen, 1995). Further evidence was obtained by *in situ* hybridization to polytene chromosomes of *Drosophila* (Dunkov *et al.*, 1996) and ultimately by the analysis of P450 genes in the complete genome sequences of *Drosophila* (Tijet *et al.*, 2001) and *A. gambiae* (Ranson *et al.*, 2002a, 2002b). The largest *Drosophila* P450 cluster carries nine genes (eight CYP6A genes and CYP317A1) at 51D on the right arm of chromosome 2. Six of these genes (Cyp6a17 to Cyp6a21) are coordinately regulated during the circadian rhythm (Ueda *et al.*, 2002). In *A. gambiae*, the largest cluster carries 14 P450 genes of the CYP6 family at 30A on the right arm of chromosome 3. A large cluster of CYP325 genes in *A. gambiae* contains 12 genes and 2 pseudogenes. In *A. gambiae*, only 22 of the 111 genes are present as singletons, with 16 clusters of 4 or more genes (Ranson *et al.*, 2002a) (Figure 2). Gene clusters are thought to arise by sequential gene duplication

events, the principal and initial mechanism of P450 diversification. In *Papilio glaucus*, CYP6B4v2 and CYP6B5v1 are clustered within 10 kb of each other (Hung *et al.*, 1996). They are 99.3% identical at the nucleotide level, with 98% identity of their single 732-bp intron and 95% identity over 616 bp of the promoter region. The proteins they encode differ by just one amino acid. CYP6B4 and CYP6B5 are thus recently duplicated genes that have not yet diverged substantially in sequence (Hung *et al.*, 1996). Similar close pairs are found in *Drosophila*, e.g., Cyp12a4/Cyp12a5; Cyp9b1/Cyp9b2; Cyp28d1/Cyp28d2. Cyp12d1 and Cyp12d2 are 2 kb apart and differ by only three nucleotides leading to three changes at the amino acid level. In *A. gambiae*, CYP6AF1 and CYP6AF2 are 99.8% identical at the nucleotide level and differ by just one amino acid (Ranson *et al.*, 2002a). CYP6D1 and CYP6D3 are clustered on chromosome 1 in the housefly (Kasai and Scott, 2001a). Although they are only 50% identical in their 500 nt UTR and their product 80% identical at the amino acid level, both genes are phenobarbital-inducible and are constitutively overexpressed in the LPR strain (Kasai and Scott, 2001b).

In the housefly, a cluster of six CYP6 genes within 24 kb of each other on chromosome 5 shows evidence of both gene duplications and chromosomal inversions (Cohen and Feyereisen, 1995). Three genes are transcribed in one direction and the next three are transcribed in the opposite direction. The six genes have a short intron at the same position in the ETLR conserved region (see Section 13.3.3.1) as many CYP6 genes. The CYP6A6 gene located at one extremity of the cluster is in fact represented only by the second exon. The first exon was not found in 2 kb of DNA upstream of this exon boundary, although the intron length of the five other genes is only 57–125 bp. CYP6A6 may therefore be a pseudogene generated during a chromosomal inversion whose breakpoint was located in the intron. The other five genes of the cluster are transcribed, with CYP6A5 being predominantly expressed in larvae (Cohen and Feyereisen, 1995).

Two recently duplicated genes may undergo gene conversion, if they do not diverge fast enough (Walsh, 1987). Gene conversion at the 5' end of the CYP6B8 and CYP6B28 genes of *Helicoverpa zea* (Li *et al.*, 2002b) has been suggested by the lower level of nt differences in the first half of the first exon, as compared to the rest of the sequence. Exon specific deficit in variation among pairs of P450 is indicative of a gene conversion event (Matsunaga *et al.*, 1990). Changes in regulatory patterns (induction, tissue specific expression) can be observed for

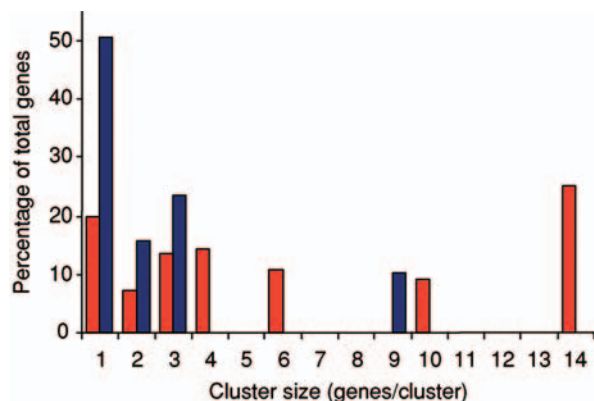


Figure 2 Clusters of P450 genes and singletons (cluster size = 1) genes in the *Drosophila melanogaster* (blue) and *Anopheles gambiae* (red) genomes. (Reproduced with permission from Ranson, H., Claudianos, C., Ortelli, F., Abgrall, C., Hemingway, J., *et al.*, 2002a. Evolution of supergene families associated with insecticide resistance. *Science* 298, 179–181; © AAAS.)

recently duplicated genes (Li *et al.*, 2002c) and may lead to their independent evolution. Extreme examples of gene conversion between duplicated P450 genes leading to their concerted evolution, of the kind seen with α -amylase genes (Hickey *et al.*, 1991), has not been reported to date.

Although events such as unequal crossing-over can lead to gene duplications, there are other mechanisms such as retrotransposition. Capture of a spliced mRNA by a retrotransposon can reintroduce an intronless sequence into the genome where it may evolve further or die as a pseudogene. This process generally occurs in germ cells, so it should be limited to genes that are expressed in those cells. The *Drosophila Cyp4g1* gene at the tip of the X chromosome (1B3) may be a case in point. It lacks introns and its closest paralog, *Cyp4g15*, has five introns and is located at 10C2-3. Furthermore, *Cyp4g1* is represented by the largest number of ESTs in *Drosophila*, and yet the apparent N-terminus of CYP4G1 is quite atypical of a microsomal P450 suggesting that an ancient 5'UTR is now part of the open reading frame. Further studies are needed to clarify the status of this gene.

There is little evidence for alternative splicing of insect P450 transcripts as additional means of generating diversity. The *Cyp4d1* gene seems to utilize two alternate first exons, and ESTs for each transcript type, CYP4D1 and CYP4D1*alt*, have been found. The first cDNA cloned (Gandhi *et al.*, 1992) uses a first exon (exon 1 prime) closest to the rest of the gene, whereas several ESTs use a more distal first exon (exon 1) instead. The two predicted proteins differ only from the N-terminal to the beginning of the first SRS (see Section 13.3.3.1 and Figure 8). The consequences of this alternative splicing in terms of catalytic competence thus remain to be examined.

13.2.3. Genomic Variation: Alleles, Pseudogenes

Apparent allelic variants of cloned P450 cDNAs and genes were already described in the earliest studies of insect P450s (Cohen *et al.*, 1992, 1994; Cohen and Feyereisen, 1995). Examples of this variation are most striking in *A. gambiae*, because the sequence released includes several "scaffolds," which are not included in the "golden path" used for genome assembly. These scaffolds represent large tracts of heterozygosity ("dual haplotype regions," Holt *et al.*, 2002), probably resulting from the mosaic nature (contributions from different *A. gambiae* cytotypes) of the strain that was sequenced. Interestingly, some of these scaffolds harbor P450 genes, even P450 gene clusters, and a comparison of the various haplotypes reveals not only considerable

differences, but also variations in the total number of P450 genes. In other words, P450 gene duplication events have occurred independently in different mosquito cytotypes. A gene recently converted to a pseudogene in one population or strain, as a result of a debilitating mutation or transposable element insertion may still be active in another population. The total number of P450 genes in a species is therefore a relative number that is genotype dependent.

In *Blattella germanica*, three pseudogenes of CYP4C21 and two pseudogenes of CYP9E2 have been described (Wen *et al.*, 2001). These pseudogenes are characterized by deletions and/or the presence of several stop codons in the open reading frame. CYP9E2P2, for instance, has just 10 nucleotide differences from CYP9E2, but two base deletions render this gene nonfunctional. The processed pseudogene CYP4W1P in the cattle tick has a 191 bp deletion, but only three other nt changes in the open reading frame (Crampton *et al.*, 1999). Another tick pseudogene, CYP319A1P, contains two DNA insertions in the open reading frame and also appears to be of recent origin (He *et al.*, 2002).

The rate of gene duplication has been reported to be extremely high, making it an event almost as frequent as point mutations at the nucleotide level (Lynch and Conery, 2000). With a rate of 31 gene duplications per genome per million years in *Drosophila*, and a half life of less than 3 million years for a duplicated gene, one can estimate that there is a P450 gene duplication event on average every 5 million years. *Drosophila* is peculiar in its ability to delete genomic DNA at a high rate, and the proportion of pseudogenes in the genome is low – 1 pseudogene for 130 proteins as compared to 1 for 19 in *Caenorhabditis elegans* (Harrison *et al.*, 2003). However the proportion of pseudogenes in the P450 family is high (five pseudogenes) in *Drosophila*, another indication of rapid turnover of P450 genes.

13.2.4. P450 Gene Orthologs

With so much gene duplication, are there still any P450 orthologs in related species? (orthology = the "same" gene in different species that has only diverged as a result of speciation). The availability of two completely sequenced genomes made it possible to identify those members of the P450 family that were truly orthologous. This is not as easy as it seems, because in some borderline cases, two formally orthologous genes may be sufficiently related to their closest formal paralog in the same species as to make the evolutionary connection between the two pairs unclear. Nonetheless, it came as a surprise

to find a very small number of 1 : 1 pairs of orthologous P450 genes between *D. melanogaster* and *A. gambiae* (Ranson *et al.*, 2002a). Zdobnov *et al.* (2002) indicate a genome wide level of 44–47% orthologous genes, but for the P450 genes only ten orthologs were found, of which five are mitochondrial P450s (Ranson *et al.*, 2002a). When two P450 genes are recognized as orthologs even though the two species have diverged about 250 million years ago, the most likely explanation is that there is a strong evolutionary constraint that has maintained this orthologous relationship. A similar or identical physiological function for the orthologous pair of P450 enzymes may represent such a constraint. Three of the five mitochondrial P450s have indeed a recognized function that is predicted to be identical in *Drosophila* and *A. gambiae*: CYP302A1, CYP314A1, and CYP315A1 are hydroxylases of the ecdysteroid biosynthetic pathway (Warren *et al.*, 2002; Petryk *et al.*, 2003; and see Section 13.4.1.1). The number of pairs of orthologs between *Drosophila* and *A. gambiae* is however higher than the number of P450 enzymes thought to participate in ecdysteroid metabolism, so that several other insect-specific or dipteran-specific conserved functions are probably carried out by the remaining pairs of orthologs. There are several cases where one gene in one species has two “orthologs” in the other – this is the case when a gene duplication event occurred in just one of the two species after speciation and 250 million years of separate evolutionary history. These cases merit special attention because knowledge of the function of one of the P450s may quickly lead to understanding the function of its alter egos. The comparative analysis of the P450s of the two dipteran species showed that the deficit of true pairs of orthologs (10 versus the predicted ± 40) was compensated by a most interesting alternative. Orthologous groups of P450 paralogs were seen, i.e., the phylogenetic analysis clearly identified several cases where one ancestral P450 gene underwent several duplication events in each of the two dipteran lineages. The CYP6A cluster on chromosome 5 of the housefly (Cohen and Feyereisen, 1995) and a cluster of CYP6A genes on the right arm of chromosome 2 in *Drosophila* are probably syntenic, in view of the synteny of these linkage groups (Weller and Foster, 1993), but the clusters have evolved separately for over 100 million years, and orthologous pairs of genes in each cluster can no longer be recognized.

Even when syntenic relationships are not maintained globally (Zdobnov *et al.*, 2002), cases of local microsynteny are observed. For example, CYP302A1 and CYP49A1 are very close on chromosome 2L

in *A. gambiae*, but their orthologs in *Drosophila* are on 3L and 2R, respectively. Nonetheless, microsynteny is maintained around CYP49A1, which is close to the adrenodoxin reductase gene and is located in both species on the negative strand of the first intron of another gene, the G protein $0-\alpha$ 47A (CG2204) in *Drosophila* and its ortholog in *A. gambiae*.

13.2.5. Intron/Exon Organization of CYP Genes

The intron/exon organization of P450 genes is a useful tool in the analysis of P450 phylogeny, as shown by the systematic studies in *C. elegans* (Gotoh, 1998), *Arabidopsis thaliana* (Paquette *et al.*, 2000), and *Drosophila* (Tijet *et al.*, 2001) (Figure 3). Intron sequence comparisons, as well as sequence comparisons of 5' flanking sequences have also helped clarify the evolutionary relationships of very closely related CYP6B genes of *Papilio* species (Li *et al.*, 2002a). Multiple events of intron loss and gain can be deduced from a comparison of the intron/exon organization of orthologous pairs of P450 genes for *Drosophila* and *A. gambiae* (Ranson *et al.*, 2002a). Intron phase is nonbiased. CYP introns follow the GT/AG rule, except the first intron in the *Drosophila* Cyp9c1 gene (Tijet *et al.*, 2001) and the first intron in the Cyp6a8 gene (Maitra *et al.*, 2002). The latter was recognized by comparison with the full length cDNA, it does not conform to usual sequence patterns, and has an AT/TC splice junction. This intron is very short (36 bp) and potentially represents 12 additional in-frame codons (Maitra *et al.*, 2002).

13.3. The P450 Enzymatic Complexes

13.3.1. Classical Biochemical Approaches

13.3.1.1. Subcellular fractions The enzymology of insect P450 can be studied in different types of environments. These are enriched subcellular organelles from insects (microsomes, mitochondria) where multiple P450 interact *in situ* with their redox partners; membranes from cellular expression systems where a cloned recombinant P450 interacts with native or engineered redox partners; and ultimately a reconstituted system of purified recombinant P450 and its redox partners in a defined system devoid of biological membranes. Transgenic expression of P450 genes is another way to study P450 biochemistry, but also regulation.

The classical preparation of microsomal fractions from insect tissue homogenates by differential centrifugation has been described extensively by Hodgson (1985) and Wilkinson (1979). It remains, with minor modifications, the most widely used first step in the biochemical characterization of insect

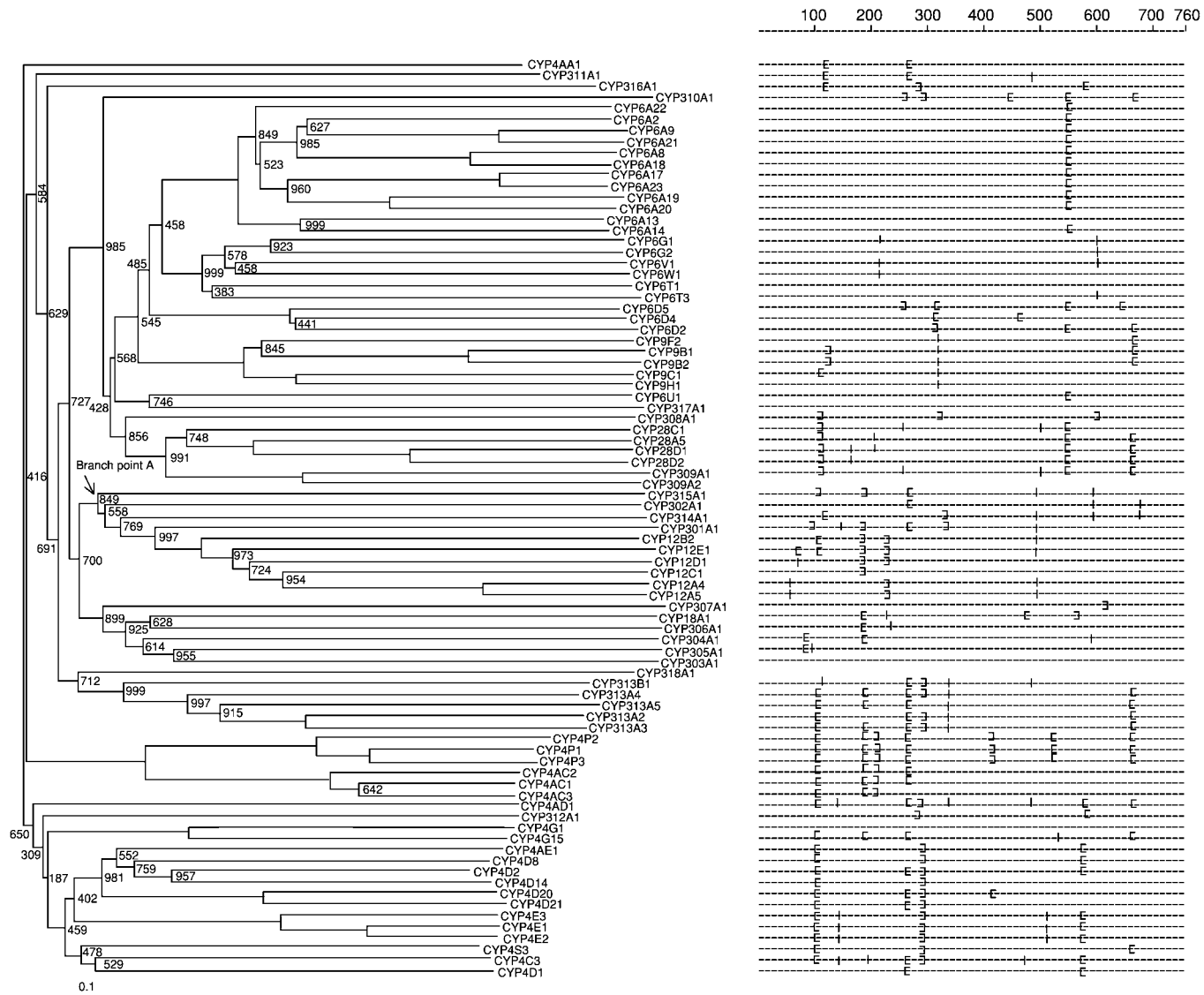


Figure 3 Neighbor-joining tree (with bootstrap value when different from 1000) and intron position of 83 P450 genes of *Drosophila melanogaster*. Branch point A indicates the mitochondrial P450 clade. Intron positions are shown schematically in the aligned open reading frames with their phase (| phase 0; [phase 1 and] phase 2). (Reprinted with permission from Tijet, N., Helvig, C., Feyereisen, R., 2001. The cytochrome P450 gene superfamily in *Drosophila melanogaster*: annotation, intron-exon organization and phylogeny. *Gene* 262, 189–198. For updated information see [<http://P450.antibes.inra.fr>].)

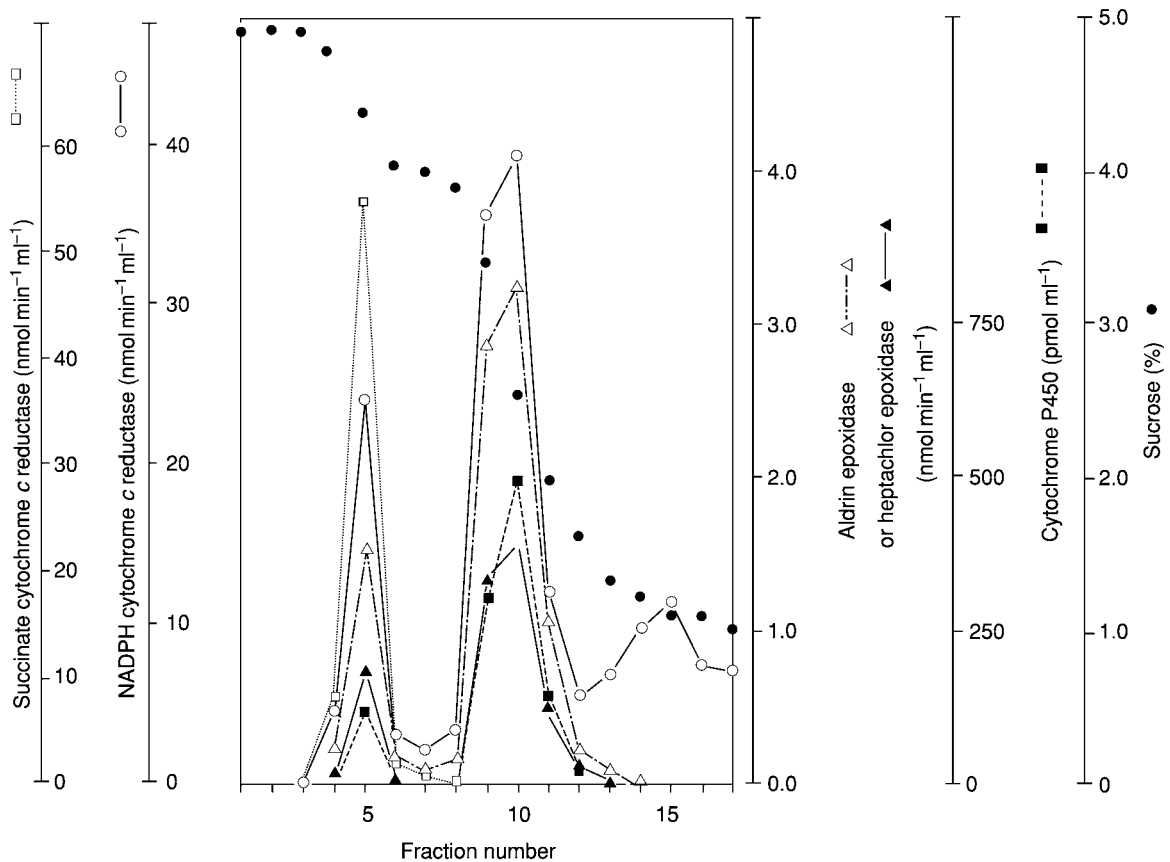


Figure 4 Sucrose density centrifugation separation of subcellular fractions of housefly larval homogenates showing the distribution of marker enzyme activities between mitochondrial and microsomal fractions. Note some P450 reductase activity in the top (soluble) fractions representing proteolytically cleaved enzyme. (Reprinted with permission from Feyereisen, R., 1983. Polysubstrate monooxygenases (cytochrome P-450) in larvae of susceptible and resistant strains of house flies. *Pestic. Biochem. Physiol.* 19, 262–269; © Elsevier.)

P450 enzymes. Linear or step gradients of sucrose for the centrifugal preparation of microsomes and mitochondria, or CaCl_2 precipitation of microsomes have been less favored. In all approaches, the careful use of marker enzymes is critical. A technique for the rapid preparation of microsomal fractions of small tissue samples relying on centrifugation at very high speed on sucrose layers in a vertical rotor has been described (Feyereisen *et al.*, 1985).

The well-documented sedimentation of P450-associated activities at low g forces in many early insect studies (reviews: Wilkinson and Brattsten, 1972; Wilkinson, 1979) has been considered a peculiar difficulty of insect biochemistry. At the time, the vertebrate toxicology and endocrinology literature had clearly identified P450 metabolism of xenobiotics as microsomal, whereas mitochondrial P450s were chiefly involved in hormone metabolism. There was therefore little incentive in probing the subcellular distribution of insect P450 activity

more carefully. The discovery of insect CYP12 enzymes and their characterization as mitochondrial P450 enzymes capable of metabolizing xenobiotics (Guzov *et al.*, 1998) has shed a new light on the early difficulties in sedimenting insect P450 activities in the “correct” fractions. It is quite probable that at least a part of the P450 activities observed in “mitochondrial” fractions were indeed carried out by CYP12 enzymes. In housefly larvae, 15–20% of the aldrin and heptachlor epoxidase activities were associated with mitochondrial fractions after sucrose density centrifugation (Feyereisen, 1983) (Figure 4).

The insect midgut is a particularly rich source of P450 activity (Hodgson, 1985), but the external brush border membrane is a significant source of membrane vesicles (BBMV) upon homogenization of the tissue. Centrifugal methods to separate the BBMV fraction from the microsomes derived from the endoplasmic reticulum have been described (Neal and Reuveni, 1992).

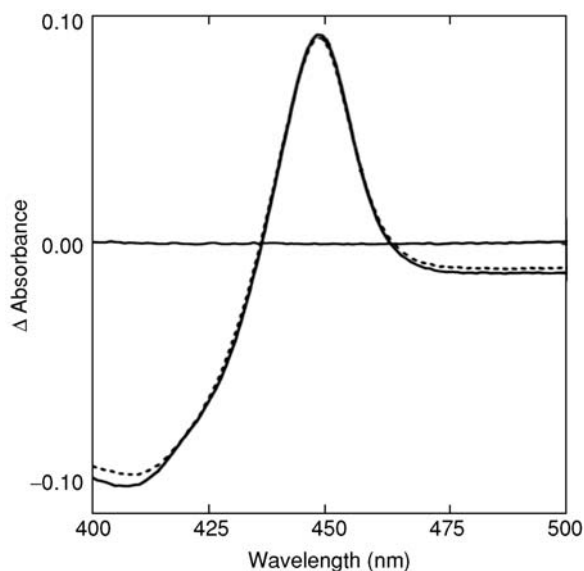


Figure 5 CO-difference spectrum of recombinant CYP12A1. The P450 was reduced with either sodium dithionite (solid line) or with bovine adrenodoxin, adrenodoxin reductase, and NADPH (dashed line).

13.3.1.2. Spectral characterization and ligand binding The analysis of P450 levels in subcellular fractions follows the original procedure of Omura and Sato (1964). A difference spectrum between reduced microsomes and reduced microsomes after gentle bubbling of CO readily displays the famous redshifted Soret peak at 450 nm (Figure 5). The concentration of P450 can be calculated from the ΔOD between 490 and 450 nm and Omura and Sato's extinction coefficient $\epsilon = 91 \text{ M}^{-1} \text{ cm}^{-1}$. This measure gives the total concentration of all forms of P450 present in the preparation. Individual P450 proteins may have peaks that are 1 or 2 nm off the 450 nm norm, and when they represent a large portion of the total P450, the total P450 peak may be shifted as a consequence. The degradation of P450 to the inactive P420 form may interfere with the measurement of P450, as already reviewed by Hodgson (1985), and the respiratory chain pigments interfere with the measurement of P450 in mitochondrial fractions. The classical Omura and Sato procedure remains the procedure of choice to measure the amount and purity of P450 proteins produced in heterologous systems (see below).

Ligand-induced spectral changes also follow classical procedures detailed in a useful review (Jefcoate, 1978). Type I spectra (peak at 380–390 nm, trough at 415–425 nm) result from ligand in the substrate binding site displacing water as sixth ligand to the heme iron (see Section 13.3.4.1, Figure 6). Type I spectra are concentration dependent, giving a spectral dissociation constant (Ks) and this titration is

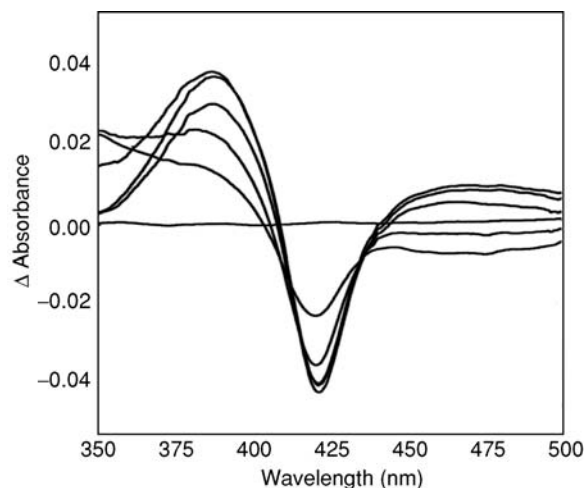


Figure 6 Type I substrate-induced difference spectrum of recombinant CYP12A1 with increasing concentrations of progesterone.

correlated with a shift of the iron from low spin to high spin. Not all type I ligands are substrates, and not all substrates are type I ligands, so this useful tool must be used with caution. A type I spectral Ks is not an enzymatic Kd.

Type II spectra (peak at 425–435 nm, trough at 390–405 nm) result from the binding of a strong ligand to the heme iron, typically the nitrogen coordination of compounds such as pyrimidines, azoles, or n-octylamine. Type II spectral titration is correlated with a shift from high spin to low spin and is a hallmark of strong inhibitors such as imidazoles (Figure 6). Other, less frequently studied spectral changes induced by ligands or their metabolism will not be discussed here (e.g., type III spectra, Hodgson, 1985; spectra of phenyl-iron complexes, Andersen *et al.*, 1997).

13.3.1.3. Assays and substrates Measurement of P450 activity is a special challenge because of the large number of different P450 enzymes, each catalyzing the metabolism of a specific (broad or narrow) range of substrates. There is therefore a very large number of assays for P450 activity. Direct assays of product appearance or substrate disappearance rely on all the tools of analytical chemistry. Indirect assays (e.g., activity of a P450 product in an enzyme or bioassay) can be useful but the strength of the claim for P450 activity depends on the purpose of the assay. The assay of a P450 produced in a heterologous system can be straightforward, but the assay of a P450 in its native microsomal or mitochondrial membrane, where it is mixed with an undetermined number and amount of other P450s, is more problematic. Metabolism of compound *M*

to product *N* in microsomes is the sum of the contributions of all P450 enzymes that catalyze the *M* to *N* reaction (and sometimes non-P450 enzymes can catalyze the same reaction!). Selective inhibitors (chemicals or antibodies) of one P450 can, by subtraction, indicate the relative contribution of that particular P450 to the reaction being measured (e.g., Wheelock and Scott, 1992a; Hatano and Scott, 1993; Korytko *et al.*, 2000b). This indirect inference is only as valid as the inhibitor is selective. Substrates that are selective for one P450 and that are easily assayed have been the object of considerable research in biomedical toxicology. The relative success of this quest (e.g., nifedipine as model probe for CYP3A4) is a result of both the limited number of major P450 expressed in human liver and the heavy investment in their study. The large number of insect P450s times the large number of insect species under study divided by the investment in their research makes a similar quest seem quixotic. By default then, but mostly by inertia of the historical development of insect P450 research, a certain number of assays have taken their place in the literature as some measure of “global” P450 activity. Most authors are now fully aware that the microsomal activity of, e.g., aldrin epoxidation, *p*-nitroanisole *O*-demethylation or 7-ethoxycoumarin *O*-deethylation is only a measure of those P450 enzymes catalyzing these reactions. But this awareness is only as recent as our understanding that there are really many P450 enzymes, and that their individual catalytic competence may be broad or narrow, overlapping with other P450 enzymes or not. Thus, the pioneers who used aldrin epoxidation as an assay did so at a time when P450 research was still strongly influenced by the dichotomy between steroid metabolism by specific P450 enzymes and drug metabolism by two major forms of liver P450. They used an analytical tool then readily available in pesticide toxicology laboratories (GC with electron capture detection for the sensitive detection of organochlorine pesticide residues), but they didn’t use it without a caveat. Quoting the classical Krieger *et al.* (1971) study: “to the extent that the rate of epoxidation of aldrin to dieldrin typifies the activity of the enzymes toward a wider range of substrates. . .” The current and still widespread use of a “model” substrate to explore P450 activities in insect subcellular fractions can be useful. If the metabolism of a randomly chosen P450 substrate (e.g., aldrin, aminopyrine, 7-methoxyresorufin) is quantitatively different between insect strains, in different tissues, following induction, etc., then this substrate has provided a clue that the qualitative or quantitative complement of P450 enzymes is changing. It is

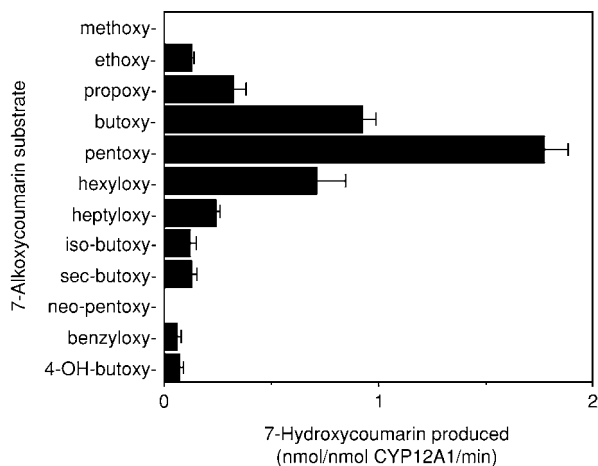


Figure 7 *O*-Dealkylation activity of *E. coli*-expressed recombinant housefly CYP12A1 in a reconstituted system. (Reprinted with permission from Guzov, V.M., Unnithan, G.C., Chernogolov, A.A., Feyereisen, R., 1998. CYP12A1, a mitochondrial cytochrome P450 from the house fly. *Arch. Biochem. Biophys.* 359, 231–240; © Elsevier.)

up to the investigator to follow up on this clue. Alkoxy coumarins and alkoxyresorufins are useful substrates for sensitive fluorometric assays and have largely replaced organochlorines as model substrates. They can be used to “map” the catalytic competence of a heterologously expressed P450 (e.g., the preference of CYP12A1 for pentoxycoumarin, Guzov *et al.*, 1998) (Figure 7). Steroids such as testosterone have multiple sites of attack by P450 enzymes and can likewise be used to characterize the activity of subcellular fractions or of heterologously expressed P450s (Amichot *et al.*, 1998; Cuany *et al.*, 1990; M.B. Murataliev, V.M. Guzov, R. Feyereisen, unpublished data).

Another approach to the study of P450 activity is to follow the consumption of the other substrates, O_2 or NADPH. This approach is certainly valid when the stoichiometry of the reaction (see eqn [1]) is under study, but its use to monitor metabolism by subcellular fractions is fraught with difficulties. Other enzymes consume O_2 and NADPH as well, and the background activity can be very high.

13.3.1.4. P450 assays in individual insects The design of assays suitable for assessing P450 activities in single insects has accompanied the need to study variations in P450 activities from the individual to the population level. Such assays allow the presentation of frequency histograms of activity levels in field-collected samples or in laboratory-selected populations and are ideally adapted to microtiter plate format. The NADPH-dependent conversion of *p*-nitroanisole to *p*-nitrophenol was followed in individual homogenates of *H. virescens* and

Pseudoplusia includens larvae (Kirby *et al.*, 1994; Rose *et al.*, 1995; Thomas *et al.*, 1996). This assay has a relatively low sensitivity, but clearly distinguished individuals from susceptible and insecticide-resistant strains. Cut abdomens of adult *Drosophila* in buffer containing 7-ethoxycoumarin can be used to measure 7-hydroxycoumarin formation in a 96-well microtiter plate format (de Sousa *et al.*, 1995). This rapid technique allows for instance the monitoring of individual variability over the course of a selection regime (Bride *et al.*, 1997). In another example, 10 000 g supernatants of individual homogenates of *Chironomus riparius* larvae were assayed for 7-ethoxyresorufin *O*-deethylation activity (Fisher *et al.*, 2003). The classical aldrin epoxidase assay adapted on single larvae of *Spodoptera frugiperda* has also been reported, but this assay is not adapted to the 96-well format (Yu, 1991, 1992).

A simple assay based on the peroxidase activity of the heme group with tetramethylbenzidine was developed for use in single mosquitoes (Brogdon, 1997). This assay, easily developed on a microtiter plate format, is an indirect assay measuring total heme content of the insect homogenate rather than P450 activity, and therefore needs to be carefully validated.

13.3.1.5. Solubilization and purification Solubilization and purification of insect P450 has generally followed the advances pioneered in the purification of vertebrate P450 (Agosin, 1985; Hodgson, 1985) and relatively a few studies since 1985 have pursued this difficult task (Ronis *et al.*, 1988; Wheelock and Scott, 1989). P450 purification from microsomes of mixed tissues (e.g., fly abdomens) can be sufficient to obtain a protein fraction suitable for antibody generation or peptide sequencing (Feyereisen *et al.*, 1989; Wheelock and Scott, 1990; Scott and Lee, 1993b).

Sequential chromatography on octylamino agarose, DEAE-cellulose, and hydroxyapatite was used to purify sodium cholate-solubilized P450s from *Drosophila* (Sundseth *et al.*, 1990). The two protein fractions obtained, P450 A and B, had only a very low 7-ethoxycoumarin *O*-deethylase activity (0.01 nmol/nmol P450/min), but the proteins were useful in generating monoclonal antibodies that allowed the subsequent cloning of CYP6A2 (Waters *et al.*, 1992).

An original approach was the purification of a locust P450 by affinity chromatography with type II and type I ligands (Winter *et al.*, 2001) that led to cloning of CYP6H1 (Winter *et al.*, 1999). In this approach, microsomes from larval *Locusta*

migratoria Malpighian tubules were first treated with the detergent synperonic NP10 to solubilize P450. The extract was then chromatographed on ω -octylamino agarose then hydroxylapatite. The third and less classical step was chromatography on a triazole agarose affinity column. The affinity ligand was a derivative of the fungicide difenoconazole, which has an affinity for ecdysone 20-monooxygenase of the same level as that of the substrate ecdysone (0.5 versus 0.2 μ M). This substituted triazole is a typical type II ligand (active site liganding of the heme) and its use on the affinity matrix led to the adsorption of all the P450 loaded on the column (Winter *et al.*, 2001). Elution from the affinity column was done by replacing the immobilized type II ligand with a soluble type I ligand, ecdysone. A major protein band of 60 kDa was thus obtained in 4% yield, with a P450 specific activity of 13.1 nmol/mg protein. Unfortunately, biochemical evidence that this P450 is in fact an ecdysone 20-monooxygenase was not obtained in this study (Winter *et al.*, 2001), so the nature of CYP6H1 (Winter *et al.*, 1999) remains conjectural.

Another variant on the classical purification schemes has been the use of immobilized artificial membrane high performance liquid chromatography (IAM-HPLC) of microsomal proteins (Scharf *et al.*, 1998). This technique allowed the 70-fold purification of a P450 from the German cockroach and the subsequent production of antibodies with this 49 kDa protein as antigen.

13.3.2. Heterologous Expression Systems

Biochemical characterization of a P450 protein and its substrate selectivity remains a *sine qua non* condition of its functional identification. Only a few P450 enzymes are characterized well enough (e.g., steroid metabolizing P450s in vertebrates) that sequence comparison can reasonably predict activity. For most other P450s, the sequence does not provide a clue to the activity, and there are now innumerable papers describing how one or a few mutations can change substrate selectivity (review: in Domanski and Halpert, 2001). Only three mutations are needed to confer to *Drosophila* CYP6A2 the ability to metabolize DDT (Bergé *et al.*, 1998; Amichot *et al.*, 2004). In the absence of significant studies on the activity of P450 proteins purified directly from insect tissues, it is the expression of P450 cDNAs in heterologous systems that has become the standard way of characterizing insect P450 proteins. A number of such expression systems have been developed over the last few years (Table 1), and the techniques are essentially similar to those used for the production of P450 proteins from mammalian or plant tissues.

Table 1 Heterologous expression systems for insect P450

<i>Expression system</i>	<i>P450 produced</i>	<i>Substrate metabolized</i>	<i>Reference</i>
<i>Escherichia coli</i>	CYP4C7	Sesquiterpenoids	Sutherland <i>et al.</i> (1998)
	CYP6A1	Aldrin, heptachlor	Andersen <i>et al.</i> (1994)
		Sesquiterpenoids	Andersen <i>et al.</i> (1997)
		Diazinon	Sabourault <i>et al.</i> (2001)
		7-Propoxycoumarin	^a
		1-Bromochlordene, chlordene, 1-hydroxychlordene, isodrin	^b
		Testosterone, progesterone, androstenedione	^c
		Pisatin	^d
		Chlorfenapyr	^e
	CYP6A2	DDT, testosterone	Amichot <i>et al.</i> (2004)
	CYP6A5	Benzphetamine, <i>p</i> -chloro- <i>N</i> -methylanilin, methoxyresorufin	^f
CYP9E1	Sesquiterpenoids	^g	
CYP12A1	Aldrin, heptachlor, diazinon, azinphosmethyl, amitraz, progesterone, testosterone, 7-alkoxycoumarins	Guzov <i>et al.</i> (1998)	
CYP15A1	<i>t t</i> , methyl farnesoate	Helvig <i>et al.</i> (2004)	
Baculovirus	CYP6A2	Aldrin, heptachlor, diazinon	Dunkov <i>et al.</i> (1997)
	CYP6B1	Furanocoumarins	Ma <i>et al.</i> (1994), Hung <i>et al.</i> (1997), Wen <i>et al.</i> (2003)
	CYP6B4	Furanocoumarins, ethoxycoumarin	Hung <i>et al.</i> (1997), Li <i>et al.</i> (2003)
	CYP6B17	Furanocoumarins, ethoxycoumarin	Li <i>et al.</i> (2003)
	CYP6B21	Furanocoumarins, ethoxycoumarin	Li <i>et al.</i> (2003)
	CYP6B25	Furanocoumarins	Li <i>et al.</i> (2003)
Yeast	CYP6A2	AflatoxinB1, 7,12-dimethylbenz[a]anthracene, 3-amino-1-methyl-5H-pyrido[4,3-b]-indole	Saner <i>et al.</i> (1996)
	CYP6D1	Methoxyresorufin	Smith and Scott (1997)
Transfected S2 cells	CYP302A1	2,22-Dideoxyecdysone	Warren <i>et al.</i> (2002)
	CYP314A1	Ecdysone	Petryk <i>et al.</i> (2003)
	CYP315A1	2-Deoxyecdysone, 2,22-dideoxyecdysone	Warren <i>et al.</i> (2002)

^aV.M. Guзов and R. Feyereisen, unpublished data.

^bJ. Walding, J.F. Andersen, and R. Feyereisen, unpublished data.

^cM.B. Murataliev, V.M. Guзов, and R. Feyereisen, unpublished data.

^dV.M. Guзов, H. VanEtten, and R. Feyereisen, unpublished data.

^eV.M. Guзов, M. Kao, B.C. Black, and R. Feyereisen, unpublished data.

^fJ.L. Stevens, J.F. Andersen, and R. Feyereisen, unpublished data.

^gJ.F. Andersen and R. Feyereisen, unpublished data.

13.3.2.1. *Escherichia coli* Bacterial production (*Escherichia coli*) of insect P450s has required several modifications of the sequence. At the 5' end of the cDNA, mutations are introduced to optimize expression (Barnes *et al.*, 1991). The second codon is replaced by Ala (Andersen *et al.*, 1994; Guзов *et al.*, 1998; Sutherland *et al.*, 1998) and silent substitutions are introduced to increase the A/T content (Sutherland *et al.*, 1998). In some cases, introduction of 4–6 His codons just before the stop codon directs the translational production of a C-terminal “histidine tag” (Guzov *et al.*, 1998; Sutherland *et al.*, 1998). P450 production can be enhanced by the addition of δ -aminolevulinic acid (a precursor for

heme biosynthesis) to the culture broth (Sutherland *et al.*, 1998). The P450 produced in bacteria is found mostly in a membrane fraction, and sometimes in a fraction of inclusion bodies that are difficult to extract. In some cases (Andersen *et al.*, 1994), a significant amount of P450 is produced as a soluble form. The *E. coli* membrane fraction carrying the recombinant P450 protein is generally suitable for analysis by difference spectroscopy for either P450 content by the Omura and Sato (1964) procedure, or for ligand binding (type I binding for potential substrates or type II binding for azoles). Although some P450 proteins (e.g., CYP17) expressed in *E. coli* can utilize an endogenous flavodoxin

reductase/flavodoxin system for catalysis, none of the insect P450 proteins tested thus far have been catalytically active in *E. coli* membrane fractions in the absence of a P450 reductase. Therefore, P450 produced in bacteria needs to be solubilized and purified by classical methods. The proteins produced with a histidine tag, once solubilized, are purified by nickel chelate affinity chromatography. Extensive dialysis is needed in both procedures to remove excess detergent or imidazole used for elution from the nickel affinity column. The P450 obtained is then suitable for reconstitution with redox partners. These partners (microsomal or mitochondrial redox partners, see Section 13.3.3) are themselves produced in *E. coli* and purified (Guzov *et al.*, 1998).

Reconstitution of a catalytically active enzyme system is then tedious or artistic, depending on one's degree of patience. It requires attention to the details of concentrations of phospholipids, detergents, proteins, and their order of addition, mixing and dilution (Sutherland *et al.*, 1998). The advantages of bacterial expression are the low cost of production of large amounts of P450, and the possibility to work with a precisely defined *in vitro* system with highly purified enzymes and their partners. A thorough characterization of the enzyme can be undertaken. The disadvantage of this formal biochemical approach is that purification and reconstitution are difficult and time-consuming, and is probably not suitable for when the goal is simply a survey of the catalytic competence of the P450, or the comparison of a large number of P450s or P450 mutants. The host organism, *E. coli*, is a rare organism devoid of P450 genes of its own while other bacteria can carry over 20.

13.3.2.2. Baculovirus Expression of P450 in lepidopteran cells by the baculovirus system requires no modification of sequence and is a widely used method for the production of proteins in an eukaryotic system. It has the potential of producing large amounts of P450 proteins for subsequent purification, but studies with insect P450 expressed with this system (Ma *et al.*, 1994; Dunkov *et al.*, 1997; Hung *et al.*, 1997; Chen *et al.*, 2002; Wen *et al.*, 2003) have relied instead on the advantage that the protein is present in a suitable milieu, the endoplasmic reticulum of an insect cell. Thus, cell lysates, briefly centrifuged to pellet cell debris, are used as enzyme source. Difference spectroscopy or immunological methods (Dunkov *et al.*, 1997) can be used to assess the amount of P450 produced. The host cells provide their endogenous P450 reductase to support the activity of the heterologous P450 when the cell lysates are incubated with an NADPH

regenerating system. Although the level of P450 reductase is sufficient to allow the measurement of a number of P450-dependent activities (Dunkov *et al.*, 1997), the stoichiometry of endogenous P450 reductase, and cytochrome *b₅*, to heterologously expressed P450 is probably not optimal. The activities measured do not represent the full potential of the P450 under study. For instance, a thirty-fold increase in CYP6A2 activity was observed when purified housefly P450 reductase and cytochrome *b₅* were added to lysates of cells expressing *Cyp6a2* (Dunkov *et al.*, 1997). An improvement of the baculovirus expression system has therefore been designed, wherein the cells are coinfecting with a virus engineered to carry the P450 and a virus engineered to carry a P450 reductase (housefly P450 reductase, Wen *et al.*, 2003). Optimal conditions were sought, and a significant increase (33-fold) in CYP6B1 activity towards the substrate xanthotoxin was achieved with the improved P450 reductase/P450 ratio. In fact, the improved conditions allowed the measurement of angelicin metabolism that was barely detectable in the absence of additional P450 reductase. Thus, in the baculovirus system, insect P450s can be studied in an insect membrane environment, without need for purification. Those are great advantages over the *E. coli* expression system. However, the interactions with its redox partners are not manipulated as easily (Wen *et al.*, 2003). The total amounts of P450 produced are also smaller, although addition of hemin to the culture medium can increase the amount of P450 produced (Dunkov *et al.*, 1997; Wen *et al.*, 2003). The total amount of P450 produced is less important in the baculovirus system than in the *E. coli* system as purification is not required for most applications, and as the highest activity of cell lysates is achieved at the correct P450/P450 reductase ratio, not at the maximal P450 production level (Wen *et al.*, 2003). The level of endogenous P450 in the control experiments, i.e., uninfected cells or cells infected with a virus carrying a non-P450 "control" cDNA, are virtually undetectable.

13.3.2.3. Transfection in cell lines Heterologous expression in transfected mammalian COS cells was first established in 1986 for bovine CYP17 (Zuber *et al.*, 1986), but it is not until later that an insect P450 was similarly expressed in an insect cell line. Thus, *Drosophila* Schneider 2 cells have been transfected with *Drosophila* P450 cDNAs (Warren *et al.*, 2002). Expression under control of the actin 5C promoter produced sufficient P450 for activity measurements. The advantage of the method is its simplicity. When the expression of the P450 is coupled

with a very sensitive assay, the method can rapidly provide qualitative data on the catalytic competence of the enzyme. However, the quantitative determination of P450 levels is more difficult to achieve, and the interaction with redox partners cannot be optimized except by coinfection. It is interesting that the CYP302A1 and CYP315A1 expressed by this method are mitochondrial P450s and the S2 cell homogenates were able to provide adequate redox partners. As used so far, it has not allowed a measurement of the amounts of P450 produced, nor have the redox partners been characterized or optimized. Cell transfection does not have the potential of the baculovirus system for large-scale production of P450 proteins.

13.3.2.4. Yeast Yeast expression systems have only started to be exploited for the production of insect P450 proteins. *Saccharomyces cerevisiae* has three P450 genes that are fully characterized, and are expressed at low levels so that inducible expression of an exogenous P450 is not hindered by the endogenous P450. Coproduction of CYP6A2 from *Drosophila* and of human P450 reductase in yeast (Saner *et al.*, 1996) generated a cell system capable of activating several procarcinogens to active metabolites that induced mitotic gene conversion or cytotoxicity. Housefly CYP6D1 was also produced in yeast but methoxyresorufin demethylation was the only marker activity obtained with microsomes of the transformed yeast (Smith and Scott, 1997). Insect P450 production in yeast has not yet achieved the success seen with plant P450 production in yeast (Schuler and Werck-Reichhart, 2003). P450 cDNAs may need to be engineered to recode the N-terminus of the protein. This has been done successfully with plant P450s to conform with the yeast codon usage (Hehn *et al.*, 2002). The replacement of the yeast P450 reductase gene with an insect P450 reductase gene by homologous recombination (Pompon *et al.*, 1996) should increase the usefulness of this yet underutilized expression system. Indeed, yeast combines the advantages of *E. coli* inducible production of large amounts of protein with the advantage of the eukaryotic cell system in which P450 enzymes can be studied in a normal membrane environment.

13.3.2.5. Transgenic insects The use of transgenic insects to study P450 function (or regulation, see Sections 13.4.5.3 and 13.5.2.2) has until now been restricted to *Drosophila*. In the first report, Gandhi *et al.* were unable to rescue by transgenesis the lethality of two complementation groups in the *Cyp4d1* region (Gandhi *et al.*, 1992). Heterologous expression of vertebrate P450s was achieved in

studies aimed at developing *Drosophila* as a genotoxicity model organism. The rat CYP2B1 gene was expressed under control of the *Drosophila* LSP1a promoter (Jowett *et al.*, 1991). This promoter ensures high levels of expression in third instar larvae. Transgenic flies expressed functional CYP2B1, as shown by increased CYP2B1-specific metabolism of 7-benzoyloxyresorufin and by increased sensitivity to cyclophosphamide, a procarcinogenic drug activated by CYP2B1. In similar experiments, canine CYP1A1 was expressed under the control of the *Drosophila* heatshock inducible *hsp70* promoter. Small amounts of CYP1A1 were produced after heatshock, sufficient to increase the sensitivity of the flies to 7,12-dimethylbenz[a]anthracene, a polycyclic aromatic hydrocarbon that is metabolized by CYP1A1 to a genotoxic metabolite (Komori *et al.*, 1993). Housefly CYP6D1 was produced in *Drosophila* under control of the heatshock inducible *hsp70* promoter, and this led to a significant increase in benzo[a]pyrene hydroxylation (Korytko *et al.*, 2000a), though heat shock decreased total P450 levels in whole body microsomes. Transgenic expression of CYP6G1 has been an important piece of evidence in demonstrating its role in DDT and neonicotinoid resistance (Daborn *et al.*, 2002; Le Goff *et al.*, 2003) as discussed below (Section 13.4.5.5). Transformation of other insects (see **Chapter 10**), notably with the piggyBac vector as in *Bombyx mori* (Tamura *et al.*, 2000) will undoubtedly increase the applications of transgenesis to P450 research.

13.3.3. P450 Enzymes and Their Redox Partners

13.3.3.1. P450 proteins The sequence identity of distantly related P450 proteins can be as low as that predicted from the random assortment of two sets of 500 or so amino acids. This is because there are very few absolutely conserved amino acids. In insect sequences available to date, these are found in five conserved motifs of the protein (**Figure 8**), the WxxxR motif, the GxE/D TT/S motif, the ExLR motif, the PxxFxPE/DRF motif and the PFxxGxRxCxG/A motif. Despite this tremendous overall sequence diversity, the increasing number of crystal structures for P450 proteins, mostly soluble forms from bacteria (Poulos *et al.*, 1995), reveals a quite high conservation of the three-dimensional structure. The description of this structure essentially follows the nomenclature of the P450cam protein, the camphor hydroxylase of *Pseudomonas putida* (Poulos *et al.*, 1985). The first motif WxxxR is located in the C-helix, and the Arg is thought to form a charge pair with the propionate of the heme. This

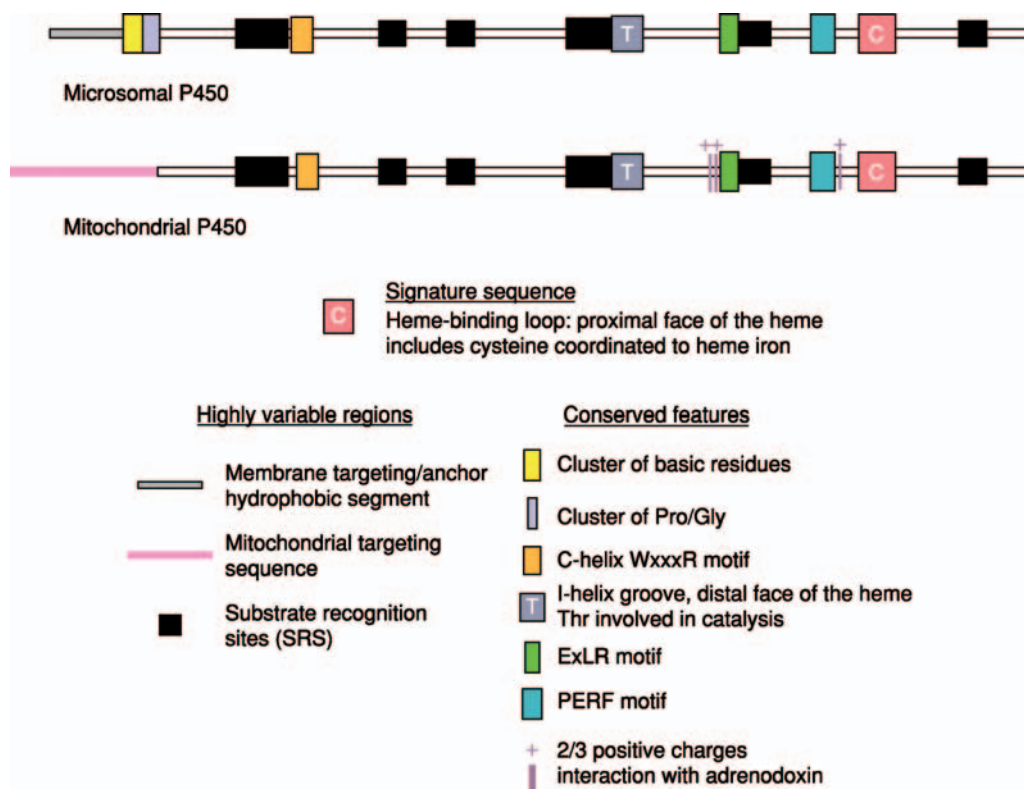


Figure 8 Conserved and variable regions of P450 proteins illustrated over their primary structure (sequence). (Adapted with permission from Werck-Reichhart, D., Feyereisen, R., 2000. Cytochromes P450: a success story. *Genome Biol.* 1, 3003.1–3003.9; © GenomeBiology.)

motif is not easily discernible, except in multiple alignments. The second conserved motif GxE/DTT/S surrounds a conserved threonine in the middle of the long helix I that runs on top of the plane of the heme, over pyrrole ring B. The third conserved motif ExLR is located in helix K. It is thought to stabilize the overall structure through a set of salt bridge interactions (E-R-R) with the fourth conserved motif PxxFxPE/DRF (often PERF, but R is sometimes replaced by H or N) that is located after the K' helix in the “meander” facing the ExLR motif (Hasemann *et al.*, 1995). The fifth conserved motif PFxxGxRxCxG/A precedes helix L and carries the cysteine (thiolate) ligand to the heme iron on the opposite side of helix I. The cysteine ligand is responsible for the typical 450 nm (hence P450) absorption of the Fe^{II}-CO complex of P450 (Mansuy and Renaud, 1995). This heme binding loop is the most conserved portion of the protein, often considered as “signature” for P450 proteins. Deviations from the consensus sequences of these five motifs deserve special attention. For instance, the CYP301A1 of both *Drosophila* and *A. gambiae* has a very unusual Tyr instead of Phe in the canonical PFxxGxRxCxG/A motif around the Cys axial ligand to the heme. These deviations may denote an atypical catalytic

function for the P450 enzyme, as seen in P450 enzymes that are not monooxygenases, such as plant allene oxide synthase (CYP74A) or vertebrate thromboxane synthase (CYP5A1) whose I helix lacks the conserved Thr. In the bacterial hydroxylase P450eryF (CYP107A), the Thr is replaced by Ala. A water molecule and a hydroxyl group of the substrate have become functional equivalents of the Thr hydroxyl (Poulos *et al.*, 1995).

P450 proteins are also characterized by their N-terminal sequence (Figure 9). Those targeted to the endoplasmic reticulum have a stretch of about 20 hydrophobic amino acids. These precede one or two charged residues that serve as halt-transfer signal and a short motif of prolines and glycines. The latter serves as a “hinge” that slaps the globular domain of the protein onto the surface of the membrane while the N-terminus is anchored through it. The presence of the PGPP hinge is necessary for proper heme incorporation and assembly of functional P450s in the cell (Yamazaki *et al.*, 1993; Chen *et al.*, 1998). A hydrophobic region between helices F and G is thought to penetrate the lipid bilayer, thus increasing the contact of the P450 with the hydrophobic environment from which many substrates can enter the active site (Williams *et al.*, 2000).

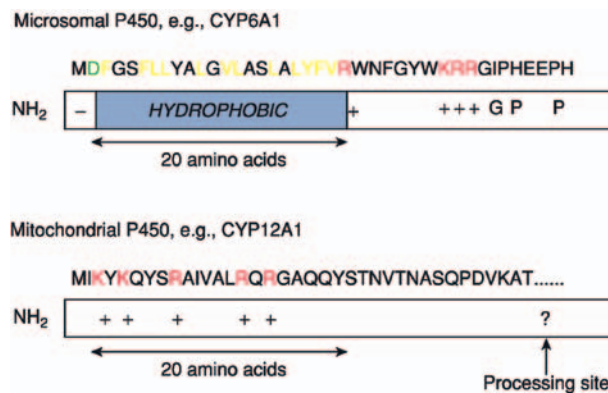


Figure 9 Scheme of the N-terminal sequence of microsomal and mitochondrial P450 proteins.

The N-terminal sequence of P450 proteins targeted to mitochondria is usually somewhat longer, and shows several charged residues (Figure 9). The mature mitochondrial protein is proteolytically cleaved at a position that has not been formally recognized for insect mitochondrial P450s to date, but is known for several mitochondrial P450s of vertebrate species. Mitochondrial P450 proteins are also characterized by a pair of charged amino acids in the K helix, R391 and K395 in CYP12A1. Homologous amino acids in mammalian P450_{scc} (K377 and K381) are responsible for the high affinity to the ferredoxin-type (adrenodoxin) electron donor (Wada and Waterman, 1992; Pikuleva *et al.*, 1999). An additional positively charged residue (R454 of CYP12A1) is homologous to R418 of CYP27A1 shown to increase affinity to adrenodoxin even further (Pikuleva *et al.*, 1999). The insect CYP12, 49, 301, 302, 314, and 315 proteins are most closely related to the mammalian mitochondrial P450 (CYP11, CYP24, and CYP27 families), to CYP44A1 from *C. elegans*, and to the pond snail CYP10. Subcellular localization of CYP12A1 by immunogold histochemistry with antibodies raised against the CYP12A1 protein produced in bacteria established the mitochondrial nature of CYP12A1 (Guzov *et al.*, 1998).

There is evidence that some vertebrate microsomal P450s, e.g., CYP1A1 and CYP2E1, are cleaved *in vivo* of their N-terminal anchor, thus revealing a cryptic mitochondrial targeting sequence, and the shortened protein is enzymatically active in mitochondria (Anandatheerthavarada *et al.*, 1999). Thus, the 1–44 residues of CYP1A1 serve a dual targeting role, with 1–32 targeting the protein to the ER cotranslationally, whereas cleavage and exposure of three basic amino acids in residues 33–44 direct the posttranslational transport to mitochondria (Bhagwat *et al.*, 1999). Whether some

insect microsomal P450 proteins (e.g., CYP314A1, see Section 13.4.1.1) behave in this fashion is currently unknown.

Interspersed throughout the globular domain of the P450 proteins are six regions with a low degree of sequence similarity, covering about 16% of the total length of the protein (Figure 8). Initially recognized in CYP2 proteins by Gotoh (1992), these are called SRS (substrate recognition sites) and this designation has been generically extended to other P450s.

P450 enzymes, whether microsomal or mitochondrial, need to interact with redox partners for their supply of reducing equivalents from NADPH. Figure 10 schematically illustrates the two types of electron transfer complexes thus formed, and the following sections provide a description of the redox partners.

13.3.3.2. NADPH cytochrome P450 reductase P450 reductase (EC 1.6.2.4) belongs to a family of flavo-proteins utilizing both FAD and FMN as cofactors. These diflavin reductases emerged from the ancestral fusion of a gene coding for a ferredoxin reductase with its NADP(H) and FAD binding domains with a gene coding for a flavodoxin with its FMN domain. This origin of the enzyme was first proposed by Porter and Kasper (1986) based on their analysis of the rat P450 reductase sequence. The fusion is dramatically illustrated by the three-dimensional structure of P450 reductase (Wang *et al.*, 1997) where the domains are clearly distinguished (Figure 11). The architecture of this domain fusion has been found in a handful of other enzymes (Murataliev *et al.*, 2004a). In some cases, further fusion with a P450 gene has led to self-sufficient P450 proteins, e.g., the fatty acid hydroxylase of *Bacillus megaterium*, P450BM3 (Nahri and Fulco, 1986) and of *Fusarium oxysporum*, P450foxy (Nakayama *et al.*, 1996).

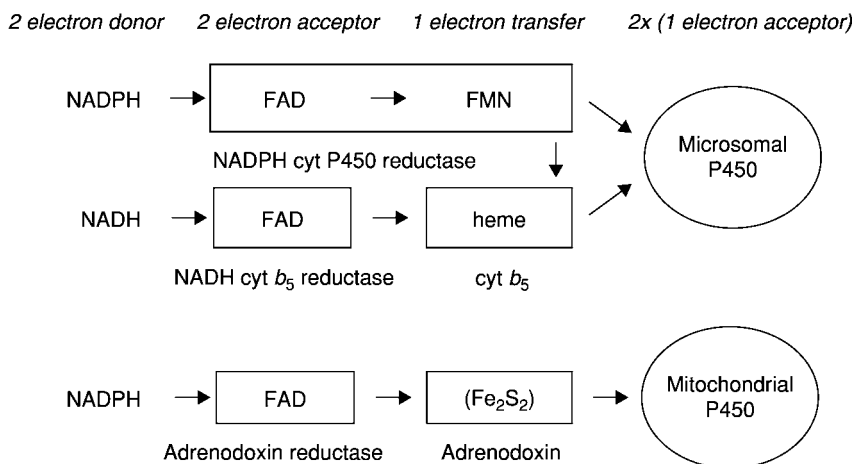


Figure 10 Mitochondrial and microsomal P450 redox partners.

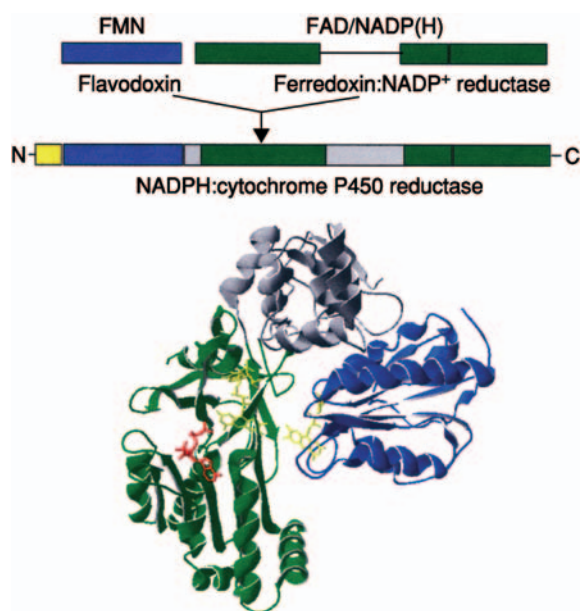


Figure 11 Structure of NADPH cytochrome P450 reductase. Top: evolutionary origin of the FMN (blue), FAD and NADP(H) (green) binding domains of the protein (yellow: membrane anchor; gray: connecting domain). Bottom: three-dimensional structure of the enzyme with the domains identified by color. The bound substrate NADPH (red) and cofactors FAD and FMN (yellow) are indicated. (Reprinted with permission from Murataliev, M.B., Feyereisen, R., Walker, F.A., 2004a. Electron transfer by diflavin reductases. *Biochem. Biophys. Acta* 1698, 1–26; © Elsevier.)

The insect P450 reductases sequenced to date are clearly orthologous to the mammalian P450 reductases, with an overall amino acid sequence identity of 54% for the housefly P450 reductase, first cloned and sequenced in 1993 (Koener *et al.*, 1993). The housefly P450 reductase gene codes for a protein of 671 amino acids, and was mapped to chromosome III. The P450 reductases of other

insects are very similar to the housefly enzyme – 82% identity for the *D. melanogaster* enzyme (Hovemann *et al.*, 1997), 57% identity for the *Bombyx mori* enzyme (Horike *et al.*, 2000), and 75% identity for the *A. gambiae* P450 reductase (Nikou *et al.*, 2003). The insect, mammalian, and yeast enzymes are functionally interchangeable in reconstituted systems of the purified proteins or in heterologous expression systems. However, no detailed study has documented how *well* a mammalian or yeast P450 reductase can support the activity of an insect P450 when compared to the cognate insect P450 reductase.

Early attempts to purify and characterize the enzyme from microsomes of housefly abdomens were hampered by the facile proteolytic cleavage of the N-terminal portion of the protein. This hydrophobic peptide anchors the reductase in the membrane, and its removal abolishes the ability of the remainder of the protein (“soluble” or “tryptic” reductase) to reduce P450s. The proteolytically cleaved reductase nonetheless retains the ability to reduce artificial electron acceptors such as cytochrome *c*, DCPIP or ferricyanide (Hodgson, 1985). Heterologous expression of the cloned P450 reductase has been achieved in *E. coli* (Andersen *et al.*, 1994) and in the baculovirus expression system (Wen *et al.*, 2003) and a purification scheme (Murataliev *et al.*, 1999) has produced quantities of enzyme sufficient for a detailed catalytic characterization of the enzyme’s functioning and of its reconstitution with redox partners (Figure 12).

P450 reductase is an obligatory partner of microsomal P450 enzymes. Antisera to *Spodoptera eridania* or housefly P450 reductase inhibit all P450-dependent activities tested (Crankshaw *et al.*,

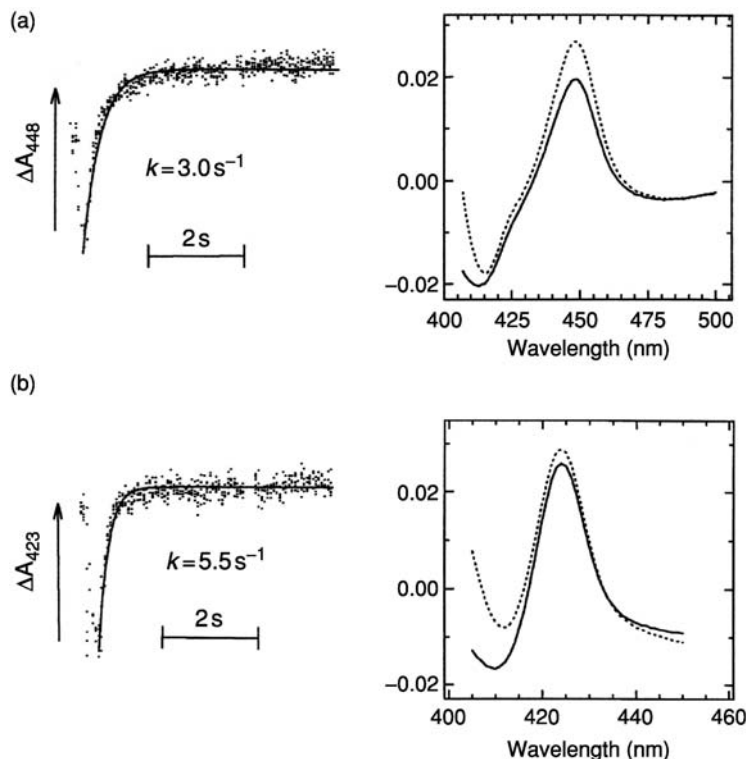


Figure 12 Reduction of housefly CYP6A1 (a) and cytochrome b_5 (b) by NADPH cytochrome P450 reductase. The recombinant proteins expressed in *E. coli* were reconstituted *in vitro*. On the left, the kinetics of reduction measured by stopped-flow spectrophotometry are shown with the calculated first-order rate constants. On the right, the end point difference spectra of CYP6A1 and cytochrome b_5 after reduction by P450 reductase (solid line) and sodium dithionite (dotted line). (Adapted from Guzov, V.M., Houston, H.L., Murataliev, M.B., Walker, F.A., Feyereisen, R., 1996. Molecular cloning, overexpression in *Escherichia coli*, structural and functional characterization of house fly cytochrome b_5 . *J. Biol. Chem.* 271, 26637–26645.)

1981; Feyereisen and Vincent, 1984). Immunoinhibition with P450 reductase antibodies serves as a strong indication of microsomal P450 involvement in NADPH-dependent activities, such as ecdysone 20-hydroxylation in the cockroach and in *B. mori* eggs (Halliday *et al.*, 1986; Horike and Sonobe, 1999; Horike *et al.*, 2000) and (*Z*)-9-tricosene biosynthesis in the housefly (Reed *et al.*, 1994). P450 reductase immunoinhibition could serve as a tool to distinguish P450 dependent activities from flavin monooxygenase (FMO) activities in microsomes from insect sources. P450 reductase also transfers electrons to cytochrome b_5 (see below) and to other microsomal enzymes such as heme oxygenase.

13.3.3.3. Cytochrome b_5 In contrast to P450 reductase, the role of cytochrome b_5 as partner in P450 dependent reactions is considerably more complex (see Section 13.3.3.3). The housefly cytochrome b_5 is a 134 amino acid protein (Guzov *et al.*, 1996) with 48% sequence identity with the orthologous rat cytochrome b_5 . Its N-terminal domain of about 100 residues is the heme-binding domain that is about 60% identical to that of the vertebrate

cytochrome b_5 . Its C-terminal portion is a hydrophobic membrane anchor. A probable fatty acid desaturase-cyt b_5 fusion protein has been misidentified as the *Drosophila* cytochrome b_5 (Kula *et al.*, 1995; Scott, 1999; Kula and Rozek, 2000), but the correct ortholog is 76% identical to the housefly cytochrome b_5 . The *H. armigera* cytochrome b_5 (Ranasinghe and Hobbs, 1999a) is 127 amino acids in length and 51% identical to the housefly cytochrome b_5 , and the *A. gambiae* cytochrome b_5 is 54% identical (Nikou *et al.*, 2003). The known insect cytochrome b_5 sequences differ at their C-terminal from both the vertebrate microsomal and outer mitochondrial membrane cytochrome b_5 sequences, so that inferences about the subcellular targeting of the insect protein (Wang *et al.*, 2003) would seem premature.

The housefly cytochrome b_5 protein was produced in *E. coli*, purified and fully characterized (Guzov *et al.*, 1996). Absorption spectroscopy and EPR revealed properties very similar to cytochromes b_5 from vertebrates. NMR spectra indicated that the orientation of the heme in the protein relative to its α , γ *meso* axis is about 1:1. This means that the

protein is present in two forms of approximately equal abundance, that result from two modes of insertion of the noncovalently bound heme in the protein between the two coordinating histidines (face up and face down). Expression of the heme-binding domain in *E. coli* revealed that the heme is kinetically trapped in a 1.2:1 ratio of the two isomers, and that this orientation results from the selective binding of heme by the apoprotein (Wang *et al.*, 2003). A redox potential of -26 mV was measured by cyclic voltammetry on a treated gold electrode in the presence of hexaminechromium(III) chloride, and was verified by classical electrochemical titration. Stopped flow spectrophotometry showed that the cytochrome b_5 is reduced by housefly P450 reductase at a high rate (5.5 s^{-1}) (Guzov *et al.*, 1996) (Figure 12).

Cytochrome b_5 can also be reduced by its own reductase, an NADH-dependent FAD flavoprotein, and can therefore provide either NADH- or NADPH-derived electrons to P450 enzymes. NADH-cytochrome b_5 reductase (EC 1.6.2.2) has been studied in *Ceratitis capitata* and *M. domestica* (Megias *et al.*, 1984; Zhang and Scott, 1996a). The N-terminus sequence of the purified housefly enzyme aligns to an internal sequence of the *Drosophila* enzyme (CG5946) indicating that it represents a proteolytically processed form. NADH-cytochrome b_5 reductase and cytochrome b_5 are also known to provide electrons to other acceptors, such as fatty acid desaturases and elongases.

13.3.3.4. Redox partners of mitochondrial P450

The redox partners of mitochondrial P450s are adrenodoxin reductase, an NADPH-dependent FAD flavoprotein and adrenodoxin, a [2Fe-2S] ferredoxin-type iron sulfur protein. These are named for the two redox partners of mammalian adrenal mitochondrial P450s, and this designation has been liberally bestowed on proteins from animals that don't have adrenals. Insect adrenodoxin reductase and adrenodoxin have not been functionally characterized, but their bovine orthologs are capable of supporting the activity of an insect mitochondrial P450, housefly CYP12A1 (Guzov *et al.*, 1998). The reduction of CYP12A1 is rapid and efficient with bovine adrenodoxin reductase/adrenodoxin while under the same conditions housefly microsomal P450 reductase is only marginally effective.

A fragment of a *Drosophila* adrenodoxin-like open reading frame is in GenBank on a stretch of DNA that also encodes a heatshock gene at 67B on the right arm of chromosome 3 (Pauli and Tonka, 1987). This stretch of 95 amino acids is similar (about 46%) to vertebrate adrenodoxin, and was initially identified

as the *Drosophila* adrenodoxin ortholog (GenBank X06542). But the correct adrenodoxin ortholog was revealed by the complete genome sequence at 64B1 as a 152 amino acid protein, 45% identical to the bovine protein. EPR spectroscopic evidence for the presence of an adrenodoxin-like protein in fat body mitochondria of *Spodoptera littoralis* has been presented (Sherrill *et al.*, 1995).

The *Drosophila* and *A. gambiae* adrenodoxin reductase and adrenodoxin genes have been annotated. The *Drosophila* P-element induced mutant *dare1* for defective in the avoidance of repellents was found to encode *Drosophila* adrenodoxin reductase (Freeman *et al.*, 1999). Strong *dare* mutants undergo developmental arrest, and this phenotype is largely rescued by feeding 20-hydroxyecdysone. Decreasing by half the wild-type expression of *dare* blocks the olfactory response. The gene is expressed at low levels in all tissues of the adult fly, including the brain and the antennae. Highest expression is found in the prothoracic gland portion of the ring gland of third instar larvae, as well as in the nurse cells of adult ovaries. These tissues are known to require mitochondrial P450s for ecdysteroid production. The 55 kDa protein encoded by *dare* is 42% identical to the human enzyme.

13.3.4. Catalytic Mechanisms

13.3.4.1. P450 reactions

Little work on insect P450 has focused on the catalytic cycle, and the mechanism derived from our understanding of the bacterial and mammalian P450 enzymes (Ortiz de Montellano, 1995b; Schlichting *et al.*, 2000) will be briefly summarized (Figure 13). The oxidized P450 is a mixture of two forms: a low spin (Fe^{III}) form with water as the sixth coordinated ligand on the opposite side of the Cys thiolate ligand, and a high spin (Fe^{III}) pentacoordinated form. Substrate binding displaces water from the sixth liganding position, leading to a shift to high spin. This shift can be observed (type I spectrum) and is accompanied by a decrease in the redox potential of P450. The P450-substrate complex receives a first electron from a redox partner (P450 reductase or adrenodoxin), and ferrous P450 (Fe^{II}) then binds O_2 . At this step CO can compete with O_2 for binding to P450, its binding leads to a stable complex, with absorption maximum at 450 nm (Figure 5), that is catalytically inactive. CO can be displaced by light irradiation at 450 nm. The P450- O_2 -substrate complex in the form of a ferric peroxide complex then accepts a second electron (from P450 reductase or in some cases cytochrome b_5 , or from adrenodoxin). Different types of activated oxygen forms of the same

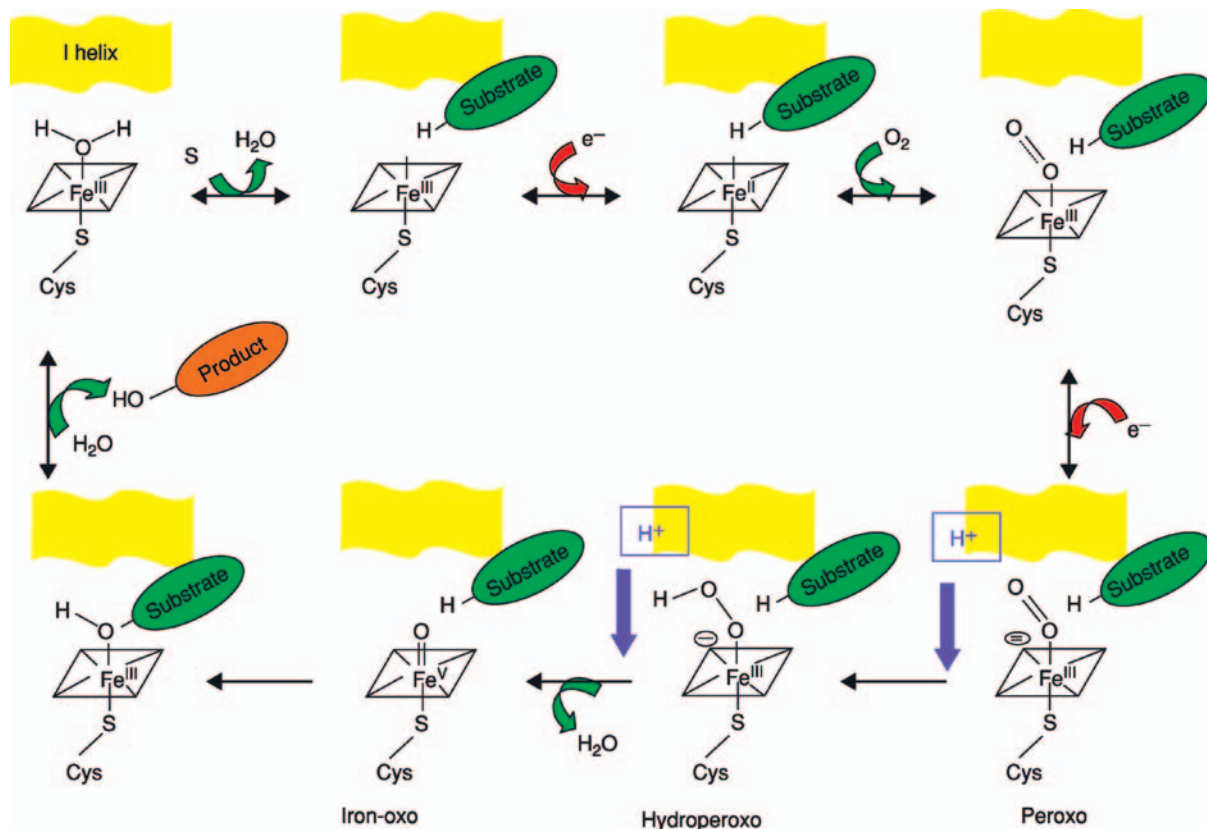


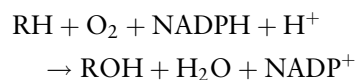
Figure 13 Catalytic cycle of P450 enzymes in monooxygenation reactions. Three possible forms of the activated oxygen species are shown. See text for details. Other reactions (reduction, isomerization, dehydration) can be catalyzed by oxygen-free forms of the enzyme. (Adapted with permission from Werck-Reichhart, D., Feyereisen, R., 2000. Cytochromes P450: a success story. *Genome Biol.* 1, 3003.1–3003.9; © GenomeBiology.)

P450 enzyme can then be formed, depending on the protonation state of the complex and on the homolytic or heterolytic cleavage of the reduced dioxygen. Although the formal reaction is the insertion of an atom of oxygen into the substrate, the other atom being reduced to water (hence the term “mixed-function oxidase”), the nature of the oxidizing species can vary. A P450 ($\text{Fe}^{\text{III}}\text{—O—O})^{2-}$ peroxy-iron form, a P450 ($\text{Fe}^{\text{III}}\text{—O—OH})^-$ hydroperoxy form, and a P450 ($\text{Fe}^{\text{V}}\text{=O}$) or P450 iron-oxo form are the preferred descriptions of the activated oxygen forms (Ortiz de Montellano, 1995b; Schlichting *et al.*, 2000; Newcomb *et al.*, 2003). The type of reaction catalyzed then depends on the substrate and substrate binding site and varies from hydroxylation to epoxidation, O-, N-, and S-dealkylation, N- and S-oxidations, or at least 60 different chemical reactions. The types of reactions currently known to be catalyzed by insect P450 enzymes are listed in Table 2. The P450(Fe^{II})–substrate complex can function as a reductase, and the P450– O_2 –substrate complex can also function as an oxidase, releasing superoxide, hydrogen peroxide, or water. Under experimental conditions, NADPH and

molecular oxygen can be substituted by organic hydroperoxides, sodium periodate, etc., in what is called the peroxide shunt (Ortiz de Montellano, 1995a).

The obligatory role of P450 reductase in catalysis of the microsomal P450 has been proven in reconstitution experiments, but the role of phospholipids is less clear and has not been specifically studied. The role of cytochrome b_5 is discussed below. Activity of the mitochondrial CYP12A1 also showed absolute dependence on reconstitution in the presence of (bovine) mitochondrial redox partners (Guzov *et al.*, 1998).

The stoichiometry (eqn [1])



that commonly describes the monooxygenase (*sensu* Hayaishi) or mixed-function oxidation (*sensu* Mason) reaction of P450 has not been confirmed experimentally for any insect P450. A more complex stoichiometry would take into account the “leakage” of activated oxygen species as superoxide, hydrogen

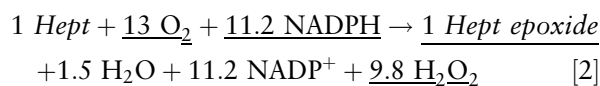
Table 2 Enzymatic reactions catalyzed by insect P450 enzymes

Reaction catalyzed	P450
Oxidase activity	
O ₂ to H ₂ O, H ₂ O ₂ , O ₂ ⁻	CYP6A1 (and probably most P450)
Monoxygenations	
Aliphatic hydroxylation	
C–H hydroxylation	CYP4C7, CYP6A1, CYP6A2, CYP6A8, CYP12A1, CYP302A1, CYP312A1, CYP314A1, CYP315A1
O-dealkylation	CYP6A1, CYP6D1 ^a , CYP12A1, CYP6A5, CYP6B4, CYP6B17, CYP6B21
Dehalogenation	CYP6A2 (DDT to DDA, DDD)
Epoxidation	CYP6A1, CYP6A2, CYP12A1, CYP15A1, CYP9E1
Aromatic hydroxylation	CYP6D1 ^a
Heteroatom oxidation and dealkylation	
Phosphorothioate ester oxidation	CYP6A1, CYP6A2, CYP12A1, CYP6D1 ^a
N-dealkylation	CYP12A1, CYP6A5
N-oxidation	+ (nicotine)
S-oxidation	+ (phorate)
Aldehyde oxidation	+ (C-26 hydroxyecdysteroids)
Complex and atypical reactions	
Carbon–carbon cleavage	+? (sterols, ecdysteroid)
Decarbonylation with C–C cleavage	+ (P450hyd)
Aromatization	+ (defensive steroids)
Dehydrogenation	+ (cholesterol)
Dehydration	
Aldoxime dehydration	+ (R-CN biosynthesis)
Reduction	–
Endoperoxide isomerization	–

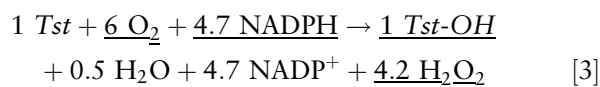
^aInference from immunoinhibition experiments.

+, indicates metabolism by microsomal P450, but specific enzyme not identified (substrate indicated), –, indicates no evidence to date.

peroxide, and water at the expense of NADPH during catalysis. In a “well coupled” reaction as in (eqn [1]) these by-products would not be formed. It is assumed, but not generally proven, that a specialized P450 metabolizing its favorite substrate would follow stoichiometry (eqn [1]). An approximate balance for CYP6A1 epoxidation of heptachlor (*Hept*) gives the following results: (M.B. Murataliev, V.M. Guzov, R. Feyereisen, unpublished data).



and for testosterone (*Tst*) hydroxylation under the same experimental conditions:



In eqns [2] and [3], the parameters that were measured are underlined. These stoichiometries show that a P450 such as CYP6A1 can be simultaneously an oxidase and a monooxygenase. These coupling stoichiometries (M.B. Murataliev, V.M. Guzov, R. Feyereisen, unpublished data) are dependent on the ratio of P450 and P450 reductase, as well as on the

presence or absence of cytochrome *b*₅ (see below). Note that those stoichiometries are not balanced, reflecting experimental error in the measurements and that the addition of superoxide dismutase did not change the amount of H₂O₂, indicating either no superoxide production, or lack of success in measuring it. The uncoupling of monooxygenation is highly likely to be a common feature of insect P450 enzymes that metabolize xenobiotics of synthetic or plant origin. The generation of reactive oxygen species is a corollary of active P450 metabolism.

13.3.4.2. P450 reductase

13.3.4.2.1. P450 interaction with P450 reductase
As seen above, the proper functioning of P450 enzymes depends on an efficient electron supply. In insect microsomes, the ratio of P450 enzymes to P450 reductase is about 6–18 to 1 (Feyereisen *et al.*, 1990). In this ratio, all P450 enzymes are summed, so that the actual ratio of one specific P450 enzyme to P450 reductase is probably smaller. The rate of the overall microsomal P450 reaction (two transfers of one electron) is relatively slow so that dissociation of the P450–P450 reductase complex is possible between the first and the second electron transfer. Indeed, cytochrome *b*₅ can replace P450 reductase

for the supply of the second electron in some cases (see below). The effect of varying the P450/P450 reductase ratio on catalytic rates was measured in a reconstituted system for heptachlor epoxidation by CYP6A1. The rate of epoxidation was determined by the concentration of the binary complex of P450 and P450 reductase, with the same high rate being observed in the presence of an excess of either protein (Figure 14). The half-saturating concentration of either protein was about 0.1 μM in the presence of cytochrome b_5 (M.B. Murataliev, V.M. Guzov, R. Feyereisen, unpublished data). This is in good agreement with the K_m of 0.14 and 0.5 μM for P450 reductase in the presence and absence of cytochrome b_5 measured previously (Guzov *et al.*, 1996). Coinfection of Sf9 cells with baculoviruses carrying CYP6B1 and P450 reductase has revealed that highest catalytic activity was achieved at an equivalent, moderate, multiplicities of infection for the two viruses (Wen *et al.*, 2003). Higher enzymatic activities of cell lysates towards furanocoumarins was not achieved when either protein was produced in excess. This result can be explained in part by documented limitations of the cell's ability to host, fold, and provide cofactors for both P450 and reductase (Wen *et al.*, 2003), but it also supports the idea that highest activity is achieved for the

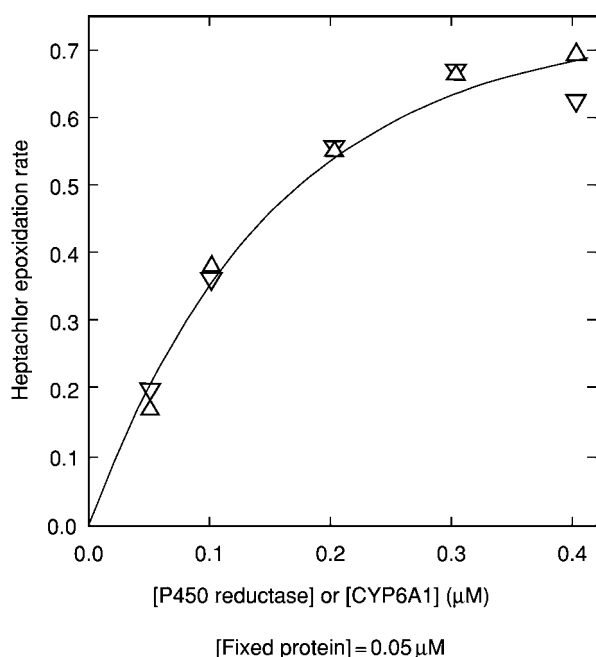


Figure 14 Heptachlor epoxidation by *E. coli*-expressed recombinant housefly CYP6A1 and NADPH cytochrome P450 reductase. A reconstituted system containing variable concentration of CYP6A1 (Δ) or P450 reductase (∇) and a fixed concentration (0.05 μM) of the reciprocal partner, and a cytochrome b_5 concentration of 1.0 μM was incubated in the presence of NADPH. (M.B. Murataliev, V.M. Guzov, R. Feyereisen, unpublished data)

highest concentration of the binary complex of the two partners.

13.3.4.2.2. P450 reductase functioning P450 reductase accepts two electrons from NADPH; more precisely, it accepts a hydride ion (one hydrogen plus one electron), and donates two electrons, one at a time, to P450 enzymes. P450 reductase is therefore an enzyme with two substrates: NADPH and the electron acceptor (P450 or artificial acceptor such as cyt *c*), and two products: NADP^+ and the reduced electron acceptor. With two bound flavins and a pathway of electron transfer $\text{NADPH} > \text{FAD} > \text{FMN} > \text{P450}$ (or cyt *c*), its reduction state during catalysis can theoretically vary between the fully oxidized state (0 el.) and the fully reduced state (4 el.). Studies with the purified recombinant housefly P450 reductase (Murataliev *et al.*, 1999; Murataliev and Feyereisen, 1999; Murataliev and Feyereisen, 2000; review: Murataliev *et al.*, 2004a) have shed light on two questions posed by this electron transfer function: what is the kinetic mechanism of this two-substrate enzyme (Ping-Pong or sequential Bi-Bi) and what are the respective reduction states of the two flavins during catalysis?

In the ping-pong mechanism, the first product of the reaction must be released before the second substrate binds to the enzyme, and no ternary complex is formed. In sequential Bi-Bi mechanisms both substrates bind to the enzyme to form a ternary complex. Although several kinetic mechanisms have been proposed (Hodgson, 1985), a careful study of the recombinant housefly P450 reductase clearly established a sequential random Bi-Bi mechanism (Murataliev *et al.*, 1999). The great sensitivity of the enzyme to ionic strength hampers the comparison of different studies (Murataliev *et al.*, 2004a). The formation of a ternary complex of NADPH, P450 reductase, and the electron acceptor suggested a role for reduced nucleotide binding in the catalysis of fast electron transfer. The rate of cytochrome *c* reduction was shown to equal the rate of hydride ion transfer from the nucleotide donor to FAD (Murataliev *et al.*, 1999). A faster electron transfer rate was observed with NADPH as compared to NADH (Murataliev *et al.*, 1999) and the 2'-phosphate was shown to contribute to more than half of the free energy of binding (Murataliev and Feyereisen, 2000). The affinity of the oxidized P450 reductase was ten times higher for NADPH than for NADP^+ (Murataliev *et al.*, 1999), and a conformational change induced by NADPH binding and important for fast catalysis was suggested by these studies.

The state of reduction of the flavins of P450 reductase during catalysis was deduced from kinetic

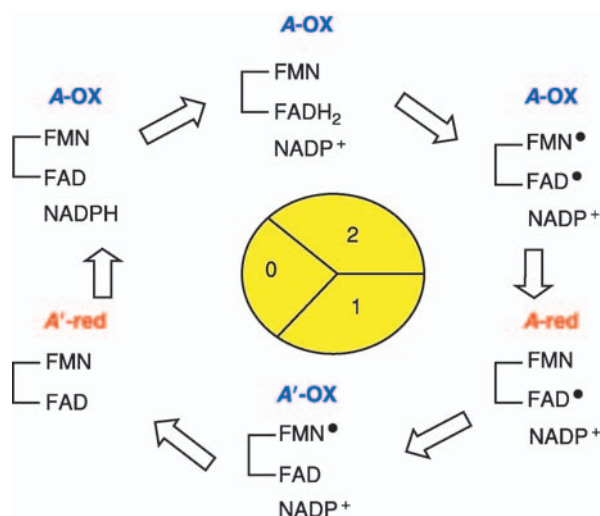


Figure 15 Reduction state of NADPH cytochrome P450 reductase during catalysis, the “0-2-1-0” cycle. The enzyme cycles between a fully oxidized state and a 2-electron reduced state. The electron acceptor (A and A’=P450 or cytochrome *c*) receives one electron at a time from an FMN semiquinone form (FMN•) of the enzyme. The release of NADP⁺ is shown here to occur at the last step but may occur earlier. Implicit additional steps are not shown, for clarity. See text for details and Murataliev *et al.* (2004a) for review.

experiments, rates of NADPH oxidation and EPR measurements of flavin semiquinone (free radical) levels. These revealed the existence of a catalytically competent FMN semiquinone, different from the “blue” neutral FMN semiquinone, known as the air-stable semiquinone that is not a catalytically relevant form of the enzyme (Murataliev and Feyereisen, 1999). Furthermore, the detailed studies of housefly P450 reductase led to a proposed catalytic cycle where the reduction state of the enzyme does not exceed 2 el., and where an FMN semiquinone, and not an FMN hydroquinone, serves as electron donor to the acceptor P450 or cytochrome *c*. This “0-2-1-0 cycle” (Figure 15) likely represents the general mechanism of P450 reductases, with strong evidence that it operates in P450BM3 and in the human P450 reductase as in the fly P450 reductase (Murataliev *et al.*, 2004a).

13.3.4.3. Role of cytochrome *b*₅ Depending on the P450 enzyme and on the reaction catalyzed, cytochrome *b*₅ may be either inhibitory, without effect, or its presence may be obligatory. Cytochrome *b*₅ can have a quantitative effect on overall reaction rates, and/or a qualitative role on the type of reaction catalyzed and the ratio of the reaction products. The role of cytochrome *b*₅ may or may not depend on its redox (electron transfer) properties. It can also influence the overall stoichiometry of the P450

reaction, in particular the “coupling rate,” i.e., the utilization and fate of electrons from NADPH relative to monooxygenation. Cytochrome *b*₅ should therefore be regarded as an important *modulator* of microsomal P450 systems. General reviews of the role of cytochrome *b*₅ in P450 reactions are available (Porter, 2002; Schenkman and Jansson, 2003) and known examples of this modulator role in insect systems follow.

The relative contribution of NADH in P450 reactions, but more importantly the NADH synergism of NADPH-dependent reactions that is occasionally observed (e.g., Ronis *et al.*, 1988; Feng *et al.*, 1992), is probably attributable to cytochrome *b*₅ as redox partner. Indeed, the *K_m* of the P450 reductase for NADH is a thousand-fold higher than for NADPH, and the *V_{max}* tenfold lower (Murataliev *et al.*, 1999), so that the contribution of NADH under normal conditions is probably channeled by NADH-cytochrome *b*₅ reductase and cytochrome *b*₅.

An anticytochrome *b*₅ antiserum severely inhibited (up to 90%) methoxycoumarin and ethoxycoumarin *O*-dealkylation and benzo[*a*]pyrene hydroxylation, but not methoxyresorufin and ethoxyresorufin *O*-dealkylation when assayed in microsomes of the housefly LPR strain (Zhang and Scott, 1994). This antiserum also inhibits cypermethrin 4'-hydroxylation by these CYP6D1-enriched microsomes (Zhang and Scott, 1996b).

Housefly cytochrome *b*₅ stimulates heptachlor epoxidation and steroid hydroxylation when reconstituted with cytochrome P450 reductase, housefly CYP6A1, and phospholipids (Guzov *et al.*, 1996; Murataliev *et al.*, 2004a). Stimulation of cyclodiene epoxidation and diazinon metabolism were also observed with *Drosophila* CYP6A2 expressed with the baculovirus system (Dunkov *et al.*, 1997). Cytochrome *b*₅ is efficiently reduced by P450 reductase (Figure 12), but it does not increase the rate of P450 reduction by P450 reductase. Because of its small redox potential (see above), cytochrome *b*₅ is unlikely to play an important role in delivering the first electron to P450 catalysis, and its stimulatory role probably involves an increased rate of transfer of the second electron. Cytochrome *b*₅ decreases the apparent *K_m* for P450 reductase and increases the *V_{max}* for epoxidation at constant CYP6A1 concentrations (Guzov *et al.*, 1996). The results suggest a role for cytochrome *b*₅ in the P450 reductase–P450 interactions.

Whereas heptachlor epoxidation by CYP6A1 was increased two- to threefold by the addition of cytochrome *b*₅, the hydroxylation of testosterone, androstenedione, and progesterone was stimulated

seven- to tenfold. The addition of cytochrome b_5 increased the ratio of 2 β -hydroxylation over 15 β -hydroxylation of testosterone. This suggests that cytochrome b_5 can have an effect on CYP6A1 conformation, probably altering the interaction of the binding site with either the C-17 hydroxyl group (decreased) or the C-3 carbonyl (increased). Interestingly, the effect of cytochrome b_5 on hydroxylation regioselectivity was also obtained with apo- b_5 (cytochrome b_5 depleted of heme and therefore redox incompetent), whereas the effect on turnover number was only much smaller with apo- b_5 . The effect of apo- b_5 is not due to heme transfer from P450 to apo- b_5 and, in fact, both apo- b_5 and (holo) cytochrome b_5 were shown to stabilize the ferrous-CO complex of CYP6A1, decreasing the rate of its conversion to P420 (M.B. Murataliev, V.M. Guzov, R. Feyereisen, unpublished data).

Cytochrome b_5 increases the coupling stoichiometry of CYP6A1 catalysis. In the heptachlor epoxidation assay, coupling (NADPH or O₂ used/heptachlor epoxide formed) increased from <8% to over 25%. This effect is even more pronounced for testosterone, where coupling efficiency in the presence of cytochrome b_5 can reach 84%. The effect of cytochrome b_5 results in a decrease in H₂O₂ production in both assays. The exact site of H₂O₂ production (P450 reductase or CYP6A1) is not known.

Coordinate induction and/or overexpression of cytochrome b_5 and P450 genes has been reported (Liu and Scott, 1996; Kasai *et al.*, 1998b; Ranasinghe and Hobbs, 1999a, 1999b; Nikou *et al.*, 2003) and this indicates that the effects of cytochrome b_5 seen *in vitro* may have significance *in vivo* as well.

13.3.4.4. Mechanisms and specificity of P450 inhibitors The common features of electron transfer, ligand binding, and catalysis described above are the features that determine the relative success of P450 inhibitors. Compounds that act as electron sinks and are readily autooxidizable can inhibit P450 reactions by inhibiting electron transfer by the respective redox partners. This mechanism is typical of the eye pigment xanthommatin that was identified as “endogenous inhibitor” in early studies (review: Hodgson, 1985). Several flavonoids may act in this way and care must be taken to distinguish P450 inhibition *per se* from inhibition of electron transfer.

Insecticide synergists (Figure 16) are among the most interesting inhibitors of P450 because of their widespread commercial use, in particular piperonyl butoxide. A landmark review paper on synergists remains that of Casida (1970). Synergists (Hodgson,

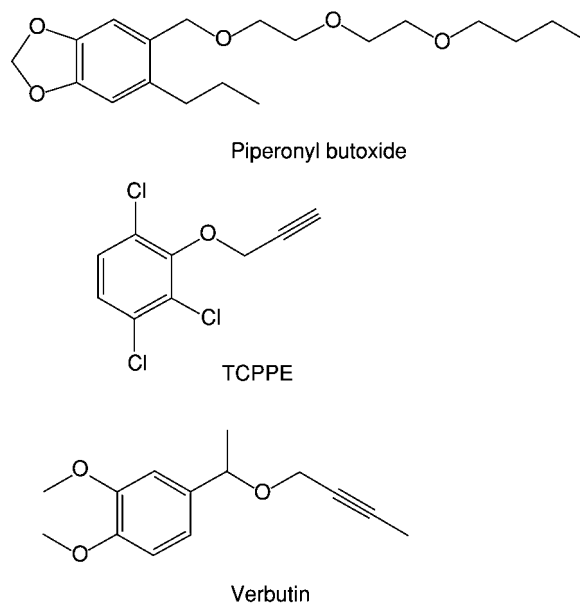


Figure 16 Structures of the synergists and P450 inhibitors piperonyl butoxide, a typical methylenedioxyphenyl (MDP) compound, TCPPE (trichlorophenylpropynyl ether) and verbutin.

1985; Bernard and Philogene, 1993) as well as P450 inhibitors in general (Ortiz de Montellano and Correia, 1995) have been covered in other insightful reviews as well. The synergism of carbaryl by piperonyl butoxide has been used in a survey of 54 insect species to estimate P450 activity *in vivo* (Brattsten and Metcalf, 1970). Although the synergistic ratio is most often presented, the usefulness of a synergistic difference has also been proposed (Brindley, 1977). The mode of action of piperonyl butoxide and other related methylenedioxyphenyl (MDP) compounds (or benzodioxole compounds) involves an initial metabolic activation by the P450 enzyme, leading to the formation of a carbene-iron complex that is virtually irreversible (Ortiz de Montellano and Correia, 1995). It follows that those P450 enzymes with (1) low affinity for the MDP compound and/or (2) low capacity to metabolize it to the carbene inhibitory form will not be inhibited, and thus piperonyl butoxide and other MDP compounds are not universal inhibitors of all P450 enzymes. Self-catalyzed destruction of P450 enzymes by terminal acetylenes or olefins and other “suicide substrates” is also, and for the same reasons, not equally effective for all P450 enzymes. The phenylpropynyl ether synergists such as TCPPE fall into this category, as well as the newer synergist verbutin (Bertok *et al.*, 2003). This nongenerality of inhibition has been well documented *in vivo*. For instance, TCPPE can be an effective synergist when piperonyl butoxide

cannot (Brown *et al.*, 1996; Zhang *et al.*, 1997). In the case of 1-aminobenzotriazole (ABT), which is metabolized to benzyne that covalently binds to the prosthetic heme, *in vitro* P450 destruction and formation of the porphyrin adduct have been measured in the housefly (Feyereisen *et al.*, 1984). P450 protein labeling by P450 inhibitors has also been achieved (Andersen *et al.*, 1995; Cuany *et al.*, 1995). The NADPH-dependent decrease in P450 caused by ABT, TCPPE, or piperonyl butoxide differs according to the induction status and fly strain, suggesting selectivity (Feyereisen *et al.*, 1984). This selectivity can be harnessed into the design of useful “suicide” inhibitors of, e.g., ecdysone biosynthesis (Luu and Werner, 1996). The synthesis of the MDP moiety seen in many plant natural products is itself dependent on a P450 activity (CYP719, Ikesawa *et al.*, 2003), and it is postulated that such compounds may be a legacy of evolutionary interactions with insect and other enemies (Berenbaum and Neal, 1987).

Another class of powerful P450 inhibitors are heterocyclic compounds with an sp² hybridized nitrogen as in pyridines, azoles, and imidazoles. These compounds bind simultaneously to the heme iron (type II ligands) and to a hydrophobic binding site of the P450 for its substrate. This “two-point binding” has therefore an intrinsic potential for selectivity, with the substrate mimic moiety of the inhibitor targeting the specific P450 and the type II-ligand moiety coordinating the heme, and inhibiting the enzyme. This reasoning has led to the design of potent inhibitors and photoaffinity labels (Andersen *et al.*, 1995) for a specific insect P450 (see Section 13.4.1.2 below). The commercial importance of this type of P450 inhibitors is emphasized by the fungicides and CYP51 inhibitors miconazole and ketoconazole.

13.3.4.5. Substrate selectivity and structure/function of P450 enzymes The substrate recognition sites (SRS) of CYP2 proteins were first described by Gotoh (1992) as highly variable regions. Subsequently, a large number of site-directed mutagenesis studies have focused on these regions to explore substrate specificity of mammalian and bacterial P450 enzymes (e.g., review: Domanski and Halpert, 2001). Although multiple sequence alignments usually show that SRS1 is highly polymorphic, the SRS1 of the CYP6B sequences of Lepidoptera are highly conserved (Berenbaum *et al.*, 1996). This suggested that in this case SRS1 may contribute to the recognition of furanocoumarins – substrates of most CYP6B enzymes studied to date. Mutagenesis of six residues of CYP6B1v1

from *Papilio polyxenes* provided some evidence for this hypothesis (Chen *et al.*, 2002). Seven mutants at the Phe116 position in SRS1 led to incorrectly folded or assembled P450 proteins that were catalytically inactive and that had absorption maxima at 420 nm instead of 450 nm in their CO-difference spectrum. One mutant, Phe116 to Trp, had a severely reduced catalytic activity. The Phe116 to Tyr mutant (insertion of a hydroxyl group on the aromatic side chain) was catalytically active and showed an altered substrate specificity towards furanocoumarins. Xanthotoxin metabolism was drastically reduced, bergapten and isopimpinellin metabolism were cut in half whereas metabolism of trioxsalen or psoralen were not affected. Homology modeling of the CYP6B1v1 structure based on a CYP102 crystal structure indicated that the Phe116 side chain projects into the active site above the plane of the heme. Four mutants at the neighboring His117 were catalytically inactive as were nine mutants at the Phe484 position, which is located in SRS6. Val368 is located in SRS5, opposite Phe116 and also in the vicinity of the active site. Its replacement by Phe resulted in a dead enzyme, whereas the mutations of Val368 to Ala or Leu did not affect CYP6B1v1 activity or specificity (Chen *et al.*, 2002). Two further replacements were either lethal (Phe206 to Leu) or without effect (His204 to Leu). These residues are outside the SRS regions, but indicated by homology modeling to be located near the substrate access channel. The overall result of this extensive set of experiments on 33 variants indicates that CYP6B1v1 is very sensitive to changes at sites that control access and geometry of the active site. Only the Phe166 to Tyr mutation in SRS1 showed a clear effect on substrate selectivity towards furanocoumarins. Only three of the inactive mutants at the Phe484 position showed a normal CO difference spectrum, and thus may have lost activity towards furanocoumarins while retaining or gaining activity towards other substrates. This residue in SRS6 is not conserved among CYP6B enzymes, however (Chen *et al.*, 2002). Further analyses of *Helicoverpa* and *Papilio* CYP6B sequences have attempted to describe ancestral CYP6B sequences and to model the geometry of their active site (Li *et al.*, 2003). The crystal structure of a CYP6B protein would contribute enormously to anchor such studies on a firm basis.

Results obtained with naturally occurring mutations situated outside the SRS regions show that substrate specificity is not encoded in the SRS alone. Three point mutations in *Drosophila* CYP6A2 (Bergé *et al.*, 1998), when combined, confer to this P450 the ability to metabolize DDT

without modifying the metabolism of testosterone (Amichot *et al.*, 2004). These mutations found in a DDT-resistant strain (see Section 13.4.5.3 below) are located between SRS4 and SRS5 in the J helix (Arg335 to Ser and Leu336 to Val) and at the end of helix L, before SRS6 (Val476 to Leu). The three mutations are located towards the “top” of helix I and may together influence the positioning of this helix and hence access to the active site (Amichot *et al.*, 2004). Significantly, the recombinant enzyme carrying only the Arg335 to Ser mutation has only a fraction of the DDT metabolizing capacity of the triple (naturally occurring) mutant, and is unstable. A better understanding of the structure–activity relationships in insect P450 enzymes is still very distant, but the diversity of insect P450 sequences may prove useful in the engineering of P450 as biocatalysts. For instance, the replacement of portions of SRS1 of P450BM3 (CYP102, a fatty acid ω -1 hydroxylase) by the homologous portions of SRS1 from CYP4C7 (a terpenoid ω -hydroxylase, see Section 13.4.1.2) modifies the regioselectivity of hydroxylation of fatty acids and terpenoids of the P450BM3 enzyme (Murataliev *et al.*, 2004b).

Information on substrate access to the active site and active site topology can also be inferred from spectral studies (Section 13.3.1.2) and biochemical studies (Figure 7). The active site topology of CYP6A1 was studied by a technique developed in Ortiz de Montellano’s group (Ortiz de Montellano and Graham-Lorence, 1993). The enzyme is first incubated with phenyldiazene to form a phenyl–iron complex. Ferricyanide-induced *in situ* migration of the phenyl group to the porphyrin nitrogens causes the formation of covalent adducts, which can then be separated by HPLC (Figure 17). The *N*-phenyl

protoporphyrin IX adducts of CYP6A1 were formed in a 17:25:33:24 ratio of the $N_B:N_A:N_C:N_D$ isomers (Andersen *et al.*, 1997). Thus in the native protein, all four pyrrole groups are somewhat exposed, whereas in several other P450s, labeling is more specific. Specific labeling indicates that the protein encumbers more space on top of the heme prosthetic group, e.g., leaving only one pyrrole ring exposed as in P450_{sc} (Pikuleva *et al.*, 1995). The type of labeling seen with CYP6A1 indicates less encumbrance by the protein on top of the heme as in CYP3A4 (Schrag and Wienkers, 2000). These experiments suggest that the active site of CYP6A1 is relatively accessible and not severely constrained. Indeed CYP6A1 metabolizes flat steroids, bulky cyclodiene insecticides, as well as a variety of sesquiterpenoids (Table 1) (Andersen *et al.*, 1994, 1997; M.B. Murataliev, V.M. Guzov, R. Feyerreisen, unpublished data). High uncoupling of electron transfer relative to monooxygenation may be a result of this broad substrate specificity.

13.4. P450 Functions

Ever since the pioneering work of Agosin and of Terriere in the early sixties on microsomal enzymes that hydroxylate DDT and naphthalene (review: Agosin, 1985), the enzymes now recognized as P450 have been best known for their role in xenobiotic metabolism in insects. They are often denoted as “detoxification enzymes.” This designation not only neglects the importance of P450 enzymes in basic physiological processes, as pointed out for drug-metabolizing enzymes in general by Nebert (1991). It also neglects the conceptual difference between metabolism (what the enzyme does to the chemical) and

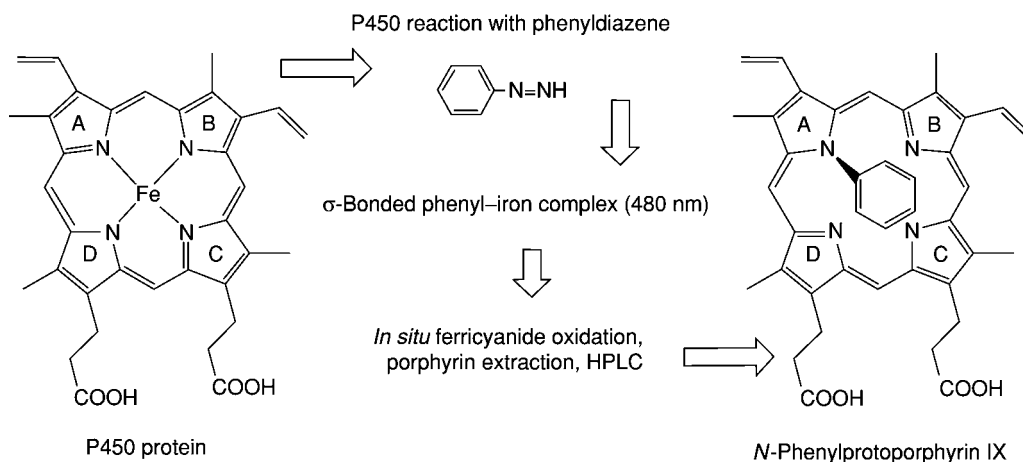


Figure 17 Active site topology of a P450 enzyme tested by formation of phenyl–porphyrin adducts. In this example, an adduct with pyrrole ring A is formed. The ratio of the four possible adducts indicates the degree of encumbrance in the native P450 protein.

toxicity (what the chemical does to the biological system). As noted before (Feyereisen, 1999):

there are many cases where P450 enzymes act as anything but “detoxification enzymes.” The easy dichotomy between biosynthetic and detoxification functions of P450s reflects more the teleological tendencies of the observer than the phylogeny or biochemistry of the enzymes.

In most studies, the P450 substrates tested are either endogenous compounds and their analogs, or xenobiotics. Rarely are both types of substrates tested on the same P450 and, indeed, the thermodynamic reality of enzyme–substrate interaction makes the quest for a formally complete description of the chemical diversity of substrates of any enzyme illusory. A future understanding of P450 functions perhaps will be based more on their evolutionary trajectories, some P450s being presently constrained by their proven physiological role (but how long have they played this role?) and some P450s being positively selected for their present adaptive role in detoxification (of plant chemicals in evolutionary time, or of insecticides in historical time). Our knowledge of insect P450 function is not sufficient to classify individual P450s or whole CYP families rationally along such evolutionary lines, so the following description may be found to be arbitrary.

13.4.1. Metabolism of Signal Molecules

13.4.1.1. Ecdysteroid metabolism

13.4.1.1.1. Ecdysone 20-monooxygenase activity (E20MO) The conversion of ecdysone to 20-hydroxyecdysone does not occur in the prothoracic glands but occurs in many peripheral tissues, such as the fat body, midgut, and Malpighian tubules. The P450 nature of the enzyme catalyzing the 20-hydroxylation of ecdysone was well established in 1985 (Smith, 1985) following the initial reports of 1977 (Bollenbacher *et al.*, 1977; Feyereisen, 1977; Johnson and Rees, 1977). An NADPH and O₂-dependent enzyme system, inhibited by typical pharmacological P450 inhibitors, was studied in several insect species. The evidence for P450 involvement was strengthened by the light-sensitive carbon monoxide inhibition observed in some of the most thorough studies (Feyereisen and Durst, 1978; Smith *et al.*, 1979; Greenwood and Rees, 1984). The agreement on the P450 nature of the reaction was accompanied by a lack of consensus on the subcellular localization of E20MO. Some studies showed a microsomal activity, other studies showed a mitochondrial activity, and yet other studies indicated the presence of both microsomal and mitochondrial activities in the same tissue (Smith,

1985). The differences in species, tissues, and experimental conditions, while real, did not permit glossing over the dual subcellular localization of E20MO.

Studies past 1985 continued to present evidence for either the mitochondrial or microsomal, or both, localizations of the E20MO activity. For instance, it has been reported to be mostly microsomal in imaginal discs of *Pieris brassicae* (Blais and Lafont, 1986) and in *Gryllus bimaculatus* midgut (Liebrich and Hoffmann, 1991). In the midgut of *Diploptera punctata* (Halliday *et al.*, 1986) and in embryos of *Bombyx mori* (Horike and Sonobe, 1999), the activity is essentially microsomal, and can be inhibited by antibodies to the insect P450 reductase. This is clear evidence that the enzyme derives its reducing equivalents from the usual microsomal redox partner in those cases.

In *Spodoptera littoralis* fat body, E20MO activity is predominantly mitochondrial, with a small amount of microsomal activity (Hoggard and Rees, 1988). The mitochondrial E20MO activity was reportedly inhibited by antibodies to vertebrate P450_{scc} (CYP11A), P450_{11β} (CYP11B), adrenodoxin, and adrenodoxin reductase (Chen *et al.*, 1994), despite the considerable sequence divergence predicted between the vertebrate and insect proteins. The immunodetection of polypeptides significantly larger than predicted (e.g., P450 at 82 kDa and adrenodoxin at 73 kDa) was also a surprising feature of that study. In whole body homogenates of third-instar *Drosophila*, and in the midgut of larval *Spodoptera frugiperda*, the E20MO activity is distributed 1:3 between mitochondrial and microsomal fractions (Smith, 1985; Yu, 1995), and it is also distributed in both fractions in larvae of the housefly, and of the flesh fly *Neobellieria bullata* (Darvas *et al.*, 1993). Weirich *et al.* (1996) compared the apparent K_m and specific activities of the mitochondrial and microsomal E20MO activities of *Manduca sexta* larval midgut. They concluded that at physiological ecdysone titers, despite a higher specific activity, the mitochondrial E20MO would contribute less than one-eighth the activity of the microsomal form.

The probable existence of several genes encoding P450s with E20MO activity was suggested (Feyereisen, 1999). This would account for the dual localization and complex regulation of the enzyme’s activity. On the other hand, alternative splicing or posttranslational modifications of a single gene product could also lead to the microsomal and mitochondrial forms. Interestingly, both microsomal and mitochondrial activities of the *Spodoptera littoralis* fat body E20MO were reported to be

reversibly activated by phosphorylation (Hoggard and Rees, 1988; Hoggard *et al.*, 1989). Cases of posttranslational modifications of P450 enzymes by reversible phosphorylation are not very common (Jansson, 1993; Oesch-Bartlomowicz and Oesch, 2003), and this observation would suggest that the *S. littoralis* fat body E20MOs are products of the same gene. The ecdysteroid 26-hydroxylases of *M. sexta* midgut and mitochondria are also both regulated by phosphorylation (Williams *et al.*, 2000b).

Petryk *et al.* (2003) identified *Drosophila* CYP314A1 as the product of the *shade* (*shd*) gene, a member of the Halloween group of developmental mutants. Expression of CYP314A1 in *Drosophila* S2 cells enabled the NADPH-dependent hydroxylation of ecdysone to 20-hydroxyecdysone by cell homogenates. RNA *in situ* hybridization shows that the *shd* gene is not expressed in the ring glands, but expression is seen in the gut, fat body, and Malpighian tubules. In embryos, *shd* is expressed primarily in epidermal cells by the time of germ band extension. Embryonic lethality of *shd* mutants indicates that *Cyp314a1* encodes the only significant E20MO activity at that stage in *Drosophila*. A CYP314A1 protein modified at the C-terminus by the addition of three copies of the hemagglutinin epitope was targeted to mitochondria of S2 cells (Petryk *et al.*, 2003). This study represents a breakthrough in our understanding of E20MO molecular genetics. *Cyp314a1* has a single clear ortholog in *A. gambiae*, but the sequence of both predicted proteins is unusual. They are clearly members of the mitochondrial P450 clade and have several intron positions in common with other mitochondrial P450 genes (Ranson *et al.*, 2002a). However, they lack two of the three positively charged residues thought to confer high affinity to adrenodoxin (Pikuleva *et al.*, 1999). Their exact N-terminal sequence is also somewhat unclear in the absence of EST sequences or proteomic data in either species to confirm the annotation prediction of the N-terminus.

Regulation of E20MO activity, developmental changes, induction, and inhibition is discussed by Lafont *et al.*

13.4.1.1.2. Ecdysone biosynthesis: biochemistry

The hydroxylation of 2-deoxyecdysone to ecdysone in *Locusta migratoria* was observed in the prothoracic glands as expected but in the Malpighian tubules, midgut, fat body, and epidermal tissues as well (Kappler *et al.*, 1986). The C-2 hydroxylation was characterized as a mitochondrial P450 activity in larval Malpighian tubules and in ovarian follicle cells of vitellogenic females (Kappler *et al.*, 1986) as

well as in the prothoracic glands (Kappler *et al.*, 1988). This P450 activity is peculiar in its very low sensitivity to CO inhibition, but virtually stoichiometric incorporation of one atom of molecular oxygen was demonstrated (Kabbouh *et al.*, 1987). The biochemical characterization of this P450 activity is supportive of the idea that the same gene product is responsible for C-2 hydroxylation in the Malpighian tubules and in the prothoracic glands. Two further hydroxylase activities have been characterized in *L. migratoria* prothoracic glands as typical P450 enzymes (Kappler *et al.*, 1988). With 2,22, 25-trideoxyecdysone as substrate for C-25 hydroxylation and 2,22-dideoxyecdysone as substrate for C-22 hydroxylation, these two activities were traced to the microsomal and mitochondrial fractions, respectively (Kappler *et al.*, 1988). The specificity of the three enzymes is suggested by their low K_m (0.5–2.5 μM) but significant competitive inhibition of the C-2- and C-22-hydroxylations by several ecdysteroids indicates that their place and substrate(s) in a grid or in a linear pathway is still conjectural, as noted by Rees (1995). Evidence towards the P450 nature of the C-25, C-22, and C-2 hydroxylases and their subcellular localization in *M. sexta* prothoracic glands has been presented (Grieneisen *et al.*, 1993). That study also suggested that the 7,8-dehydrogenation of cholesterol was a microsomal, NADPH-dependent enzyme. Inhibition of the reaction in “mildly disrupted” prothoracic glands by fenarimol ($\text{EC}_{50} = 0.1 \text{ mM}$) and carbon monoxide (63% inhibition at $\text{CO}:\text{O}_2$ of 19:1) was taken as evidence for P450 involvement. There is to date no convincing evidence for the involvement of P450 enzymes in the biosynthetic steps that occur in Dennis Horn’s famed “black box” between the 7,8-dehydrogenation and the ultimate hydroxylations.

Furthermore, there is equally little biochemical information on the enzymes responsible for the dealkylation of phytosterols to cholesterol. The reactions involved (desaturation, epoxidation, C–C bond cleavage) have been elegantly described in terms of their chemistry (Ikekawa *et al.*, 1993). They are well within the catalytic competence of P450 enzymes, and whether these dealkylation reactions are catalyzed by P450 enzymes or not, this remains a challenging area of research in view of their central role in the nutritional physiology of phytophagous species.

13.4.1.1.3. Ecdysone biosynthesis: molecular genetics

The study of *Drosophila* Halloween mutants that identified CYP314A1 as an E20MO also identified the C-2 and the C-22 hydroxylase, and may identify further P450 enzymes of the

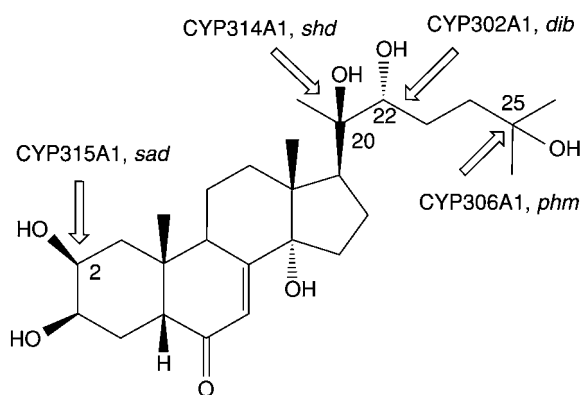


Figure 18 The insect molting hormone 20-hydroxyecdysone with the sites of P450 hydroxylation of its precursors identified to date in *Drosophila melanogaster*. P450 and gene names are shown.

ecdysteroid pathway (Figure 18). The *disembodied* (*dib*) gene encodes CYP302A1 identified as the C22 hydroxylase (Chavez *et al.*, 2000; Warren *et al.*, 2002). Similarly, the *shadow* (*sad*) gene encodes CYP315A1, the C2 hydroxylase (Warren *et al.*, 2002). The *dib* gene was identified by classical molecular genetics approaches starting from the genetic locus, ultimately leading to a mitochondrial P450 sequence (Chavez *et al.*, 2000). The identification of the *sad* gene (as that of *shd*, see above) exploited the match between genetic map and the sequence from the *Drosophila* genome project (Warren *et al.*, 2002). In all three cases, mutations leading to stop codons in the sequence confirmed the identification of the gene. Both *dib* and *sad* genes are expressed in embryos, as well as in the larval ring gland and follicle cells of adult ovaries. Transient expression in S2 cells coupled with the use of radiolabeled substrates, 2-deoxyecdysone and 2,22-dideoxyecdysone, was used to identify the reaction catalyzed (Warren *et al.*, 2002). Additional mutants such as *phantom* (*phm*) and *spook* (*spo*) that have a similar phenotype are thought to encode additional P450s involved in ecdysone biosynthesis, CYP306A1, a 25-hydroxylase, and CYP307A1, respectively (J.T. Warren *et al.*, personal communication). The *without children* (*woc*) mutant has identified a transcription factor that controls the 7,8-dehydrogenation of cholesterol or 25-hydroxycholesterol (Warren *et al.*, 2001) and may help identify the enzyme involved in this early step of ecdysone biosynthesis. Little is known of P450 expression in ecdysteroidogenic tissues of other insects. Interestingly, an RT-PCR fragment of a CYP4G-like transcript (GenBank AY635178) was isolated from *Manduca sexta* prothoracic glands. Northern hybridization shows high expression of a 2.3-kb message between days 2 and 5

of the last larval instar (before and during the first peak of ecdysteroid synthesis), with no detectable expression in fat body, midgut, nerve cord, or ovary (M.J. Snyder, V.M. Guzov, J.L. Stevens, and R. Feyereisen, unpublished data). The function of this member of the CYP4 family in prothoracic glands is unknown, but may be related to that of crayfish CYP4C15 (Aragon *et al.*, 2002) or *Drosophila* CYP4G15 (Maibeche-Coisne *et al.*, 2000).

13.4.1.1.4. Ecdysteroid catabolism The C-26 hydroxylation of 20-hydroxyecdysone has been characterized as a typical microsomal P450 activity in an epithelial cell line of *Chironomus tentans* (Kayser *et al.*, 1997). However, a dual localization (microsomal and mitochondrial) was reported for ecdysteroid 26-hydroxylase in *M. sexta* midgut (Williams *et al.*, 1997, 2000b). The reaction has a low K_m for 20-hydroxyecdysone ($\sim 1 \mu\text{M}$). The *C. tentans* cell line metabolizes 20, 26-dihydroxyecdysone further to two less polar metabolites produced in a constant 3:1 ratio. This metabolism is NADPH-dependent and inhibited byazole compounds. The two metabolites are diastereomers of a cyclic hemiacetal formed (nonenzymatically) by the reaction of the C-22 hydroxyl group with a C-26 aldehyde (Kayser *et al.*, 2002). It is not clearly established whether the P450 conversion of the C-26 hydroxyl to the C-26 aldehyde is carried out by the same enzyme that initially hydroxylates 20-hydroxyecdysone. The C-26 aldehyde is a presumed intermediate in the conversion of C-26 ecdysteroids to C-26 ecdysonic acids, a common inactivation product of ecdysteroids, but phosphate conjugation of the C-26 hydroxyl is observed in some insects (Rees, 1995). P450-mediated hydroxylations followed by oxidations to the carboxylic acid are known, e.g. CYP701A3 of *A. thaliana* converting *ent*-kaurene to *ent*-kaurenoic acid or CYP27 in the biosynthesis of bile acids.

Side-chain cleavage of ecdysteroids has been reported as an inactivation route, but despite the analogy to the reaction catalyzed by vertebrate P450_{scc} (CYP11A), i.e., the C–C bond cleavage at a vicinal C-20,C-22 diol, there is currently no information on the enzymology of this reaction. P450_{scc} is a unique enzyme in that it catalyzes not just the C–C bond cleavage, but also the two preceding hydroxylations of cholesterol at C-20 and C-22 (in that order).

13.4.1.2. P450 and juvenile hormone metabolism (see also Chapter 8) The landmark chemical feature of juvenile hormones (JHs) is the presence of the epoxide group, and by 1985 it was already well

established that epoxidation of the JH precursors in the corpora allata (CA) was catalyzed by a P450 enzyme. The biochemical evidence was presented in two studies on the allatal enzyme, one with *Blaberus giganteus* CA homogenates (Hammock, 1975) and the other with *L. migratoria* CA (Feyereisen *et al.*, 1981) that established its microsomal nature. A number of compounds that took advantage of the P450 nature of the epoxidase have been tested as inhibitors of juvenile hormone biosynthesis (Hammock and Mumby, 1978; Brooks *et al.*, 1985; Pratt *et al.*, 1990; Unnithan *et al.*, 1995). The allatal epoxidase was viewed as an attractive new target for “biorational” insecticides, because it is an insect-specific target, and because its inhibition should lead to desirable effects – precocious metamorphosis and adult sterility akin to the effects of precocenes (Bowers *et al.*, 1976). A serious pursuit of this goal would only be possible with a molecular characterization of the epoxidase and its heterologous expression that would permit screening at a scale not possible by the laborious dissection of the glands. Photoaffinity labeling was first developed as a technique that could facilitate purification and subsequent cloning of the epoxidase. Bifunctional compounds with a substituted imidazole to coordinate the heme iron and a diazirine or a benzophenone group to label the substrate binding site were synthesized and tested (Andersen *et al.*, 1995). These compounds were potent inhibitors of methyl farnesoate epoxidation to JH III in the CA of *D. punctata*, and selectively labeled a 55 kDa protein.

13.4.1.2.1. CYP15A1 – the epoxidase However, eventual cloning of the epoxidase was achieved by a less subtle route, the sequencing of ESTs from *D. punctata* CA. Sequencing of >900 ESTs from the CA of vitellogenic females yielded three ESTs matching a P450 sequence by BLAST analysis. A full-length cDNA of this P450 was expressed in *E. coli* (Helvig *et al.*, 2004). This P450 termed CYP15A1, has a high affinity for methyl farnesoate, showing a type I spectrum with a K_s of 6 μ M. The enzyme, when reconstituted with fly P450 reductase, catalyzed the NADPH-dependent epoxidation of 2*E*, 6*E*-methyl farnesoate to JH III. The epoxidation is highly stereoselective (98:2) to the natural 10*R* enantiomer over its diastereomer. The enzyme also has a high substrate specificity, epoxidizing the 2*E*, 6*E* isomer preferentially over the 2*Z*, 6*E* isomer, and accepting no other substrate tested including farnesoic acid. CYP15A1 is expressed selectively in the CA, and the rank order of inhibition of CYP15A1 activity by substituted imidazoles is

identical to that of JH biosynthesis by isolated CA (Helvig *et al.*, 2004).

13.4.1.2.2. CYP4C7 – ω -hydroxylase Expression of the CYP15A1 gene is high when JH synthetic levels are high, but another P450 gene, CYP4C7, also selectively expressed in the CA of *D. punctata* (Sutherland *et al.*, 1998), has an expression pattern that mirrors the pattern of JH synthesis. The recombinant CYP4C7 enzyme produced in *E. coli* metabolized a variety of sesquiterpenoids but not mono- or diterpenes. In addition to metabolizing JH precursors, farnesol, farnesal, farnesoic acid, and methyl farnesoate, it also metabolized JH III to a major metabolite identified as (10*E*)-12-hydroxy-JH III (Sutherland *et al.*, 1998). Although this ω -hydroxylated JH III has not been identified as a product of the CA, *L. migratoria* CA are known to produce several hydroxylated JHs in the radiochemical assay *in vitro* (Darrouzet *et al.*, 1997; Mauchamp *et al.*, 1999). These hydroxy-JHs, 8'-OH-, 12'-OH (Darrouzet *et al.*, 1997), and 4'-OH-JH III (Mauchamp *et al.*, 1999) may be major products of the CA after JH III itself and their role is unknown. Their presence suggests that locust CA have a P450 homologous to cockroach CYP4C7 that has a lower regioselectivity. Indeed, the hydroxy-JHs can be synthesized by locust CA from JH III, and this hydroxylation is inhibited by CO and piperonyl butoxide (Couillaud *et al.*, 1996).

The tight physiological regulation of CYP4C7 expression in adult female *D. punctata* (Sutherland *et al.*, 2000) indicates that the terpenoid ω -hydroxylase has an important function to play at the end of vitellogenesis and at the time of impending chorionation and ovulation. It was hypothesized (Sutherland *et al.*, 1998, 2000) that this hydroxylation was a first step in the inactivation of the very large amounts of JH and JH precursors present in the CA of this species after the peak of JH synthesis.

The study of JH metabolism has long been dominated by esterases and epoxide hydrolases (see **Chapter 8**), but early work on insecticide-resistant strains of the housefly revealed oxidative metabolism as well (review: Hammock, 1985). Evidence that housefly CYP6A1 efficiently metabolizes sesquiterpenoids, including JH to its 6,7-epoxide, confirms these early studies (Andersen *et al.*, 1997). Hydroxylation and epoxidation of JHs are thus confirmed P450-mediated metabolic pathways for these hormones.

A comparison of four P450 enzymes that metabolize methyl farnesoate (**Table 3**) shows their

Table 3 Specificity of insect P450 enzymes towards methyl farnesoate (MF)

P450	Substrate	Reaction	Product formed	Reference
CYP15A1	2E, 6E-MF	Epoxidation	10R-epoxide	Helvig <i>et al.</i> (2004)
CYP4C7	Sesquiterpenoids	ω -Hydroxylation	12E-OH	Sutherland <i>et al.</i> (1998)
CYP9E1	2Z > 2E-MF (1.5:1)	Epoxidation	10-Epoxide 10R:10S (1:1)	^a
CYP6A1	MF or JH all 4 isomers	Epoxidation	6 or 10-epoxide 10S:10R (3:1)	Andersen <i>et al.</i> (1997)

^aJ.F. Andersen, G.C. Unnithan, J.F. Koener, and R. Feyereisen, unpublished data. Data from Helvig, C., Koener, J.F., Unnithan, G.C., Feyereisen, R., 2004. CYP15A1, the cytochrome P450 that catalyzes epoxidation of methyl farnesoate to juvenile hormone III in cockroach *corpura allata*. *Proc. Natl Acad. Sci. USA* 101, 4024–4029; © National Academy of Sciences, USA.

catalytic versatility. One is extremely substrate specific (CYP15A1), another is not (CYP6A1). Three of them are epoxidases, one is a hydroxylase on this substrate (CYP6A1 also has activity as a hydroxylase – but not on this substrate). One is a stereoselective epoxidase (CYP15A1), another lacks product enantioselectivity (CYP9E1).

13.4.1.3. Biosynthesis of long-chain hydrocarbons

The cuticle of insects can be characterized by its hydrocarbon composition, and this can serve as a subtle tool in chemical taxonomy (Lockey, 1991). In some insects, cuticular or exocrine alkanes and alkenes can serve as allomones, or even pheromones. To cite but one example, the Dufour gland secretions of the leaf-cutting ant *Atta laevigata* are deposited on foraging trails. This trail pheromone comprises n-heptadecane, (Z)-9-nonadecene, 8,11-nonadecadiene, and (Z)-9-tricosene (Salzemann *et al.*, 1992). Despite their apparently simple structure, the biosynthesis of these hydrocarbons is complex and the enzymes involved are still under intense study. Schematically (Figure 19), long chain (18–28 carbons) fatty acid CoA esters are first reduced to their aldehyde by an acyl-CoA reductase, and they are subsequently shortened to form C_{n-1} hydrocarbons (Reed *et al.*, 1994). The conversion of (Z)-tetracosenoyl-CoA (from a C24:1 fatty acid) to (Z)-9-tricosene (a C23:1 alkene) has been characterized in housefly epidermal microsomes. The role of the aldehyde tetracosenal as an intermediate is evidenced by trapping experiments with hydroxylamine. The next step is NADPH- and O₂-dependent, and is truly an oxidative decarbonylation reaction, which releases the terminal carbon as CO₂ and not as CO (Reed *et al.*, 1994). Inhibition by carbon monoxide and anti-P450 reductase antibodies are strongly indicative of a P450 reaction, and this most peculiar enzyme was called P450hyd (Reed *et al.*, 1994). The reaction does not involve a terminal desaturation, because both C-2 protons of the

aldehyde are retained in the hydrocarbon product, and the aldehydic proton is transferred to the product (Reed *et al.*, 1995). NADPH and molecular oxygen can be replaced by “peroxide shunt” donors, suggesting that this reaction involves a perferryl iron-oxene species of activated oxygen (Ortiz de Montellano, 1995a), and that it proceeds through an alkyl radical intermediate (Reed *et al.*, 1995). This type of oxidative decarbonylation reaction may be widespread in insects because a mechanistically identical conversion of octadecanal to heptadecane has been documented in flies, cockroaches, crickets, and termites (Mpuru *et al.*, 1996).

13.4.1.4. Pheromone metabolism

In female houseflies, the (Z)-9-tricosene produced by P450hyd is a major component of the sex pheromone, being responsible for inducing the courtship ritual and the males' striking activity. Additional components of the pheromone are the sex recognition factors (Z)-9,10-epoxytricosane and (Z)-14-tricosen-10-one. These compounds are obviously derived from (Z)-9-tricosene by oxidative metabolism (Blomquist *et al.*, 1984). A microsomal P450 was shown to oxidize the alkene from either side at a distance of 9/10 carbons in-chain (Ahmad *et al.*, 1987), but the structural requirements of the enzyme for its substrate are otherwise strict (Latli and Prestwich, 1991). This P450 activity is found in various tissues of the male and female, including the epidermis and fat body, but the highest specific activity is found in male antennae (Ahmad *et al.*, 1987). The C23 epoxide and ketone are absent internally in females, but accumulate on the surface of the cuticle (Mpuru *et al.*, 2001), suggesting the localization of this P450 activity in epidermal cells of female flies.

More generally, P450 enzymes are probably involved in the biosynthesis of many insect pheromones and allomones, e.g., epoxides of polyunsaturated hydrocarbons in arctiid moths, disparlure of the gypsy moth (Brattsten, 1979a), or monoterpenes

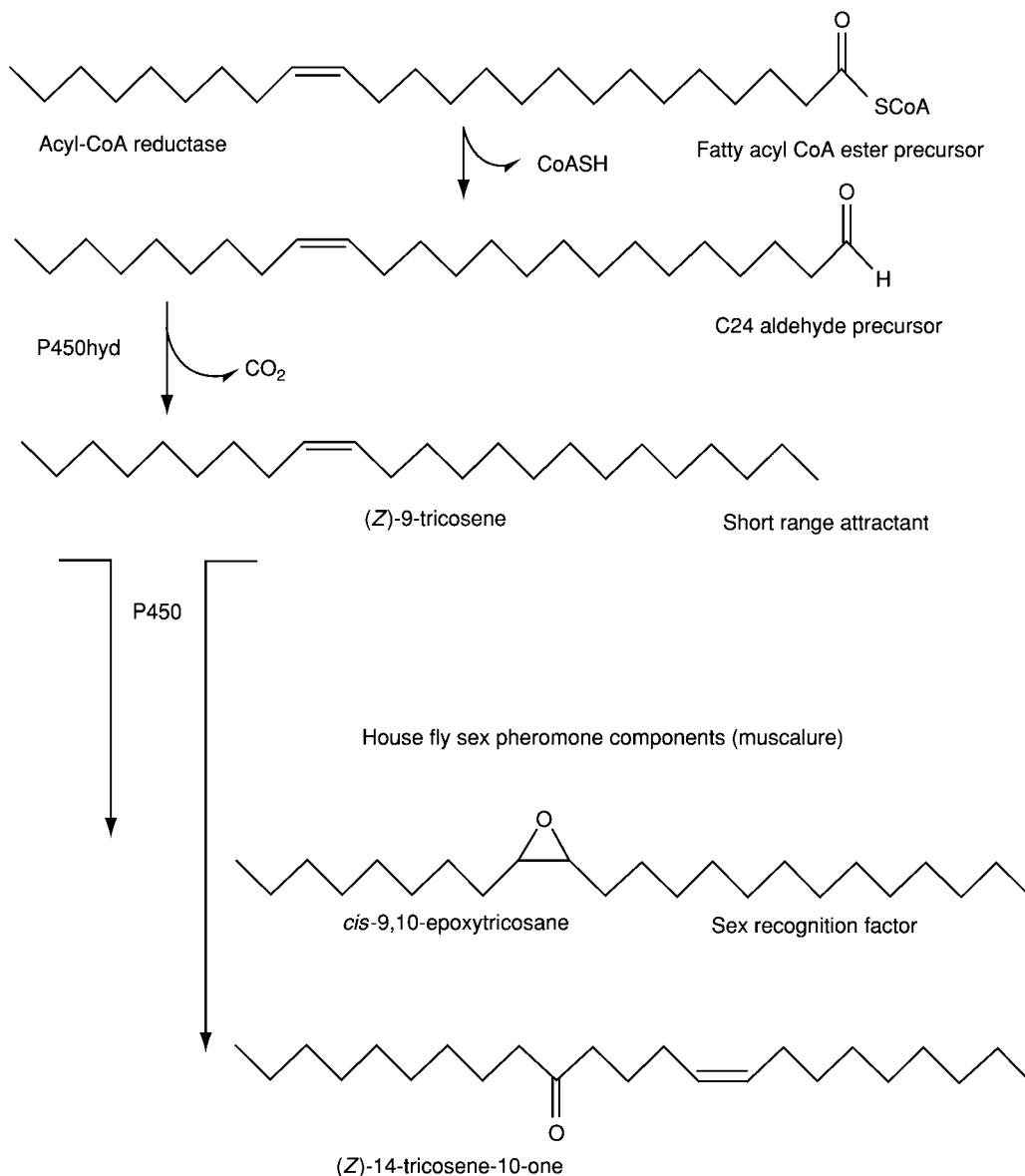


Figure 19 Biosynthesis of (Z)-9-tricosene and components of the housefly sex pheromone. P450hyd acts as a decarbonylase to produce (Z)-9-tricosene from an aldehyde precursor. Another P450 attacks (Z)-9-tricosene from either side to give the two components of muscalure.

in bark beetles (Brattsten, 1979a; White *et al.*, 1979; Hunt and Smirle, 1988). In the honeybee, caste-specific ω and $\omega-1$ hydroxylations of fatty acids to mandibular pheromones have all the characteristics of P450 reactions (Plettner *et al.*, 1998). These P450s involved in pheromone biosynthesis are likely to be exquisitely specific.

Evidence for pheromone catabolism by P450 enzymes is also accumulating. As shown for the metabolism of (Z)-9-tricosene in the housefly, a distinction between biosynthesis and catabolism can be purely semantic in the case of a biogenetic

succession of chemicals that have different signaling functions. Compound B that is a metabolite of compound A may have less or none of compound A's activity, but may have its own specific biological activity. Nonetheless, pheromones as signal molecules need to be metabolically inactivated and this catabolism may occur in the antennae themselves. Several P450 mRNAs have been identified in insect antennae. In *Drosophila*, a partial P450 cDNA, along with a UDP-glycosyltransferase and a short chain dehydrogenase/reductase, were found by northern blot analysis to be preferentially expressed

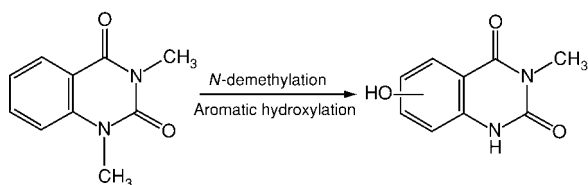


Figure 20 P450-dependent *N*-demethylation and ring hydroxylation of the *Phyllopertha diversa* sex pheromone by antennal microsomes of male scarab beetles. See text and Wojtasek and Leal (1999) for details.

in the third antennal segments, with lower expression in legs (Wang *et al.*, 1999). This P450 cDNA corresponds to *Cyp6w1* for which many ESTs have been identified in a head cDNA library. In *Mamestra brassicae*, two P450 cDNAs were cloned from antennae, *CYP4L4* and *CYP4S4* (Maibeche-Coisne *et al.*, 2002). Both genes are strongly expressed in the sensilla trichodea as shown by *in situ* hybridization. Whereas *CYP4S4* expression is restricted to the antennae, *CYP4L4* is also expressed in proboscis and legs. Five P450 ESTs representing three P450 genes were found in a small EST project using male *Manduca sexta* antennae (Robertson *et al.*, 1999), which is further evidence that antennae may harbor several P450 enzymes. High levels of P450 reductase expression have been noted in *Drosophila* antennae (Hovemann *et al.*, 1997), and adrenodoxin reductase is also expressed in antennae (Freeman *et al.*, 1999). Antennal P450s are therefore an active enzyme system, complete with their redox partners. The physiological role of the P450s thus found in antennae is still formally unknown, but the presence of other enzymes generally associated with detoxification processes (Robertson *et al.*, 1999; Wang *et al.*, 1999) are indeed suggestive of a large category of odorant-metabolizing enzymes. In addition to the presence of P450 transcripts in antennae, there is at least one report where a P450 reaction has been characterized in antennae, but the CYP gene coding for the P450 catalyzing this reaction is still unknown. Antennal microsomes of the pale brown chafer, *Phyllopertha diversa*, metabolize the alkaloid sex pheromone by a P450 enzyme (Wojtasek and Leal, 1999). This enzyme is specifically produced in male antennae, and its activity is not detected in other scarab beetles or lepidopteran species (Figure 20). P450 enzymes may have found in insects a role as odorant degrading enzymes (ODE) along with esterases, aldehyde oxidases, glucosyl-transferases, etc.

13.4.1.5. Metabolism of fatty acids and related compounds P450-catalyzed fatty acid ω -hydroxylation has been reported in insects (Feyereisen and

Durst, 1980; Ronis *et al.*, 1988; Clarke *et al.*, 1989; Cuany *et al.*, 1990; Rose *et al.*, 1991). Clofibrate selectively induces ω -hydroxylation (and not ω -1) of lauric acid by housefly and *Drosophila* microsomes, just as it induces the rat CYP4A1 ω -hydroxylase (Clarke *et al.*, 1989; Amichot *et al.*, 1998). No clear peroxisome proliferator activated receptor (PPAR, Issemann and Green, 1990) gene ortholog but several paralogs are present in the *Drosophila* genome. In contrast to clofibrate, phenobarbital induces *Drosophila* ω , ω -1, and ω -2 hydroxylations (Amichot *et al.*, 1998), indicating the presence of several fatty acid hydroxylases. By homology to vertebrate CYP4 enzymes it is thought that cockroach CYP4C1 has a ω -hydroxylase function as well, but evidence is lacking. CYP4C7, CYP6A1, and CYP12A1 lack lauric acid hydroxylase activity (Andersen *et al.*, 1997; Guzov *et al.*, 1998; Sutherland *et al.*, 1998), but CYP6A8 has laurate ω -1 hydroxylase activity (C. Helvig, personal communication). Although the role of vertebrate P450 enzymes in the metabolism of arachidonic acid and eicosanoids is well established (Capdevila *et al.*, 2002), there is currently no indication for a similar function of P450 in insects.

The biosynthesis of volicitin, an elicitor of plant volatile production found in the oral secretions of caterpillars occurs in the insect from the plant-derived fatty acid linolenic acid (Pare *et al.*, 1998). This biosynthesis involves C-17 hydroxylation and glutamine conjugation. It is likely that this hydroxylation will be shown to be catalyzed by a P450 enzyme.

13.4.1.6. Defensive compounds The tremendous variety of chemicals used for defense of insects against predation is well documented, but the enzymes involved in their *de novo* synthesis or in their transformation from ingested precursors are less studied. It is very likely that P450 enzymes may have found there a fertile ground for their chemical prowess. Just two examples are described.

The biosynthesis of cyanogenic compounds found as defensive compounds in many insect species (Nahrstedt, 1988) may well involve P450 enzymes. For instance, the cyanogenic glucosides linamarin and lotaustralin found in *Heliconius* butterflies and *Zygaena* moths, are clearly derived from the amino acids valine and isoleucine. The pathway probably involves N-hydroxylation of the amino acids, further metabolism to the aldoximes and nitriles, and final C-hydroxylation before conjugation to the glucoside (Figure 21). The aldoximes and nitriles are efficiently incorporated *in vivo* (Davis and Nahrstedt, 1987; Holzkamp and Nahrstedt, 1994).

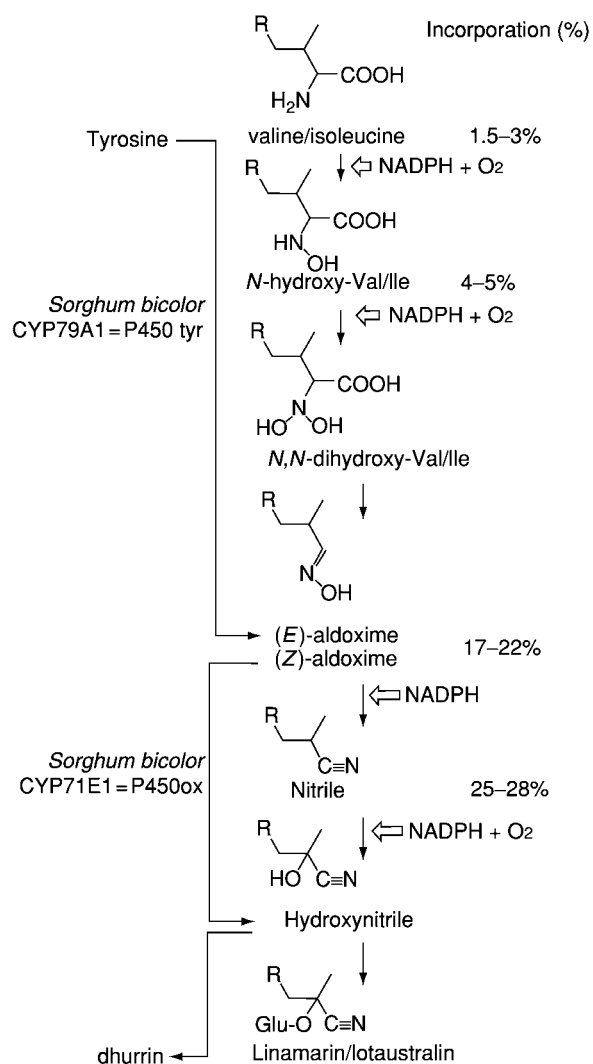


Figure 21 Biosynthesis of linamarin and lotaustralin from valine (R=H) and isoleucine (R=CH₃), indicating possible sites of P450 metabolism. The efficiency of incorporation of intermediates *in vivo* is shown on the right. The homologous reactions catalyzed by two multifunctional *Sorghum* P450 enzymes converting tyrosine to dhurrin are shown on the left.

The pathway resembles that found in plants, where two multifunctional P450 enzymes are sufficient to convert the amino acid to the hydroxynitrile substrate of the conjugating enzyme (Figure 21). For dhurrin biosynthesis from tyrosine in sorghum, these are CYP79A1 and CYP71E1 (Kahn *et al.*, 1997). Whereas the plant pathway appears to “channel” the substrates through the two P450s with little escape of the aldoxime intermediate, nothing is known of the number and functioning of the cyanogenic pathway enzymes used by Lepidoptera. Characterization of the P450 enzymes involved should allow a comparison of the plant and insect solutions to this biosynthetic challenge.

Another comparison of interest will be that of enzymes involved in the biosynthesis of insect defensive steroids with the well characterized steroid metabolizing enzymes of vertebrates. A number of aquatic Coleoptera (Dysticidae) and Hemiptera (Belastomatidae) synthesize a variety of steroids, mostly pregnanes (Scrimshaw and Kerfoot, 1987). With cholesterol as the presumed precursor in these carnivorous insects, one may envisage the evolution of an insect side-chain cleavage enzyme, of a C-21 hydroxylase and of a C17-C21 lyase that would catalyze reactions identical to those of CYP11A, CYP21, and CYP17. Some defensive steroids also have an aromatic A-ring, 7 α , or 15 α hydroxyl groups. A pregnene-3 β , 20 β -diol glucoside is synthesized from cholesterol in female pupae of *M. sexta* (Thompson *et al.*, 1985). The role of this compound is unknown, but its synthesis strongly suggests the existence of a C20-C22 side chain cleavage enzyme in Lepidoptera as well.

13.4.2. Xenobiotic Metabolism: Activation and Inactivation

13.4.2.1. Natural products The metabolism of plant toxins by insects has been reviewed extensively (see, e.g., Brattsten, 1979b; Dowd *et al.*, 1983; Ahmad, 1986; Ahmad *et al.*, 1986; Mullin, 1986; Yu, 1986). Relatively a few studies have directly assessed the role of P450 enzymes in the metabolism of natural compounds by more than one or two criteria such as microsomal localization, NADPH and O₂ dependence, and inhibition by piperonyl butoxide. In most cases, metabolism is associated with detoxification, e.g., the metabolism of xanthotoxin in *Papilio polyxenes*, *S. frugiperda*, and *Depressaria pastinacella* (Bull *et al.*, 1986; Nitao, 1990), the metabolism of α -terthienyl in larvae of three lepidopteran species (Iyengar *et al.*, 1990), and the metabolism of nicotine in *Manduca sexta* larvae (Snyder *et al.*, 1993). Alpha- and beta-thujones are detoxified by P450 as evidenced by synergism of their toxicity by three P450 inhibitors in *Drosophila* and by the lower toxicity of six of their metabolites (Hold *et al.*, 2001). Studies with flavone (Wheeler *et al.*, 1993), monoterpenes (Harwood *et al.*, 1990) and the alkaloid carnegine (Danielson *et al.*, 1995) show clearly however that evidence for metabolism by P450 *in vitro* may not be sufficient to define an *in vivo* toxicological outcome.

Natural products in the diet can act as inducers of P450 as well of other enzymes (e.g., glutathione S-transferases) and as a result of this induction (see below), the metabolism of the inducing compound or of coingested plant compounds can change dramatically over time (Brattsten *et al.*, 1977).

Differences between acute and chronic toxicity are thus often the result of altered expression patterns (quantitative and qualitative) of P450 genes. This was demonstrated, for instance, in studies on *Spodoptera* larvae (Brattsten, 1983; Gunderson *et al.*, 1986). The monoterpene pulegone and its metabolite menthofuran are more acutely toxic to *S. eridania* than to *S. frugiperda*, but the reverse is true for chronic toxicity.

A study by Yu (1987) compared the metabolism of a large number of plant chemicals of different chemical classes by *S. frugiperda* and *Anticarsia gemmatalis* (velvetbean caterpillar) microsomes. Two indirect methods were used, on the one hand, the NADPH-dependent decrease in substrate and on the other hand, the substrate-induced NADPH oxidation. This metabolism is inhibited by piperonyl butoxide and by carbon monoxide, and induced by a number of chemicals, particularly indole-3-carbinol, strongly suggesting P450 involvement. Such indirect methods are very useful as screening tools, as a first step towards a more thorough characterization of metabolism. However, they give no qualitative indication of the chemical fate of the substrate, nor quantitative indication of the levels of metabolism, as NADPH consumption is correlated to the coupling rate of the reaction, rather than to the rate of product formation (see Section 13.3.4.1). Clearly, insect P450 enzymes as a whole are capable of metabolizing a tremendous variety of naturally occurring chemicals, but the role of individual enzymes and their catalytic competence still needs a better description.

Heterologously expressed P450 enzymes of the CYP6B subfamily from *Papilio* species (see Section 13.4.3 and Table 1) are well characterized for their ability to metabolize furanocoumarins (Hung *et al.*, 1997; Li *et al.*, 2003; Wen *et al.*, 2003). They fit the description of enzymes with “broad and overlapping specificity” towards these compounds. Their range of catalytic competence is quite variable. For instance, CYP6B21 and CYP6B25 metabolize the angular furanocoumarin angelicin at a similar rate (0.4–0.5 nmol/min/nmol P450), but whereas CYP6B21 also metabolizes 7-ethoxycoumarin at a similar rate (0.5 nmol/min/nmol P450), CYP6B25 does not have appreciable 7-dealkylation activity (Li *et al.*, 2003).

Natural products are not just an endless catalog of P450 substrates and inducers, but they also comprise a varied and complex set of inhibitors of P450 enzymes. These inhibitors range from “classical” reversible inhibitors to substrates that are activated to chemically reactive, cytotoxic forms (e.g., Neal and Wu, 1994).

13.4.2.2. Insecticides and other xenobiotics The metabolism of insecticides by P450 enzymes is very often a key factor in determining toxicity to insects and to nontarget species, but it can also represent a key step in the chain of events between contact, penetration, and interaction at the target site. The classical example is probably the metabolism of phosphorothioate insecticides. In many cases, the active ingredients of organophosphorus insecticides are phosphorothioate (P=S) compounds (a.k.a. phosphorothionates), whereas the molecule active at the acetylcholinesterase target site is the corresponding phosphate (P=O). It has long been recognized that the P=S to P=O conversion is a P450-dependent reaction. In the case of diazinon, this desulfuration has been studied for three heterologously expressed insect P450 enzymes (Dunkov *et al.*, 1997; Guzov *et al.*, 1998; Sabourault *et al.*, 2001). All three P450s metabolized diazinon not just to diazoxon, the metabolite resulting from desulfuration, but also to a second metabolite resulting from oxidative ester cleavage. Similarly, antibodies to housefly CYP6D1 inhibit the microsomal desulfuration of chlorpyrifos as well as its oxidative ester cleavage (Hatano and Scott, 1993). The mechanism of P450-dependent desulfuration is believed to involve the initial attack of the P=S bond by an activated oxygen species of P450, leading to an unstable and therefore hypothetical phosphoxythiirane product (Figure 22). The collapse of this product can lead to two possible outcomes: (1) the replacement of sulfur by oxygen in the organophosphate product with the release of a reactive form of sulfur; and (2) the cleavage of the phosphate ester (or thioester) link with the substituent of highest electron-withdrawing properties, the “leaving group.”

Outcome (1) can be viewed as “activation” because the P=S to P=O desulfuration produces an inhibitor of acetylcholinesterase often several orders of magnitude more potent than the P=S parent compound. However, the fate of this product of “activation” depends on the histological proximity to the target, sequestration, excretion, and further metabolism of the phosphate (by oxidative or hydrolytic enzymes). Kinetic evidence with the heterologously expressed CYP6A1, CYP6A2, and CYP12A1, as well as immunological evidence with CYP6D1 indicate that these P450 enzymes do not metabolize the P=O product of the parent P=S compound they metabolize.

Outcome (2) or “dearylation” is without question a detoxification because the oxidative cleavage of the “leaving group” yields compounds unable to inhibit acetylcholinesterase, the dialkylphosphorothioate,

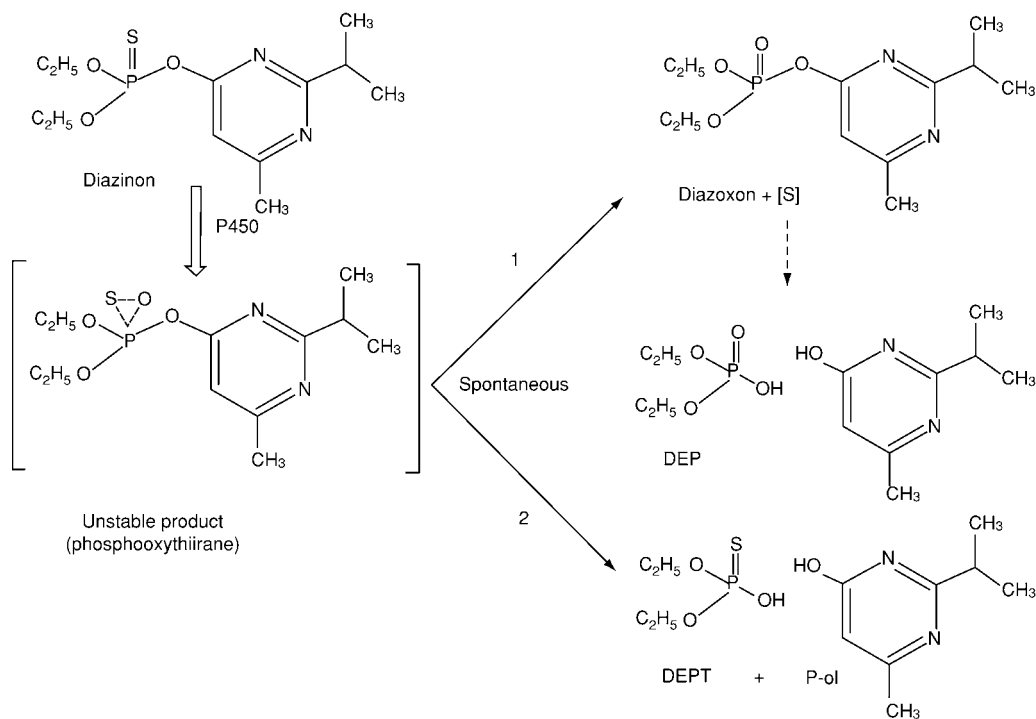


Figure 22 Metabolism of diazinon by cytochrome P450. Following an insertion of oxygen into the substrate, a reactive intermediate collapses (1) by desulfuration or (2) by cleavage of the ester linkage (dearylation). DEP: diethylphosphorothioate, P-ol: 2-isopropoxy-4-methyl-6-hydroxypyrimidine, [S]: reactive form of sulfur released during the reaction. Diazoxon can be further converted to DEP and P-ol by the same or another P450 in a subsequent reaction, or by a phosphotriester hydrolase. DEP may also be formed by spontaneous degradation of the initial product of diazinon monooxygenation. The ratio of outcomes 1 and 2 and the fate of the reactive sulfur depends on the P450 enzyme and on the type of OP substrate (see **Table 4**).

and/or dialkylphosphate. The ratio of outcome (1) and (2) appears P450-specific and substrate specific (**Table 4**) suggesting that the collapse of the unstable initial product of P450 attack is influenced by the active site environment. Theoretically, some P450 enzymes may very strongly favor outcome (1) or (2) or vice versa, thus qualifying as relatively “clean” activators or detoxifiers, but there is to date little direct evidence from the insect toxicological literature for such P450 enzymes (see however Oi *et al.*, 1990).

Furthermore, the sulfur released by the reaction can bind covalently either to neighboring proteins thus leading to cellular damage, or to the P450 protein itself (at least in vertebrate liver where this specific aspect has been studied, Kamataki and Neal, 1976). Parathion causes NADPH-dependent inhibition of methoxyresorufin *O*-demethylation activity (a P450Lpr-selective activity) in the housefly whereas chlorpyrifos does not (Scott *et al.*, 2000). Therefore, it is not just the fate of the initial P450 metabolite of the P=S compound that depends on the P450 and the OP, but it is also the fate of the sulfur released that can vary.

Changes in the level of expression of P450 genes or P450 point mutations may be sufficient to change this delicate balance between activation and inactivation *in vivo*. For instance, fenitrothion resistance in the Akita-f strain of the housefly is related to an increase in oxidative ester cleavage over desulfuration measured in abdominal microsomes (Ugaki *et al.*, 1985). In *H. virescens*, methyl parathion resistance in the NC-86 strain, which has an unchanged level of total P450, is related to a replacement of a set of P450 enzymes with high desulfuration activity by a set of P450 enzymes that metabolize less parathion, and do so with a lower desulfuration/oxidative ester cleavage ratio (Konno and Dauterman, 1989) (see **Table 4**).

P450 enzymes that metabolize OPs can metabolize other insecticides as well and this sometimes leads to potentially useful interactions. Thus, enhanced detoxification of dicofol in spider mites can lead to enhanced chlorpyrifos activation, hence negative cross-resistance (Hatano *et al.*, 1992). Similarly, permethrin resistance in horn flies is suppressible by piperonyl butoxide and negatively related to diazinon toxicity (Cilek *et al.*, 1995). In *H. armigera*

Table 4 Desulfuration and oxidative ester cleavage of organophosphorus insecticides by P450 enzymes and microsomes

P450 enzyme	Ratio of OP desulfuration/oxidative ester cleavage	Reference
Human CYP2C19	8.5 d, 1.30 p, 0.14 c	Kappers <i>et al.</i> (2001), Tang <i>et al.</i> (2001), Mutch <i>et al.</i> (2003)
Human CYP3A4	3.0 d, 0.50 p, 0.66 c	Kappers <i>et al.</i> (2001), Mutch <i>et al.</i> (2003)
Human CYP2B6	0.7 d, 0.01 p, 3.38 c	Kappers <i>et al.</i> (2001), Mutch <i>et al.</i> (2003)
Human liver microsomes	0.29 d, 0.37 p, 0.57 c	Kappers <i>et al.</i> (2001), Mutch <i>et al.</i> (2003)
Housefly CYP6A1	0.37 d	Sabourault <i>et al.</i> (2001)
<i>Drosophila</i> CYP6A2	0.92 d	Dunkov <i>et al.</i> (1997)
Housefly CYP12A1	0.69 d	Guzov <i>et al.</i> (1998)
Housefly CYP6D1	2.0 c	Hatano and Scott (1993)
Housefly <i>CSMA</i> microsomes	0.95 f	Ugaki <i>et al.</i> (1985)
Housefly <i>Akita-f^a</i> microsomes	0.59 f	Ugaki <i>et al.</i> (1985)
<i>Heliothis virescens</i> microsomes	1.90 mp	Konno and Dauterman (1989)
<i>Heliothis virescens</i> NC-86 ^a microsomes	1.32 mp	Konno and Dauterman (1989)

^aResistant strain.

d, diazinon; p, ethyl parathion; c, chlorpyrifos; f, fenitrothion; mp, methyl parathion.

populations from West Africa, triazophos shows negative cross-resistance with pyrethroids, and in this case the synergism shown by the OP towards the pyrethroids appears to be due to an enhanced activation to the oxon form (Martin *et al.*, 2003). These interactions were observed *in vivo* or with microsomes, but it is likely that they do reflect the properties of single P450 enzymes with broad substrate specificity rather than the fortuitous coordinate regulation of different P450 enzymes with distinct specificities.

Organophosphorus compounds such as disulfoton and fenthion can also be activated by thioether oxidation (formation of sulfoxide and sulfone), but it is not clear whether these reactions are catalyzed in insects by a P450 or by a flavin monooxygenase (FMO). Further examples of oxidative bioactivation of organophosphorus compounds have been discussed (Drabek and Neumann, 1985).

The toxicity of fipronil to house flies is increased sixfold by the synergist piperonyl butoxide, whereas the desulfinyl photodegradation product is not detoxified substantially by P450 (Hainzl and Casida, 1996; Hainzl *et al.*, 1998). Conversion of fipronil to its sulfone appears to be catalyzed by a P450 enzyme in *Ostrinia nubilalis* (Durham *et al.*, 2002) and in *Diabrotica virgifera* (Scharf *et al.*, 2000). In the latter, the toxicity of fipronil sulfone is about the same as that of the parent compound, and piperonyl butoxide has only a marginal effect as synergist. In contrast, synergists antagonize the toxicity of fipronil in *Blattella germanica*, suggesting that oxidation to the sulfone represents an activation step in this species (Valles *et al.*, 1997).

The now banned cyclodiene insecticides aldrin, heptachlor, and isodrin are epoxidized by P450

enzymes to environmentally stable, toxic epoxides, dieldrin, heptachlor epoxide, and endrin (Brooks, 1979; Drabek and Neumann, 1985). Recombinant CYP6A1, CYP6A2, and CYP12A1 can catalyze these epoxidations (see Table 1). Examples of proinsecticide metabolism include the activation of chlorfenapyr by *N*-dealkylation (Black *et al.*, 1994) and of diafenthiuron by *S*-oxidation (Kayser and Eilinger, 2001). In each case, the insect P450-dependent activation is a key in the selective toxicity of these proinsecticides that target mitochondrial respiration. Recombinant housefly CYP6A1 catalyzes the activation of chlorfenapyr (V.M. Guzov, M. Kao, B.C. Black, and R. Feyereisen, unpublished data). In *H. virescens*, toxicity of chlorfenapyr is negatively correlated with cypermethrin toxicity (Pimprale *et al.*, 1997). Genetic analysis indicates that a single factor is involved so the same P450 that activates chlorfenapyr may also detoxify cypermethrin in this species (Figure 23). A similar case of negative cross-resistance of chlorfenapyr in a pyrethroid-resistant strain has been reported in the hornfly *Haematobia irritans* (Sheppard and Joyce, 1998).

The metabolism of imidacloprid is also of interest in this respect. Although not extensively studied to date, there is evidence that piperonyl butoxide can synergize the toxicity of imidacloprid, but P450-dependent metabolism can also lead to several bioactive metabolites in some insects. How these are further metabolized and how resistance can be caused by P450 attack on this molecule remains unclear (see however Section 13.4.5.5).

In vivo synergism by piperonyl butoxide, a typical inhibitor of P450 enzymes (see Section 13.4.5.1), is often used to implicate a P450-mediated detoxification, and there are innumerable such examples in

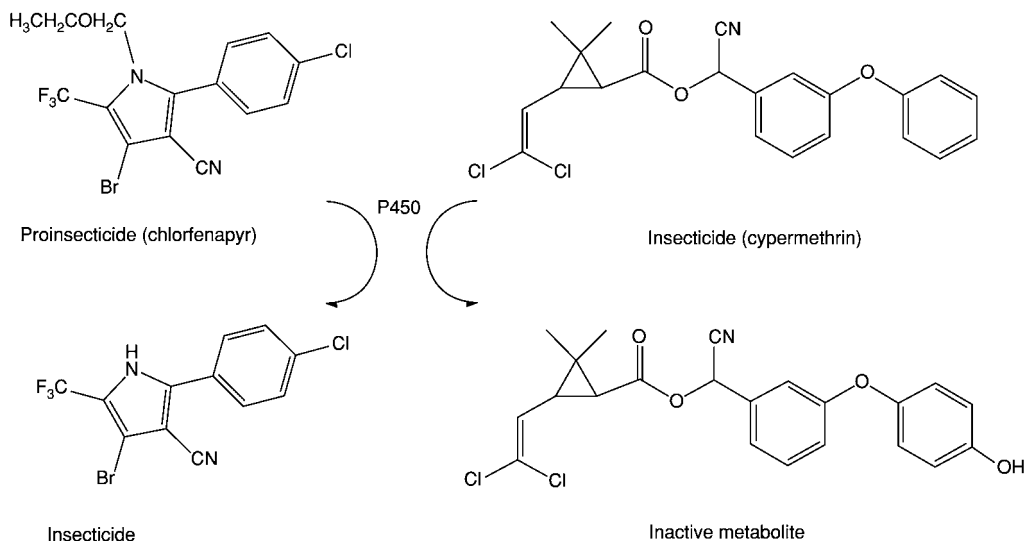


Figure 23 Chlorfenapyr and cypermethrin metabolism. The same P450 in *Heliothis virescens* probably activates the pyrrole and inactivates the pyrethroid, resulting in negative cross-resistance.

the literature. The inference is much stronger when two unrelated synergists are used *in vitro*, and when metabolites of the pesticide are identified. For instance, pyriproxyfen is hydroxylated by fat body and midgut microsomes of larval house flies to 4'-OH-pyriproxyfen and 5'-OH-pyriproxyfen and these activities are inhibited by PB and TCPPE (Zhang *et al.*, 1998).

The study of xenobiotic metabolism by individual P450 enzymes expressed in heterologous systems has barely begun (Table 1). Whereas the CYP6 enzymes clearly comprise some enzymes with "broad and overlapping" substrate specificity, even closely related enzymes of this family can differ substantially in their catalytic competence. The task of predicting which xenobiotic or natural product will be metabolized by which type of P450 is currently not possible.

13.4.3. P450 and Host Plant Specialization

13.4.3.1. The Krieger hypothesis and beyond

The interactions of plants and insects, and more specifically the role of plant chemistry on the specialization of phytophagous insects have generated a vast literature. "Secondary" plant substances are variously seen to regulate insect behavior and/or to serve as weapons in a coevolutionary "arms race" (Dethier, 1954; Fraenkel, 1959; Ehrlich and Raven, 1964; Jermy, 1984; Bernays and Graham, 1988). In chemical ecology alone, "no other area is quite so rife with theory" (Berenbaum, 1995). Many of the theories and some of the experiments implicitly or explicitly deal with the insect's ability to metabolize plant secondary substances by P450 and other

enzymes. In the case of behavioral cues, we are far from understanding the true importance of P450 enzymes in the integration of chemosensory information, e.g., as "odorant degrading enzymes." In the case of detoxification, however, the landmark paper of Krieger *et al.* (1971) can be seen as echoing the Fraenkel (1959) paper, by exposing the *raison d'être* of P450 enzymes. They stated that "higher activities of midgut microsomal oxidase enzymes in polyphagous than in monophagous species indicates that the natural function of these enzymes is to detoxify natural insecticides present in the larval food plants." In that 1971 study, aldrin epoxidation was measured in gut homogenates of last instar larvae from 35 species of Lepidoptera. Polyphagous species had on average a 15 times higher activity than monophagous species. This trend was seen in sucking insects as well, with a 20-fold lower aldrin epoxidase activity in the oleander aphid *Aphis nerii* when compared to the potato aphid *Myzus euphorbiae* or to the green peach aphid *M. persicae* (Mullin, 1986). The former is a specialist feeder on two plant families, Asclepiadaceae and Apocynaceae, whereas the latter two are generalists found on 30–72 plant families. The concept extended to other detoxification enzymes and was broadened to cover prey/predator, e.g., in mites where the predatory mite has a five times lower aldrin epoxidase activity than its herbivorous prey (Mullin *et al.*, 1982). The toxicity of the natural phototoxin α -terthienyl is inversely proportional to the level of its metabolism in Lepidoptera and is related to diet breadth. Metabolism (4.0 nmol/min/nmol P450) is highest in *O. nubilalis* that feeds on numerous

phototoxic Asteraceae, lower in *H. virescens* that has a broad diet, including some Asteraceae that are nonphototoxic, and lowest in *M. sexta*, a specialist of Solanaceae (Iyengar *et al.*, 1990).

The conceptual framework of Krieger *et al.* has been challenged (Gould, 1984) and defended (Ahmad, 1986). An alternative view (Berenbaum *et al.*, 1992) proposes that aldrin epoxidation represents “P450s with broad substrate specificity [that] are most abundant in insects that encounter a wide range of host plant metabolites.” A careful repetition of the Krieger experiments on lepidopteran larvae from 58 species of New South Wales failed to show significant differences in aldrin epoxidation between monophagous and polyphagous species (Rose, 1985). High activity in both monophagous and polyphagous species was invariably linked to the presence of monoterpenes in the host diet. The evidence presented in the sections below indicates that polyphagous and oligophagous species alike rely on the ability to draw on a great diversity of P450 genes, encoding a great diversity of specific and less specific enzymes and regulated by a great diversity of environmental sensing mechanisms – induction. The ability to induce P450 enzymes and deal with a wide range of toxic chemicals in the diet has been thought to present a “metabolic load” for polyphagous species, with specialists restricting their “detoxification energy” to one or a few harmful substrates (e.g., Whittaker and Feeny, 1971). However, careful studies in both oligophagous and polyphagous species have refuted this concept of metabolic load (e.g., Neal, 1987; Appel and Martin, 1992).

13.4.3.2. Host plant chemistry and herbivore P450 Cactophilic *Drosophila* species from the Sonoran desert are specialized to specific columnar cactus hosts by their dependency on unusual sterols (*D. pachea*) or by their unique ability to detoxify their host's allelochemicals, notably isoquinoline alkaloids and triterpene glycosides (Frank and Fogleman, 1992; Fogleman *et al.*, 1998). P450-mediated detoxification was shown by the loss of larval viability in media that contained both allelochemicals and piperonyl butoxide, and by the induction of total P450 or alkaloid metabolism by the cactus allelochemicals or by phenobarbital (Frank and Fogleman, 1992; Fogleman *et al.*, 1998). Several P450s of the *CYP4*, *CYP6*, *CYP9*, and *CYP28* families are induced by cactus-derived isoquinoline alkaloids and by phenobarbital, but not by triterpene glycosides; only a *CYP9* gene was induced by alkaloids and not by phenobarbital (Danielson *et al.*, 1997, 1998; Fogleman *et al.*, 1998). The capacity to detoxify isoquinoline alkaloids was not related to

DDT or propoxur tolerance, and while phenobarbital induced P450s capable of metabolizing the alkaloid carnegine in *D. melanogaster*, this was not sufficient to produce *in vivo* tolerance (Danielson *et al.*, 1995). Selection of *D. melanogaster* with Saguaro alkaloids over 16 generations, however, led to P450-mediated resistance to the cactus alkaloids (Fogleman, 2000). These studies suggest the evolution of specific responses in the cactophilic species involving the recruitment of a phylogenetically unrelated subset of P450 genes in each instance of specialization of a fly species on its host cactus.

The oligophagous tobacco hornworm (*M. sexta*) feeds essentially on Solanaceae and its adaptation to the high levels of insecticidal nicotine found in tobacco depends largely on metabolic detoxification, although other tolerance mechanisms may be contributing as well (Snyder and Glendinning, 1996). Hornworm larvae fed an nicotine-free artificial diet (naive insects) are rapidly poisoned by the ingestion of a nicotine-supplemented diet, but this diet is not deterrent. Poisoning is evidenced by convulsions and inhibition of feeding. The small amount of ingested nicotine induces its own metabolism, so that approximately 36 h later the larvae resume feeding normally, without further signs of poisoning. The inhibition of feeding and its resumption after nicotine exposure is directly related to P450 induction. Indeed, treatment with piperonyl butoxide, which itself has no effect on feeding, inhibits the increase in nicotine-diet consumption that occurs once nicotine metabolism has been induced (Snyder and Glendinning, 1996). Naive insects metabolize nicotine to nicotine 1-N-oxide at a low level, whereas nicotine-fed insects metabolize it further to cotinine-N-oxide at a higher level (Snyder *et al.*, 1994). These reactions are catalyzed by one or more P450 enzymes (Snyder *et al.*, 1993). The effects of nicotine on marker P450 activities are complex: the metabolism of three substrates is induced at low nicotine levels, seven are only induced at higher levels, and three are unaffected (Snyder *et al.*, 1993). *CYP4M1* and *CYP4M3* are moderately induced in the midgut but not in the fat body, but *CYP4M3* and *CYP9A2* are not affected by nicotine (Snyder *et al.*, 1995; Stevens *et al.*, 2000).

P450 induction has also been inferred in the polyphagous spider mite *Tetranychus urticae*, where the performance of a bean-adapted population on tomato was severely compromised by piperonyl butoxide (Agrawal *et al.*, 2002). The P450 inhibitor did not reduce acceptance of tomato as a host, nor did it reduce the performance of the bean-adapted population on bean, strongly suggesting a postingestive induction of P450 as a mechanism of acclimation

to the novel host. In the polyphagous noctuid *Spodoptera frugiperda*, ingestion of indole 3-carbinol increases once the continuous exposure to this toxic compound has induced P450 enzymes (Glendinning and Slansky, 1995).

13.4.3.3. *Papilio* species and furanocoumarins

The adaptation of specialist herbivores to toxic components of their host plants is best documented in the genus *Papilio*. The black swallowtail, *P. polyxenes*, feeds on host plants from just two families, the Apiaceae (Umbelliferae) and the Rutaceae. These plants are phytochemically similar, particularly in their ability to synthesize furanocoumarins. Biogenetically derived from umbelliferone (7-hydroxycoumarin) the linear furanocoumarins (related to psoralen) and angular furanocoumarins (related to angelicin) are toxic to nonadapted herbivores (Berenbaum, 1990). This toxicity is enhanced by light as furanocoumarins are best known for their UV photoreactivity leading to adduct formation with macromolecules, particularly DNA. *Papilio polyxenes* has become a model in the study of adaptation to dietary furanocoumarins. Xanthotoxin, a linear furanocoumarin, induces its own metabolism in a dose-dependent fashion when added to the diet of *P. polyxenes* larvae (Cohen *et al.*, 1989). This P450-dependent metabolism proceeds probably by an initial epoxidation of the furan ring followed by further oxidative attack and opening of the ring, leading to nontoxic hydroxylated carboxylic acids (Ivie *et al.*, 1983; Bull *et al.*, 1986). Inducible xanthotoxin metabolism is observed in all leaf-feeding stages of *P. polyxenes*, and is higher in early instars (Harrison *et al.*, 2001). Xanthotoxin induces its own metabolism in the midgut, but also in the fat body and integument (Petersen *et al.*, 2001). The metabolism of bergapten and sphondin is also induced by dietary xanthotoxin. Levels of total midgut microsomal P450s are unaffected by xanthotoxin, and photoactivation is not required for induction (Cohen *et al.*, 1989). The metabolism of xanthotoxin is 10 times faster in *P. polyxenes* than in the nonadapted *S. frugiperda* (Bull *et al.*, 1986). *Papilio polyxenes* microsomal P450s are also less sensitive to the inhibitory effects of xanthotoxin. NADPH-dependent metabolism of xanthotoxin leads to an uncharacterized reactive metabolite that can covalently bind P450 or neighboring macromolecules, i.e., xanthotoxin can act as a "suicide substrate" (Neal and Wu, 1994). This NADPH-dependent covalent labeling of microsomal proteins is seven times higher in *M. sexta* than in *P. polyxenes*. Inhibition of aldrin epoxidation and *p*-nitroanisole O-demethylation by xanthotoxin is also 6- and 300-fold higher, respectively, in *M. sexta*

than in *P. polyxenes* (Zumwalt and Neal, 1993). Myristicin, a methylene dioxyphenyl compound (see Section 13.3.4.4) found in the host plant parsnip is less inhibitory to *P. polyxenes* than to *H. zea* (Berenbaum and Neal, 1985; Neal and Berenbaum, 1989).

A distinct protein band of 55 kDa appears in midgut microsomes of xanthotoxin-treated *P. polyxenes* larvae and its microsequencing led to the cloning of CYP6B1, a P450 shown to be inducible by xanthotoxin or parsnip (Cohen *et al.*, 1992). Several variants of CYP6B1 have been cloned that presumably represent different alleles, v1, v2, and v3. The three variants differ from each other at 3, 6, or 9 amino acid positions (Cohen *et al.*, 1992; Prapaipong *et al.*, 1994). The CYP6B1 gene is selectively induced by linear furanocoumarins. Initial studies suggested that additional, related P450 transcripts were present in *P. polyxenes* and inducible by angular furanocoumarins (Hung *et al.*, 1995b). Expression in the baculovirus system revealed that CYP6B1 v1 and v2 metabolize the linear furanocoumarins bergapten, xanthotoxin, isopimpinellin, and psoralen (Ma *et al.*, 1994). Little metabolism of the angular furanocoumarin angelicin was observed in this early study but improvements in the heterologous expression system by coexpression of insect P450 reductase increased rates of metabolism sufficiently to confirm the role of CYP6B1 in the metabolism of angelicin as well (Wen *et al.*, 2003). The furanocoumarins were metabolized in the improved expression system with the following preference: xanthotoxin > psoralen > angelicin. The latter is less efficiently metabolized *in vivo* (Li *et al.*, 2003) and *P. polyxenes* is less adapted to it (Berenbaum and Feeny, 1981).

A second P450 was cloned from *P. polyxenes*; it encodes CYP6B3 that is 88% identical to CYP6B1 (Hung *et al.*, 1995a). CYP6B3 is expressed at lower basal levels than CYP6B1, but both CYP6B1 and CYP6B3 are inducible by xanthotoxin, sphondin, angelicin, and bergapten in the midgut. CYP6B3 responds more readily to the angular furanocoumarins than CYP6B1 (Hung *et al.*, 1995a), and CYP6B1 is more inducible than CYP6B3 (Harrison *et al.*, 2001). A later study showed that CYP6B1 is induced by xanthotoxin in the midgut, fat body, and integument, but CYP6B3 is induced by xanthotoxin only in the fat body (Petersen *et al.*, 2001).

The presence of CYP6B-like transcripts in species related to *P. polyxenes* was suggested early on by positive signals on northern blots with RNA from *Papilio brevicauda* and *Papilio glaucus* that were treated with xanthotoxin (Cohen *et al.*, 1992). *Papilio brevicauda* is like *P. polyxenes*, a species

that feeds on furanocoumarin-containing Apiaceae, but *Papilio glaucus* is a generalist that encounters furanocoumarin-containing plants (e.g., hoptree, *Ptelea trifoliata*) only occasionally. Xanthotoxin, nevertheless, induces its own metabolism in all three species (Cohen *et al.*, 1992). *Papilio glaucus* is highly polyphagous and is reported to feed on over 34 plant families, and therefore offers an interesting contrast to *P. polyxenes*. Esterase, glutathione S-transferase, and P450 activities are highly variable and dependent on the species of deciduous tree foliage that this species feeds on (Lindroth, 1989). *Papilio glaucus* has significant levels of linear and angular furanocoumarin metabolism, that are highly inducible by xanthotoxin (Hung *et al.*, 1997). A series of nine CYP6B genes and some presumed allelic variants were cloned from *P. glaucus* (Hung *et al.*, 1996, 1997; Li *et al.*, 2001, 2002a). The first two genes CYP6B4 and CYP6B5 are products of a recent gene duplication event, and their promoter region is very similar (Hung *et al.*, 1996). Six additional and closely related members of the CYP6B subfamily were cloned from *Papilio canadensis*, another generalist closely related to *P. glaucus* but not known to feed on plants containing furanocoumarins (Li *et al.*, 2001, 2002a). Xanthotoxin induced CYP6B4-like and CYP6B17-like genes in both species, but the level of furanocoumarin metabolism was lower in *P. canadensis* (Li *et al.*, 2001). This wide spectrum of CYP6B enzymes represents a wide range of activities towards furanocoumarin substrates. Whereas CYP6B4 of *P. glaucus* expressed in the baculovirus system efficiently metabolizes these compounds (Hung *et al.*, 1997), CYP6B17 of *P. glaucus*, and CYP6B21 and CYP6B25 from *P. canadensis* have a more modest catalytic capacity (Li *et al.*, 2003). *Papilio troilus*, a relative of *P. glaucus* that specializes on Lauraceae that lack furanocoumarins, has undetectable basal or induced xanthotoxin metabolism (Cohen *et al.*, 1992).

The genus *Papilio* thus offers a complete range of situations: (1) specialists that deal efficiently with furanocoumarins by inducible expression of CYP6B genes; (2) generalists that also carry related CYP6B genes, but whose inducibility and metabolism are less efficient; and (3) nonadapted specialists that appear to have lost the inducible CYP6B panoply (Berenbaum *et al.*, 1996; Berenbaum, 1999, 2002; Li *et al.*, 2003).

13.4.3.4. Furanocoumarins and other insects

The metabolism of furanocoumarins or the inducibility of P450 by these compounds is not restricted to Papilionidae. Xanthotoxin induces its own metabolism in the parsnip webworm, *Depressaria*

pastinacella (Nitao, 1989). This species belongs to the Oecophoridae, and is a specialist feeder on three genera of furanocoumarin-containing Apiaceae. It is highly tolerant to these compounds and metabolizes them not just by opening the furan ring, but in the case of sphondin, it is also capable of O-demethylation (Nitao *et al.*, 2003). Although furanocoumarin metabolism is inducible, the basal (uninduced) activity is high (Nitao, 1989), and the response is a general one, with little discrimination of the type of furanocoumarin inducer or the type of furanocoumarin metabolized (Cianfrogna *et al.*, 2002). The P450 enzymes involved in furanocoumarin metabolism by *D. pastinacella* are unknown. Low stringency northern hybridization failed to elicit a signal with a CYP6B1 probe (Cohen *et al.*, 1992).

A species that does not encounter furanocoumarins, the solanaceous oligophage *M. sexta*, responds to xanthotoxin by inducing CYP9A4 and CYP9A5 (Stevens *et al.*, 2000). The generalist *S. frugiperda* also induces P450 as well as glutathione S-transferases in response to xanthotoxin (Yu, 1984; Kirby and Ottea, 1995). It has low basal P450-mediated xanthotoxin metabolism, but this metabolism is inducible by a variety of compounds including terpenes and flavone (Yu, 1987). In the highly polyphagous *H. zea*, a similar situation is encountered. Xanthotoxin metabolism is low, but inducible by itself as well as by phenobarbital and α -cypermethrin (Li *et al.*, 2000b). A number of CYP6B genes have been cloned from these *Helicoverpa* species. CYP6B8 of *H. zea* is very close in sequence to CYP6B7 from *H. armigera*, and it is inducible by xanthotoxin and phenobarbital (Li *et al.*, 2000a). The high conservation of sequence in the SRS1 region suggests that the CYP6B enzymes of *Helicoverpa* are competent in furanocoumarin metabolism as indeed, these species occasionally encounter furanocoumarins in their diet. The CYP6B9/B27 and B8/B28 genes are pairs of recently duplicated genes (Li *et al.*, 2002b). Their tissue and developmental pattern of expression is subtly different as is their pattern of induction by a variety of chemicals (Li *et al.*, 2002c).

13.4.4. Host Plant, Induction and Pesticides

The adaptive plasticity conferred by the inducibility of P450 enzymes on different diets can have important consequences for insect control and the economics of pest insects. It is far from being just an ecological oddity or an interesting set of tales of insect natural history. It is well recognized that the same insect species fed different (host) plants will show differences in their response to pesticides

(Ahmad, 1986; Yu, 1986; Lindroth, 1991), and that these differences often reflect the induction of P450 enzymes, as well as of other enzymes, glutathione *S*-transferases, epoxide hydrolases, etc. The complexity of plant chemistry makes it difficult to account for the contribution of each individual chemical to this response and key components are often analyzed first (e.g., Moldenke *et al.*, 1992). Similarly, the multiplicity of P450 genes and the range of P450 enzyme specificity makes it difficult to predict the outcome of exposure to a plant chemical. The toxicological importance of the plant diet on the herbivore's P450 status (induction, inhibition) is well recognized in pharmacology where the joint use of chemical therapy and traditional herbs can have unpredicted outcomes (Zhou *et al.*, 2003).

Larvae of the European corn borer, *Ostrinia nubilalis*, fed leaves from corn varieties with increasing DIMBOA content and thus increasing levels of resistance to leaf damage had correspondingly increased levels of total midgut P450 and *p*-nitroanisole *O*-demethylation activity (Feng *et al.*, 1992). These studies suggest that constitutive host plant resistance may affect the insect response to xenobiotics. In addition, the induction of host plant defense by insect damage may itself be a signal for induction of herbivore P450 enzymes, as shown in *H. zea*. The plant defense signal molecules jasmonate and salicylate induce *CYP6B8*, *B9*, *B27*, and *B28* (Li *et al.*, 2002d) in both fat body and midgut. The response to salicylate is relatively specific as *p*-hydroxybenzoate, but not methylparaben, also acts as an inducer.

Treatment with 2-tridecanone, a toxic allelochemical from trichomes of wild tomato, protects *H. zea* larvae against carbaryl toxicity (Kennedy, 1984) and *H. virescens* larvae against diazinon toxicity (Riskallah *et al.*, 1986b). In *H. virescens* larvae, the compound caused both qualitative and quantitative changes in P450 spectral properties (Riskallah *et al.*, 1986a), an induction confirmed by its effect on specific P450 genes in the gut of *M. sexta* larvae (Snyder *et al.*, 1995; Stevens *et al.*, 2000). Larvae of *H. virescens* with a genetic resistance to 2-tridecanone have increased P450 levels and P450 marker activities (benzphetamine demethylation, benzo[*a*]pyrene hydroxylation, phorate sulfoxidation), and these can be further increased by feeding 2-tridecanone (Rose *et al.*, 1991). A laboratory population of *H. zea* can rapidly display increased tolerance to α -cypermethrin by selection of an increased P450 detoxification ability with a high dose of dietary xanthotoxin (Li *et al.*, 2000b).

Beyond the host plant, it is the whole biotic and chemical environment that determines the response

of an insect to pesticide exposure. Herbicides and insecticide solvents can serve as inducers (Brattsten and Wilkinson, 1977; Kao *et al.*, 1995; Miota *et al.*, 2000). Aquatic larvae are exposed to natural or anthropogenic compounds that alter their P450 detoxification profile (David *et al.*, 2000, 2002; Suwanchaichinda and Brattsten, 2002). Virus infection affects P450 levels (Brattsten, 1987), and the expression of several P450 genes is affected during the immune response (see Section 13.5.1.2).

13.4.5. Insecticide Resistance

13.4.5.1. Phenotype, genotype, and causal relationships Insecticide resistance is achieved in a selected strain or population by: (1) an alteration of the target site; (2) an alteration of the effective dose of insecticide that reaches the target; or (3) a combination of the two. The resistance phenotypes have long been analyzed according to these useful biochemical and physiological criteria. At the molecular genetic level, several classes of mutations can account for these phenotypes (Taylor and Feyereisen, 1996) and a causal relationship between a discrete mutation and resistance has been clearly established for several cases of target site resistance (ffrench-Constant *et al.*, 1999). The molecular mutations responsible for P450-mediated insecticide resistance are only beginning to be explored. In contrast to CYP51, which is a target for a major class of fungicides, no insect P450 has been recognized as a primary target for a commercial insecticide. Thus, biochemical changes in P450 structure or activity can lead to changes in insecticide sequestration, activation, or inactivation, so that all the classes of molecular mutations (structural, up- or downregulation, see Taylor and Feyereisen, 1996) can be theoretically involved in P450-mediated resistance. When the number of P450 genes is taken into account, it is little wonder that P450 enzymes are so often involved in insecticide resistance, and that it has been so difficult finding, and establishing, the role of resistance mutations for P450 genes.

Traditionally, the first line of evidence for a role of a P450 enzyme in resistance has been the use of an insecticide synergist (e.g., piperonyl butoxide), a suppression or decrease in the level of resistance by treatment with the synergist being diagnostic. In cases too many to list here, this initial and indirect evidence is probably correct, however there are cases where piperonyl butoxide synergism has not been explained by increased detoxification (Kennaugh *et al.*, 1993). Piperonyl butoxide may also be a poor inhibitor of the P450(s) responsible for resistance, so that the use of a second, unrelated synergist may be warranted (Brown *et al.*, 1996; Zhang *et al.*,

1997). In addition, the synergist as P450 inhibitor can decrease the activation of a proinsecticide, so that lack of resistance suppression can be misleading. Chlorpyrifos resistance in *D. melanogaster* from vineyards in Israel maps to the right arm of chromosome 2 (see Section 13.4.5.5) and is enhanced by piperonyl butoxide rather than suppressed (Ringo *et al.*, 1995).

An independent and additional line of evidence is the measurement of total P450 levels or metabolism of selected model substrates. An increase in either or both being viewed as diagnostic. Again, such evidence is tantalizing but indirect, and the absence of change uninformative. The validation of a model substrate for resistance studies requires substantial knowledge about the P450(s) involved, and is therefore best assessed a posteriori.

An increase in the metabolism of the insecticide itself in the resistant strain is more conclusive. For instance, permethrin metabolism to 4'-hydroxypermethrin was higher in microsomes from *Culex quinquefasciatus* larvae that are highly resistant to permethrin (Kasai *et al.*, 1998b) than in their susceptible counterparts. Total P450 and cytochrome *b*₅ levels were 2.5 times higher in the resistant strain. Both permethrin toxicity and metabolism were inhibited by two unrelated synergists, TCPPE and piperonyl butoxide. A similarly convincing approach was taken to show P450 involvement in the resistance of housefly larvae of the YPPF strain to pyriproxyfen. Gut and fat body microsomes were shown to metabolize the IGR to 4'-OH-pyriproxyfen and 5''-OH-pyriproxyfen at higher rates than microsomes of the susceptible strains and this metabolism was synergist-suppressible (Zhang *et al.*, 1998). The major, dominant resistance factor was linked to chromosome 2 in that strain (Zhang *et al.*, 1997).

Increased levels of transcripts for one or more P450 genes in insecticide-resistant strains has now been reported in many cases (see Table 5). This suggests that overexpression of one or more P450 genes is a common phenomenon of metabolic resistance but does not by itself establish a causal relationship with resistance. In some cases, the increased mRNA levels have been related to increased transcription (Liu and Scott, 1998), or increased protein levels (Liu and Scott, 1998; Sabourault *et al.*, 2001). Genetic linkage between increased mRNA or protein levels for a particular P450 and resistance has been obtained to the chromosome level (*CYP6A1*, *Cyp6a2*, *Cyp6a8*, *CYP6D1*, *CYP9A1*, *CYP12A1*: Cariño *et al.*, 1994; Liu and Scott, 1996; Rose *et al.*, 1997; Guzov *et al.*, 1998; Maitra *et al.*, 2000), and closer to marker genes (*Cyp6g1*, *CYP6A1*: Daborn

et al., 2001; Sabourault *et al.*, 2001). Linkage is just the first step in establishing a causal link between a P450 gene and resistance.

The following is a discussion of specific cases of P450 genes associated with insecticide resistance that have been studied in greater detail. Evidence for mutations causing constitutive overexpression in *cis* and *trans*, as well as an example of point mutations in a P450 coding sequence are currently available. The variety of mechanisms, even in a single species in response to the same insecticide, is striking. The paucity of available data on the molecular definition of the resistant genotype and on its causal relationship to resistance is also striking when compared to the wealth of data on target site resistance (French-Constant *et al.*, 1999).

13.4.5.2. CYP6A1 and diazinon resistance in the housefly Rutgers strain *CYP6A1* was the first insect P450 cDNA to be cloned, and the gene was shown to be phenobarbital-inducible and constitutively overexpressed in the multiresistant Rutgers strain (Feyereisen *et al.*, 1989). A survey of 15 housefly strains (Cariño *et al.*, 1992) showed that *CYP6A1* is constitutively overexpressed at various degrees in eight resistant strains, but not in all resistant strains, notably R-Fc known to possess a P450-based resistance mechanism. Thus, the first survey with a P450 molecular probe confirmed the results of the first survey of housefly strains with marker P450 activities (aldrin epoxidation and naphthalene hydroxylation; Schonbrod *et al.*, 1968): there is no simple relationship between resistance and a molecular marker, here the level of expression of a single P450 gene. That different P450 genes would be involved in different cases of insecticide resistance was a sobering observation (Cariño *et al.*, 1992), even before the total number of P450 genes in an insect genome was known. The constitutive overexpression of *CYP6A1* was observed in larvae and in adults of both sexes. Overexpression was shown in both developmental stages to be linked to a semidominant factor on chromosome 2 (Cariño *et al.*, 1994), but the *CYP6A1* gene was mapped to chromosome 5 (Cohen *et al.*, 1994). The gene copy number being identical between Rutgers and a standard susceptible strain (*sbo*), gene amplification could not be invoked to explain overexpression (Cariño *et al.*, 1994), and the existence of a chromosome 2 *trans*-acting factor(s) differentially regulating *CYP6A1* expression in the Rutgers and *sbo* strains was implied. Competitive ELISA using purified recombinant *CYP6A1* protein as standard showed that the elevated mRNA levels were indeed translated into elevated protein levels (Sabourault

Table 5 P450 overexpression in insecticide-resistant strains

<i>P450 overexpressed</i>	<i>Strain</i>	<i>Resistance pattern</i>	<i>Reference</i>
<i>Musca domestica</i>			
<i>CYP6A1</i>	Rutgers and other strains	OP, carbamates, IGR	Feyereisen <i>et al.</i> (1989), Cariño <i>et al.</i> (1992)
<i>CYP6A1</i> ^a	Rutgers		Sabourault <i>et al.</i> (2001)
<i>CYP6D1</i>	LPR	Pyrethroids	Liu and Scott (1996)
P450Lpr/ <i>CYP6D1</i> ^a	LPR		Liu and Scott (1996)
<i>CYP6D1/CYP6D1</i> ^a	NG98, Georgia	Pyrethroids	Kasai and Scott (2000)
<i>CYP6D1</i>	YPER	Pyrethroids	Shono <i>et al.</i> (2002)
<i>CYP6D3</i>	LPR		Kasai and Scott (2001b)
<i>CYP12A1</i>	Rutgers		Guzov <i>et al.</i> (1998)
<i>Drosophila melanogaster</i>			
<i>Cyp4e2</i>	RaleighDDT	DDT	Amichot <i>et al.</i> (1994)
<i>Cyp6a2</i>	91R	DDT	Waters <i>et al.</i> (1992)
	RaleighDDT	DDT	Amichot <i>et al.</i> (1994)
	MHIII-D23	Malathion	Maitra <i>et al.</i> (2000)
<i>CYP6A2</i> ^a	Several strains	DDT, pyrethroids	Bride <i>et al.</i> (1997)
<i>Cyp6a8</i>	91R		Maitra <i>et al.</i> (1996)
	MHIII-D23		Maitra <i>et al.</i> (2000)
	Wis-1lab	DDT	Le Goff <i>et al.</i> (2003)
<i>Cyp6g1</i>	Hikone R and 20 strains	DDT	Daborn <i>et al.</i> (2001, 2002)
	WC2	Lufenuron, propoxur	Daborn <i>et al.</i> (2002)
	EMS1	Imidacloprid	Daborn <i>et al.</i> (2002)
	Wisconsin-1, 91-R	DDT	Brandt <i>et al.</i> (2002)
<i>Cyp12d1/2</i>	Wisconsin-1, 91-R	DDT	Brandt <i>et al.</i> (2002), Le Goff <i>et al.</i> (2003)
<i>Drosophila simulans</i>			
<i>CYP6G1</i> ^b	OV1	DDT, imidacloprid, malathion	Le Goff <i>et al.</i> (2003)
<i>Heliiothis virescens</i>			
<i>CYP9A1</i>	Macon Ridge	Thiodicarb	Rose <i>et al.</i> (1997)
<i>Helicoverpa armigera</i>			
<i>CYP4G8</i>		Pyrethroids	Pittendrigh <i>et al.</i> (1997)
<i>CYP6B7</i>		Pyrethroids	Ranasinghe and Hobbs (1998)
<i>Lygus lineolaris</i>			
<i>CYP6X1</i>		Permethrin	Zhu and Snodgrass (2003)
<i>Anopheles gambiae</i>			
<i>CYP6Z1</i>	PSP	Pyrethroids	Nikou <i>et al.</i> (2003)
<i>Culex quinquefasciatus</i>			
<i>CYP6F1</i>	JPal-per	Pyrethroids	Kasai <i>et al.</i> (2000)
<i>Culex pipiens pallens</i>			
<i>CYP4H21, H22, H23 CYP4J4, CYP4J6</i>	RR	Deltamethrin	Shen <i>et al.</i> (2003)
<i>Diabrotica virgifera</i>			
<i>CYP4</i>		Me-parathion, carbaryl	Scharf <i>et al.</i> (2001)
<i>Blattella germanica</i>			
P450MA ^a		Chlorpyrifos	Scharf <i>et al.</i> (1999)

^aP450 protein level increased.^bPresumed CYP6G1 ortholog of *D. simulans*.

et al., 2001; see **Table 6** for comparison of Rutgers and *sbo*). A comparison of the coding sequence of CYP6A1 between Rutgers and two susceptible strains showed no (*sbo*) or little (*aabys*) sequence variation. Five amino acid changes were noted in *aabys*, two at the far N-terminus and three at the far C-terminus, well outside the regions (SRS) thought to influence enzyme activity (Cohen *et al.*, 1994). The lack of CYP6A1 sequence difference between

Rutgers and *sbo* indicated that if CYP6A1 was implicated in diazinon resistance in the Rutgers strain, it was through elevation of enzyme activity alone. Reconstitution of recombinant CYP6A1 expressed in *E. coli* with its redox partners (Sabourault *et al.*, 2001) provided the conclusive evidence for its role in diazinon resistance, as CYP6A1 metabolizes the insecticide with a high turnover (18.7 pmol/pmol CYP6A1/min), and a

Table 6 Comparison of a susceptible and a resistant strain of the housefly

Fly strain	<i>sbo</i> (S)	Rutgers (R)
Diazinon contact toxicity: LC50 ($\mu\text{g}/\text{pint jar}$)	4.4	167.8
Resistance ratio	1	37.8
Diazinon topical toxicity: LD50 ($\mu\text{g}/\text{fly}$)	0.059 ^a	7.1
Resistance ratio	1	120
P450 level (nmol/mg protein)	0.14	0.29
Aldrin epoxidation (pmol/min/pmol P450)	4.4 ^a	15.6
CYP6A1 mRNA relative level	1	27.5
CYP12A1 mRNA relative level	1	15
CYP6A1 protein level (fmol/abdomen)	36	565
Diazinon metabolized by CYP6A1		
Oxidative cleavage (pmol/min/fly)	0.5	7.7
Desulfuration to oxon (pmol/min/fly)	0.2	2.9
OP oxon metabolized by mutant ali-esterase (pmol/min/fly)	0.0	2.2–2.5
NADPH-dep. diazinon metabolism by microsomes (pmol/min/abdomen)	2.9 ^a	15.5

^aNAIDM susceptible strain.

favorable ratio (2.7) between oxidative ester cleavage and desulfuration (see Section 13.4.2.2 and Table 4).

The nature of the chromosome 2 *trans*-acting factor and of the mutation leading to resistance in the Rutgers strain has long remained enigmatic despite considerable circumstantial evidence for a major resistance factor on chromosome 2 (Plapp, 1984). Diazinon resistance and high CYP6A1 protein levels could not be separated by recombination in the short distance between the *ar* and *car* genes (3.3–12.4 cM). This region carries an ali-esterase gene (*MdxE7*) implicated as its *Lucilia cuprina* ortholog in organophosphorus insecticide resistance (Newcomb *et al.*, 1997; Claudianos *et al.*, 1999). A Gly137 to Asp mutation in this ali-esterase abolishes carboxylesterase activity towards model compounds such as methylthiobutyrate, and confers a measurable phosphotriester hydrolase activity towards an organophosphate (“P=O”), chlorfenvinphos. Chromosome 2 of the Rutgers strain carries this Gly137 to Asp mutation, and low CYP6A1 protein levels are correlated with the presence of at least one wild-type (Gly137) allele of *MdxE7*. Recombination in the *ar-car* region could not dissociate diazinon susceptibility, low CYP6A1 protein level, and the presence of a Gly137 allele of the ali-esterase (Sabourault *et al.*, 2001). It was therefore hypothesized that the wild-type ali-esterase metabolizes an (unknown) endogenous substrate into a negative regulator of CYP6A1 transcription. Removal of this regulator (by loss-of-function of

the ali-esterase) would increase CYP6A1 production and, hence, diazinon metabolism. Nature seems to have found the optimal loss-of-function mutation (Gly137 to Asp) as the Rutgers haplotype has swept through global populations of the housefly (C. Claudianos, J. Brownlie, V. Taskin, M. Kence, R.J. Russell, J.G. Oakeshott, personal communication). The mutant ali-esterase probably helps clearing the activated form (P=O) of the insecticide (Sabourault *et al.*, 2001). The negative regulation by the Gly137 allele and the diazinon resistance-enhancing effect of the Asp137 allele may explain the incomplete dominance of the diazinon resistance trait. Housefly strains that are susceptible or that are not known/shown to overexpress CYP6A1 predictably carry at least one Gly137 allele (Scott and Zhang, 2003). The LPR strain that overexpresses CYP6A1 (Cariño *et al.*, 1992) and has increased OP metabolism (Hatano and Scott, 1993), as well as another resistant strain (NG98) carry other alleles (Scott and Zhang, 2003) that have impaired ali-esterase activity, Trp251 to Ser or Leu (Campbell *et al.*, 1998; Claudianos *et al.*, 2001). Corroborating, but indirect evidence for the hypothesis is the predicted pleiotropic effect of a *trans*-acting regulator. Constitutive overexpression of CYP12A1 whose product metabolizes diazinon as well (Guzov *et al.*, 1998) and *GST-1* in the Rutgers strain are also controlled by a chromosome 2 factor, possibly the same as the one controlling CYP6A1 expression.

Although alternative hypotheses can be advanced, such as the fortuitous genetic closeness between the ali-esterase and a factor that increases the level of CYP6A1, a diazinon metabolizing enzyme, such hypotheses do not have the benefit of elegance. The mutant ali-esterase cannot account for the carbamate and JHA resistance so is not the sole factor of resistance seen in many housefly strains. Diazinon-resistant *L. cuprina* have provided evidence for Oppenoorth’s mutant ali-esterase hypothesis (Newcomb *et al.*, 1997), but the role of P450 in OP resistance cannot be ignored. Indeed, the Q strain of the sheep blowfly is more resistant to parathion than to paraoxon (Hughes and Devonshire, 1982) and indirect evidence for a P450 involvement in *L. cuprina* diazinon resistance has also been presented (Kotze, 1995; Kotze and Sales, 1995).

13.4.5.3. *Cyp6a2* in *Drosophila*: overexpression and mutant enzyme Insecticide-resistant strains of *D. melanogaster* have been studied at the molecular genetic level and the *Cyp6a2* gene has been implicated in the metabolic resistance of several of

them. *Cyp6a2* is constitutively overexpressed in the 91R strain that is resistant to malathion and DDT by a factor of 20–30 relative to the susceptible 91C strain (Waters *et al.*, 1992). DDT resistance maps to 56 cM on the left arm of chromosome 2 in the 91R strain (Dapkus, 1992), which is at or near the chromosomal location of *Cyp6a2* (43A1-2). Initially, the presence of a 96 bp insertion in the 3' UTR of the gene was proposed to confer a low level of expression to the *Cyp6a2* gene (Waters *et al.*, 1992), but this insertion (or rather, the lack of it) was neither correlated with DDT resistance (Delpuech *et al.*, 1993) nor confirmed to be linked to overexpression in resistant strains (Dombrowski *et al.*, 1998). *Cyp6a8* is also constitutively overexpressed in the 91R strain (Maitra *et al.*, 1996) and the expression of both *Cyp6a2* and *Cyp6a8* is repressed in 91R/91C hybrids (Maitra *et al.*, 1996; Dombrowski *et al.*, 1998; Maitra *et al.*, 2000). Flies (*rosy*⁵⁰⁶, insecticide susceptible and with constitutively low expression of *Cyp6a2*) were transformed with a P-element carrying the *Cyp6a2* gene of the 91R strain driver by its own promoter. These flies express the transgene at higher levels than the endogenous *Cyp6a2* but at lower levels than in their native 91R background (Dombrowski *et al.*, 1998). The expression of both *Cyp6a2* and *Cyp6a8* is also constitutively higher in the MHIII-D23 strain initially selected for malathion resistance (Maitra *et al.*, 2000). Genetic crosses and chromosome substitution experiments conclusively showed that the expression of both genes (located on the 2R chromosome) is repressed by factors on the third chromosome of the insecticide-susceptible 91C and *rosy*⁵⁰⁶ strains. In contrast, the third chromosome of the MHIII-D23 and 91R strains carries a loss-of-function mutation for this negative *trans* regulator, allowing the constitutive overexpression of the two genes (Maitra *et al.*, 2000). Further careful examination of the promoter activity of the *Cyp6a8* gene by fusion with a luciferase reporter gene in transgenic flies identified a –11/–761 bp region of the *Cyp6a8* gene of the 91R strain that was sufficient to respond to the negative regulation by the *rosy*⁵⁰⁶ (wild-type) *trans* acting factor (Maitra *et al.*, 2002). CYP6A2, similar to CYP6A1, appears to have a broad substrate specificity (see Table 1). Whether CYP6A2 of the 91R strain is capable of metabolizing DDT is unknown, as its sequence differs from that of the baculovirus produced CYP6A2 from the *iso-1* strain, which does not metabolize DDT, and from that of the Raleigh-DDT strain, which does (see below). Nonetheless, the studies with the 91R and MHIII-D23 strains are clear indications for loss-of-function mutations in gene(s) encoding

negative regulators of P450 gene expression on chromosome 3. Genetic analyses of malathion resistance and of P450 expression (electrophoretic bands and marker activities) (Hallstrom, 1985; Hallstrom and Blanck, 1985; Houpt *et al.*, 1988; Waters and Nix, 1988) have pointed to one or more loci between 51 and 61 cM on the right arm of chromosome 3, and it is interesting that this chromosome arm is thought to be orthologous to chromosome 2 of the housefly (Weller and Foster, 1993). The *Drosophila* ortholog of the *MdαE7* gene (*Est23*) is located at 84D9.

Resistance to DDT in the RaleighDDT strain offers a different picture. This strain has very high DDT resistance (>10 000-fold; Cuany *et al.*, 1990). Its piperonyl butoxide-suppressible resistance is polyfactorial but the major, dominant resistance factor maps to 55 cM on the second chromosome as in the 91R strain. At least two P450 genes, *Cyp6a2* and *Cyp4e2*, are constitutively overexpressed in this strain (Amichot *et al.*, 1994). The genetic localization of resistance matches the locus of *Cyp6a2* (A. Brun-Barale, S. Tares, J.M. Bride, A. Cuany, J.B. Bergé, M. Amichot, personal communication), and the RaleighDDT allele was sequenced. Three point mutations, Arg335 to Ser, Leu336 to Val, and Val476 to Leu, were found (Bergé *et al.*, 1998). Overexpression was separated from the point mutations by repeated backcrossing to a marked susceptible strain and DDT selection. The resulting strain called 152 retained the mutant *Cyp6a2* gene and high monofactorial resistance to DDT (>1000-fold), which also maps to 54.4 cM. Both RaleighDDT and 152 flies are characterized by elevated *in vitro* DDT metabolism, as well as elevated ethoxycoumarin and ethoxyresorufin O-deethylase activities. Strain 152 lost the constitutive expression of *Cyp6a2*, which was therefore caused by an unlinked *trans*-acting factor (A. Brun-Barale, S. Tares, J.M. Bride, A. Cuany, J.B. Bergé, M. Amichot, personal communication).

The wild-type CYP6A2 and five engineered versions (each individual mutation, a double mutant carrying the first two and a triple mutant corresponding to the RaleighDDT allele) were expressed in *E. coli* and assayed for activity. The mutant enzymes were all characterized by a decreased stability when compared to the wild-type enzyme. The enzyme production in *E. coli* was significantly lower and the ratio of holoenzyme produced (measured by the Fe^{II}–CO complex) to apoenzyme produced (measured with an anti-CYP6A2 antibody) was also lower. However, the triple mutant was uniquely capable of metabolizing DDT to DDA, DDD, and dicofol at rates that were

9–13 times higher than the wild-type enzyme whose DDT metabolism was barely measurable. In addition to the triple mutant, only the Arg335 to Ser single mutant had the capacity to hydroxylate DDT to dicofol at a rate significantly different from wild-type. In contrast to DDT metabolism, the 16 β hydroxylation of testosterone was not affected in the various single and multiple mutants. Thus, the three mutations found in *Cyp6a2* of the RaleighDDT (and its derivative 152) strain collectively confer DDT-metabolizing ability to the mutant CYP6A2 enzyme. Genetic localization of DDT resistance to the *Cyp6a2* gene locus is therefore explained. When the point mutations of the 152 strain are combined with overexpression as in the parent RaleighDDT strain, the very high level of resistance can be rationalized. This is the first example of point mutations in an insect P450 enzyme that contribute to insecticide resistance.

13.4.5.4. CYP6D1, the housefly LPR strain, and pyrethroid resistance The LPR strain of the housefly is highly resistant to pyrethroids with a phenoxylbenzyl moiety. This permethrin-selected strain has several resistance mechanisms, with P450-based detoxification as a major contributor. An abundant form of P450 (P450Lpr) was purified from abdomens of adult LPR flies, and immunological data indicated that P450Lpr represents 67% of the P450 in microsomes from LPR flies, a tenfold (Wheelock and Scott, 1990) increase over the reference-susceptible strain. Peptide sequences from the purified protein allowed PCR amplification and cloning of the P450Lpr gene, *CYP6D1* (Tomita and Scott, 1995). This gene is located on chromosome 1 of the housefly (Liu *et al.*, 1995), and is constitutively overexpressed by about tenfold in the LPR strain. This overexpression is not caused by gene amplification, but by increased transcription. It has been claimed that increased transcription of *CYP6D1* causes insecticide resistance (Liu and Scott, 1998), but transgenic expression of *CYP6D1* in *Drosophila* (Korytko *et al.*, 2000a) has not been reported to confer resistance, and *CYP6D1* produced in yeast (Smith and Scott, 1997) has not been reported to metabolize pyrethroids. Instead, the evidence for the role of *CYP6D1* in pyrethroid resistance is based on the inhibition of microsomal deltamethrin and cypermethrin metabolism by anti-P450Lpr antibodies (Wheelock and Scott, 1992b; Korytko and Scott, 1998). The metabolism of pyrethroids by *CYP6D1* has been studied in its microsomal environment of the LPR strain. Deltamethrin is metabolized (<0.5pmol/min/pmol *CYP6D1*) preferentially

at the gem-dimethyl group on the acid moiety (Wheelock and Scott, 1992b) whereas cypermethrin is mainly hydroxylated at the 4' position on the alcohol moiety, at a very low rate (4.17 pmol/min/pmol *CYP6D1*, or less than 1 *CYP6D1* turnover per 2 h) (Zhang and Scott, 1996b). Whether overexpression or point mutations of *CYP6D1* or both (as for *Cyp6a2* of *Drosophila* RaleighDDT) are involved in pyrethroid resistance in the LPR strain is yet unknown. Indeed, the *CYP6D1* gene sequence from 5 strains shows a high polymorphism, with 57 variable sites in the coding region alone, of which 12 are nonsilent (Tomita *et al.*, 1995). Six amino acid changes are specific to the LPR strain (*CYP6D1v1*) when compared to pyrethroid-susceptible strains and several of these mutations appear to align with SRS3.

The characterization of the resistance mutation(s) is made difficult by its almost completely recessive nature, and by the important contribution of the genes *pen* (for reduced penetration) and *kdr* (for target site resistance) of chromosome 3 (Liu and Scott, 1995). The absence of this resistant chromosome 3 decreases permethrin resistance from 16 000-fold to 170-fold. The remaining resistance is entirely suppressible by piperonyl butoxide, and is conferred by a combination of the resistant chromosomes 1 and 2 from the parent LPR strain in the homozygous condition (Liu and Scott, 1995). There is no substantial resistance or *CYP6D1* overproduction conferred by isolated LPR chromosomes 1 or 2, or by their subsequent combination (Liu and Scott, 1995, 1996). Thus, permethrin selection of a P450-mediated resistance requires both copies of the LPR chromosomes 1 and 2. The resistance and *CYP6D1* overexpression linked to chromosome 1 are dominant, whereas the contributions of chromosome 2 are mostly recessive (Liu and Scott, 1996, 1997a). Furthermore, cytochrome *b₅* is essential for *CYP6D1*-metabolism of cypermethrin (Zhang and Scott, 1996b), and the overexpression of cytochrome *b₅* in LPR maps to the same chromosomes as permethrin resistance and *CYP6D1* overexpression (Liu and Scott, 1996). These data suggest a unique combination in the LPR strain of chromosome 2 *trans*-acting factor(s) with at least a matched *cis*-factor on chromosome 1. Sequence differences between the 5'UTR of the *CYP6D1* gene of LPR and of other strains have been documented (Scott *et al.*, 1999).

CYP6D1 is also overexpressed, but cytochrome *b₅* is not, in the permethrin-resistant strain NG98 from Georgia, USA (Kasai and Scott, 2000). These permethrin-resistant flies from Georgia carry virtually the same *CYP6D1v1* haplotype as LPR, with

only 2 nt changes in introns in a sequence spanning from 658 nt upstream of the ATG to the stop codon (Seifert and Scott, 2002). However, in a strain (ALHF) from neighboring Alabama with high permethrin resistance, chromosomes 1 and 2 play little role but chromosome 5 plays a major piperonyl butoxide-suppressible role (Liu and Yue, 2001). *CYP6D1* is overexpressed by about 2.4-fold and cytochrome *b*₅ by about 1.5-fold in the Japanese strain YPER (Shono *et al.*, 2002). This strain does not carry the *CYP6D1v1* allele, and chromosome 2 has a major role in this permethrin-resistant strain. Permethrin resistance thus appears largely recessive in several housefly strains, with multiple mutations, both regulatory and structural, potentially playing a role in the P450 contribution to the multifactorial resistance.

A gene closely linked to *CYP6D1* on chromosome 1 codes for a similar (78% identity) P450, *CYP6D3* (Kasai and Scott, 2001a). It is 12-fold overexpressed in adult flies of the LPR strain, but it is also expressed in larvae (Kasai and Scott, 2001b), as opposed to *CYP6D1*, which has an adult-specific pattern of expression.

13.4.5.5. *Drosophila Cyp6g1*, the *Rst(2)DDT* gene at 64.5 cM on chromosome 2 The power of *Drosophila* genetics coupled with the tools made possible by the complete genome sequence has provided the most detailed, yet complex molecular genetic detail about a P450-based insecticide resistance mechanism. The resistance gene *Rst(2)DDT* has been genetically characterized for over 40 years (reviews: Daborn *et al.*, 2001; Wilson, 2001). The position of this gene around 64.5 cM on the left arm of chromosome 2 has become almost mythical, as a number of phenotypes were linked to this locus, from dominant DDT resistance to phenylthiourea susceptibility, from organophosphorus to carbamate resistance, from various P450-dependent activities to vinyl chloride activation. EMS mutagenesis of a wild-type stock and selection with imidacloprid led to two strains with moderate imidacloprid resistance and moderate cross-resistance to DDT (Daborn *et al.*, 2001). Conversely, two DDT-resistant strains (Hikone-R and Wisconsin-1) were shown to be cross-resistant to imidacloprid. Fine scale mapping of this dominant resistance localized *Rst(2)DDT* to a region from 48D5-6 to 48F3-6 on the polytene chromosome map. Of three candidate P450 genes in this region, *Cyp6g1*, *Cyp6g2*, and *Cyp6t3*, only the first one showed constitutive overexpression in the DDT- and imidacloprid-resistant strains tested (Daborn *et al.*, 2001). A DNA microarray comprising probes for all the

Drosophila P450 genes was addressed with target cDNAs from susceptible strains and from the DDT-resistant Hikone-R strain and the propoxur-resistant WC2 strain. In both cases, *Cyp6g1* was the only P450 gene showing constitutive overexpression (Daborn *et al.*, 2002). Microarray data thus offer, at the transcriptional level, a complete analysis of all P450 genes, thereby facilitating the further rational study of one or more P450 genes (Figure 24). This is in contrast to the classical approaches that address single genes simply because they are the only ones for which a molecular probe is available. Overexpression of *Cyp6g1* was confirmed by quantitative (RT)PCR in 20 strains, and DDT, imidacloprid, nitenpyram, and lufenuron resistances were all independently mapped to the *Cyp6g1* locus in the Hikone-R and WC2 strains. The insertion of a terminal direct repeat of the transposable element *Accord* was systematically found in the 5' UTR of 20 different resistant strains from across the globe. Phylogenetic analysis of the first intron sequence of the gene showed a unique haplotype in resistant strains versus a large diversity of susceptible haplotypes, suggesting that a selective sweep had occurred in global *Drosophila* populations (Daborn *et al.*, 2002). Transgenic flies producing CYP6G1 under control of a heatshock promoter in the GAL4/UAS system showed both increased transcription of *Cyp6g1* and survival to a discriminating dose of DDT after heatshock. Similarly, transgenic expression under the tubulin promoter showed overexpression of only the *Cyp6g1* gene, and larval survival to discriminating doses of acetamiprid, imidacloprid, and nitenpyram (Le Goff *et al.*, 2003). *Cyp6g1* overexpression was confirmed in the DDT-resistant Wisconsin and 91R strains, and the adjacent *Cyp12d1* (or *Cyp12d2*) gene is overexpressed, as well as DDT-inducible in both strains (Brandt *et al.*, 2002). In the Wisconsin-1 strain, *Cyp6g1* and *Cyp12d1/2* are the only P450 genes that are overexpressed, whereas in the Wis1lab strain, in which DDT selection was applied after genetic removal of the *Cyp6g1* region, only *Cyp6a8* is overexpressed (Le Goff *et al.*, 2003). In all the strains discussed above, DDT resistance is significant but low compared to the RaleighDDT strain suggesting that while *Cyp6g1* (overexpressed in the RaleighDDT strain; S. Tares, personal communication) may constitute a first line of defense seen in field populations, further insecticide pressure in the laboratory may select additional mechanisms.

In a Brazilian strain of *D. simulans* resistant to DDT, imidacloprid, and malathion, only the *Cyp6g1* probable ortholog is overexpressed (Le Goff *et al.*, 2003). In a California population of

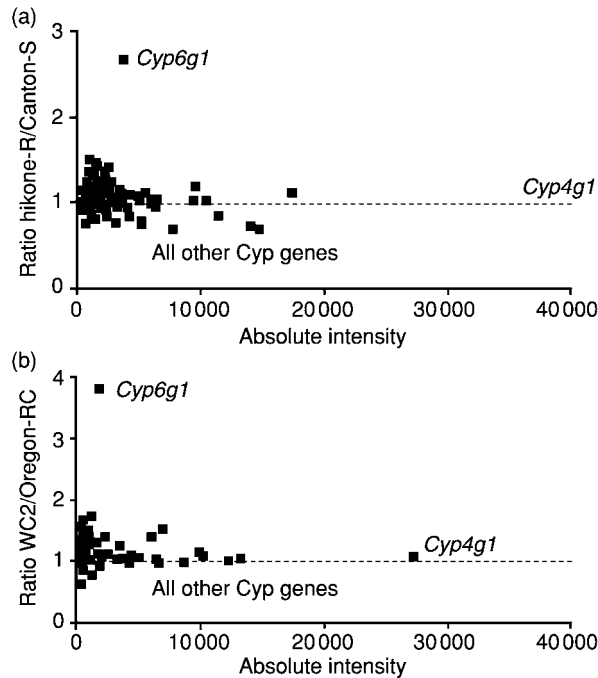


Figure 24 *Cyp6g1* overexpression in two insecticide-resistant strains, Hikone-R(a) and WC2(b), of *Drosophila melanogaster*. DNA microarray analysis revealed overexpression of just one P450 gene. (Reproduced with permission from Daborn, P.J., Yen, J.L., Bogwitz, M.R., Le Goff, G., Feil, E., *et al.*, 2002. A single P450 allele associated with insecticide resistance in *Drosophila*. *Science* 297, 2253–2256; © AAAS.)

D. simulans the 5'-flanking sequence of the *Cyp6g1* ortholog is nearly fixed for a *Doc* transposable element insertion. This insertion is absent from African populations and is associated with increased transcript abundance of *Cyp6g1* and resistance in a remarkable analogy with the Accord case of *D. melanogaster Cyp6g1* (Schlenke and Begun, 2004).

13.4.5.6. P450-mediated resistance: evolution
Resistance-conferring mutations in insecticide target sites (Rdl, AchE, sodium channel) have a remarkable pattern of orthology in widely divergent species. The hypothesis that a few paralogous, perhaps orthologous P450 genes would repeatedly be found to be responsible for metabolic resistance in various insect species has not been confirmed, beyond the single example of *Cyp6g1* in *D. melanogaster* and *D. simulans* (that are only 2.5 million years apart). Furthermore, when resistance due to constitutive overexpression of a P450 is caused by a mutation is *trans*, the effect can be pleiotropic with more than one P450 gene from more than one family being overexpressed (e.g., *CYP12A1* and *CYP6A1*). In Diptera as well as in Lepidoptera, members of the *CYP4* family also appear to be involved in resistance, and there is evidence for involvement of *CYP9* genes in the latter (Rose *et al.*,

1997). Therefore, an alternative hypothesis is that the multitude of P450 genes, whose expression is inducible and therefore not strongly expressed in most developmental stages/tissues, constitute a “reservoir” in which mutations affecting expression levels can be selected by insecticide exposure. In the field, this can lead to selective “sweeps” of these most adapted mutations as seen for the global predominance of the Rutgers and *Cyp6g1*/Accord haplotypes in house flies and fruit flies. These mutations would typically be loss-of-function mutations, which inactivate the fine level regulation of expression and therefore increase overall expression of a random P450 gene. If its product happens to metabolize the insecticide, even marginally, this may constitute a selective advantage for the organism. Loss-of-function mutations in the large target of negative regulatory sequences are predicted to be more frequent than gain-of-function mutations (in the smaller open reading frame) that would improve the catalytic efficiency of a P450 enzyme towards the insecticide (Taylor and Feyereisen, 1996). However, polymorphisms in the P450 sequence may be less detrimental to fitness than (often pleiotropic) changes in expression (see, e.g., Halpern and Morton, 1987), so that more examples of point mutations in P450 coding sequences are likely to emerge. The study of insecticide resistance has

clearly entered the age of genomics (Oakeshott *et al.*, 2003) and much progress is expected in the coming years.

13.5. Regulation of P450 Gene Expression

13.5.1. Spatial and Temporal Patterns of P450 Gene Expression

13.5.1.1. Enzyme activities and P450 expression
P450-dependent enzyme activities had been detected in virtually all insect tissues and developmental stages studied as early as the first edition of this series (Hodgson, 1985). Many studies have continued to document these patterns and changes (e.g., Feyereisen and Farnsworth, 1985; Ahmad, 1986; Gunderson *et al.*, 1986; Scott and Lee, 1993a). With molecular probes, the expression patterns of individual P450 genes have now been presented in different tissues, developmental stages or induction regimes with varying degrees of detail, and with probes of varying degree of specificity. Early studies relied on northern blots and dilution dot-blot for quantification (e.g., Cariño *et al.*, 1992, 1994), with later ones relying on the highly specific ribonuclease protection assays (e.g., Hung *et al.*, 1995b; Sutherland *et al.*, 1998). **Table 7** shows a summary of those studies reporting a specific

pattern of expression. The many studies in which the expression of a particular P450 was studied episodically or by simply rounding up the usual suspects (midgut, fat body, etc.) are not specifically listed.

In situ mRNA hybridization for the *Cyp6a2* gene has been used in *Drosophila* to show that in adults treated with phenobarbital, this gene is transcribed in the midgut, the pericuticular fat bodies, and the Malpighian tubules (Brun *et al.*, 1996). *Cyp6a2* expression is seen in a large number of tissues by immunohistochemistry (Saner *et al.*, 1996), and peaks in the third larval and pupal stages as shown by northern blots. The *Cyp6a2* promoter linked to a GFP reporter gene has been used in transgenic flies to document the expression of this gene (G.C. Unnithan, unpublished data). *In situ* hybridization has been used to document the expression patterns of *sad*, *dib*, and *shd* (Warren *et al.*, 2002; Petryk *et al.*, 2003). Systematic surveys (Tomancak *et al.*, 2002) will eventually document the expression patterns of all P450 genes.

Many unanswered questions remain about P450 expression patterns. For instance, sublethal infection by cytoplasmic polyhedrosis viruses in *H. virescens*, *M. sexta*, and *S. frugiperda* depresses P450 levels and P450-dependent activities, and increases the toxicity of insecticides (Brattsten, 1987). What are the mechanisms, the P450 genes involved and the significance of these observations? Changes in P450 levels are observed after a blood meal in *Culex pipiens*

Table 7 Specific spatial or temporal expression of P450 genes

P450	Species	Tissue/comments	Reference
CYP4C7	<i>Diptera punctata</i>	Corpora allata	Sutherland <i>et al.</i> (1998)
CYP4G15	<i>Drosophila melanogaster</i>	Larval brain	Maibeche-Coisne <i>et al.</i> (2000)
CYP4D21	<i>Drosophila melanogaster</i>	Head fat cells in males under control of <i>transformer</i> and <i>doublesex</i>	Fujii and Amrein (2002)
CYP4L4	<i>Mamestra brassicae</i>	Antennae, proboscis, legs	Maibeche-Coisne <i>et al.</i> (2002)
CYP4S4	<i>Mamestra brassicae</i>	Antennae	Maibeche-Coisne <i>et al.</i> (2002)
CYP6B2	<i>Helicoverpa armigera</i>	Larval specific	Ranasinghe <i>et al.</i> (1997)
CYP6D1	<i>Musca domestica</i>	Adult specific	Scott <i>et al.</i> (1996)
CYP6D1	<i>Musca domestica</i>	Thoracic ganglia	Korytko and Scott (1998)
CYP6L1	<i>Blattella germanica</i>	Adult male (probably accessory glands, may be testes as well)	Wen and Scott (2001a)
CYP6Z1	<i>Anopheles gambiae</i>	Adult specific	Nikou <i>et al.</i> (2003)
CYP15A1	<i>Diptera punctata</i>	Corpora allata	Helvig <i>et al.</i> (2004)
CYP302A1	<i>Drosophila melanogaster</i>	(Embryo) prothoracic gland portion of the ring gland, follicle cells	Chavez <i>et al.</i> (2000); Warren <i>et al.</i> (2002)
CYP310A1	<i>Drosophila melanogaster</i>	Overexpressed in embryos of Toll10B mutants (Dorsal target in the mesoderm)	Stathopoulos <i>et al.</i> (2002)
CYP312A1	<i>Drosophila melanogaster</i>	Male specific	Kasai and Tomita (2003)
CYP314A1	<i>Drosophila melanogaster</i>	(Embryo) ovary, gut, Malpighian tubules, fat body	Petryk <i>et al.</i> (2003)
CYP315A1	<i>Drosophila melanogaster</i>	(Embryo) prothoracic gland portion of the ring gland, follicle cells, nurse cells	Warren <i>et al.</i> (2002)
CYP315A1	<i>Drosophila melanogaster</i>	One of 30 significantly downregulated genes in eyes overexpressing TIGR/MYOC	Borras <i>et al.</i> (2003)

(Baldridge and Feyereisen, 1986), but too little is known of P450 expression patterns in blood-feeding or phloem-feeding insects. What is the significance of the patterns of expression seen in *Drosophila* where about 20% of the genes are in groups of 10–30 genes spread over 20–200 kb that are coordinately expressed (Spellman and Rubin, 2002). Are the P450s present in some of these 200 groups, for instance the *CYP6A* cluster at 55A on chromosome 2R (Ueda *et al.*, 2002), functionally linked?

13.5.1.2. DNA microarrays and P450 gene expression The genomic approach to biology has transformed the study of genes one by one to a study of very large numbers of genes in parallel, and ultimately to the study of the whole transcriptome of an organism (see **Chapter 10**). This approach, using tools such as DNA microarrays and SAGE, will rapidly change our understanding of the role of P450 in the life of insects. The interpretation of transcriptome studies is in its infancy and “data mining” will require a considerable interplay of experimental design and *in silico* analysis. In the case of P450 genes, the initial EST-based DNA microarrays (e.g., White *et al.*, 1999; Arbeitman *et al.*, 2002) were biased by the poor representation of P450 genes in the early EST collections (Tijet *et al.*, 2001). Careful attention to the potential for cross hybridization between closely related genes is needed (Xu *et al.*, 2001), but the design and validation of thematic arrays (e.g., with all the P450 genes) can allay this concern. The massive amount of data can reveal a link between a P450 gene and a physiological response; it is easy to overstate the relationship that may be a remote effect in a cascade of events. Genomic physiology has nonetheless provided much descriptive information on P450 gene expression already, and a few *Drosophila* examples are listed here. An exhaustive,

P450-focused study will be possible in the near future, so these examples are just an exciting preview.

The earliest developmental study in which 534 sequences showed developmental variations (White *et al.*, 1999, Arbeitman *et al.*, 2002) revealed four genes repressed by premature expression of the ecdysone-inducible DHR3 nuclear receptor (*Cyp12a2*, *Cyp6a2*, *Cyp4d2*, and *Cyp4ad1*). The link between *Cyp6a2* and the ecdysteroid cascade confirms earlier experimental (Spiegelman *et al.*, 1997) and *in silico* (Dunkov *et al.*, 1997; Dombrowski *et al.*, 1998) data. Two other genes, *Cyp28d1* and *Cyp9f2*, were repressed at the onset of metamorphosis (White *et al.*, 1999). *Cyp310a1* was overexpressed in Toll10B mutant embryos (Furlong *et al.*, 2001), identified as a Dorsal target (Stathopoulos *et al.*, 2002) and its pattern of expression confirmed in a high-throughput *in situ* hybridization project (Tomancak *et al.*, 2002). The Toll pathway represses seven P450 genes (*Cyp316a1*, *Cyp4ac1*, *Cyp6g1*, *Cyp18a1*, *Cyp28d1*, *Cyp6w1*, and *Cyp4d14*) in response to bacterial infection in adults, while *Cyp4e2* is also repressed in the acute response (De Gregorio *et al.*, 2001, 2002). Profiles of circadian rhythms have revealed the coregulation of six *Cyp6a* genes that are adjacent in a cluster at 51D5 on chromosome 2R (Ueda *et al.*, 2002) as well as the control of *Cyp4e2* expression by the *Clk* gene. A number of other P450 genes including *Cyp18a1* and *Cyp4p1* are also cycling with different phases (Claridge-Chang *et al.*, 2001; McDonald and Rosbash, 2001), with *Cyp4d21*, *Cyp6a21*, and *Cyp304a1* identified in both studies (**Figure 25**). Aging and oxidative stress (paraquat treatment) have revealed the responsiveness of a number of P450 genes, with *Cyp6a17* and *Cyp28a5* upregulated by paraquat and *Cyp6g1*

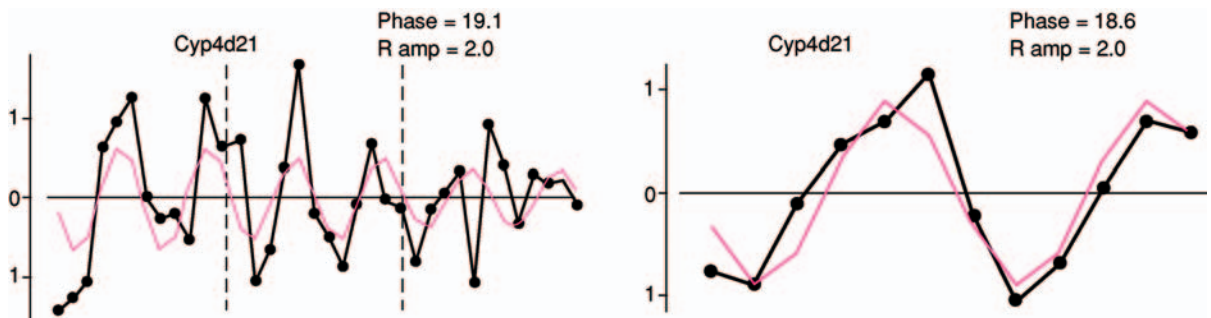


Figure 25 Circadian rhythmicity of *Cyp4d21* expression in *Drosophila melanogaster* expressed as a log ratio of change. Left: 36 time points collected over 6 days (Affymetrix microarray data); right: 12 time points collected over 2 days. Estimated phases and log ratio amplitudes are indicated. Red lines are 24-h guidelines. (Reprinted with permission from Claridge-Chang, A., Wijnen, H., Naef, F., Boothroyd, C., Rajewsky, N., *et al.*, 2001. Circadian regulation of gene expression systems in the *Drosophila* head. *Neuron* 32, 657–671; © Elsevier.)

downregulated under those conditions (Zou *et al.*, 2000). In another study, 18 P450 genes were found to respond to paraquat treatment by either increased or decreased expression. Those increased by paraquat treatment were also increased by H₂O₂ treatment. Four genes are increased by treatment with tunicamycin that blocks N-glycosylation and thus causes a stress at the level of the endoplasmic reticulum (ER). *Cyp28a5* is strongly induced by the three treatments, suggesting it may be a general stress-responsive gene (F. Girardot, V. Monnier, and H. Tricoire, personal communication). The subtle interactions of sex, age, and genotype have been discussed (Jin *et al.*, 2001), with *Cyp4c3* and *Cyp6g1* as two examples. *Cyp4c3* is upregulated by starvation in larvae, an effect that can be attributed to lack of sugar (Zinke *et al.*, 2002). This effect points to a conserved physiological function of some CYP4C genes, as cockroach CYP4C1 is also induced by starvation and is upregulated by hypertrehalosemic hormone (Lu *et al.*, 1995).

DNA microarray analysis of the transcriptome of transgenic flies has also led to the validation of *Drosophila* as a model for human disease (Borras *et al.*, 2003). Flies transformed with a human gene associated with glaucoma show a phenotype of distorted ommatidia and fluid discharge. Fifty transcripts have an altered expression profile in the head and, interestingly, *Cyp315a1* is suppressed significantly in these experiments (Borras *et al.*, 2003). Specific or thematic microarrays have also started to change research in insecticide resistance (Oakeshott *et al.*, 2003; see Section 13.4.5.5).

Serial analysis of gene expression (SAGE) analysis identified *Cyp4d21* as one of three genes expressed preferentially in the fat cells of the head, and specifically expressed in males under control of the sex determination genes *transformer* and *doublesex* (Fujii and Amrein, 2002). The male-specific expression of *Cyp312a1* in microarray experiments has also been reported and confirmed by real-time PCR (Kasai and Tomita, 2003).

13.5.2. P450 Induction, Inducers and Signal Transduction

13.5.2.1. Induction by hormones *Drosophila* *Cyp18* was discovered as the ecdysone-inducible *Eig17-1* gene (Hurban and Thummel, 1993). The expression of this gene clearly pulses closely after each ecdysteroid peak (Bassett *et al.*, 1997) (Figure 26), but the mechanism of its ecdysone inducibility has not been studied in detail. The *Cyp6a2* gene is also inducible by ecdysone. The arrest of ecdysone production decreases CYP6A2

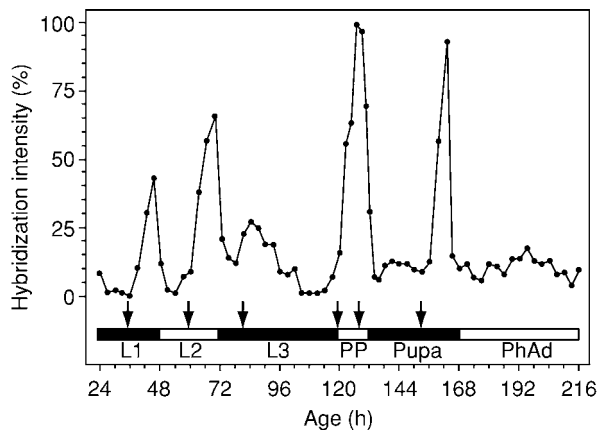


Figure 26 Developmental pattern of *Cyp18* expression in *Drosophila*. Peaks of *Cyp18* expression follow each surge in endogenous molting hormone level. (Reprinted with permission from Bassett, M.H., McCarthy, J.L., Waterman, M.R., Sliter, T.J. 1997. Sequence and developmental expression of *Cyp18*, a member of a new cytochrome P450 family from *Drosophila*. *Mol. Cell. Endocrinol.* 131, 39–49; © Elsevier.)

levels and slightly increases DDT toxicity (Spiegelman *et al.*, 1997). Ecdysone inducibility of *Cyp6a2* may be mediated by Broad-Complex Z1 and Z4 transcription factor binding sites or ecdysone response elements (EcRE) (see Chapter 7) seen in the promoter of the gene (Dunkov *et al.*, 1997; Dombrowski *et al.*, 1998).

CYP4C1 is inducible by hypertrehalosemic hormone (HTH) and starvation in the fat body of *Blaberus discoidalis* (Bradfield *et al.*, 1991). The dose-response for CYP4C1 induction (ED₅₀ = 3 pmol/insect, 8 h after injection) corresponds to the physiological range of HTH-dependent biosynthesis of trehalose and this induction appears to be a direct effect of the hormone (Lu *et al.*, 1995). JH inhibits CYP4C1 expression in adult females, but not in males; this JH inhibition controls the decrease in CYP4C1 transcript levels in the fat body. Yet HTH can override the JH inhibition (Lu *et al.*, 1999). The dual regulation of CYP4C1 expression is indicative of a fine regulation of this P450, presumed to be involved in fatty acid ω-hydroxylation (by analogy to the function of mammalian CYP4 enzymes). If confirmed, such a function might be related to gluconeogenesis from fatty acid oxidation (an HTH stress and starvation response) as opposed to (and thus inhibited by) the JH-controlled metabolism of the fat body cell towards vitellogenesis (Lu *et al.*, 1999) (see Chapter 8).

13.5.2.2. Xenobiotic-inducible genes One remarkable feature of P450 genes is that the transcription rate of many of them (sometimes gene batteries

that also include genes other than P450s) is induced by foreign chemicals or “xenobiotics.” Genetic models have provided much of our current understanding of induction of the mammalian *CYP1* genes by aryl hydrocarbons, such as dioxin, through the Ah receptor pathway (Denison and Nagy, 2003). For instance, dioxin and other aromatic hydrocarbons bind to the Ah receptor, which itself dimerizes with a protein called Arnt. The dimer of these two bHLH-PAS proteins is thought to induce a battery of genes including the *CYP1A1* gene. However, the molecular mechanism is not well understood for many other classes of inducers. Nuclear receptors play a key role at the interface between physiological responses and environmental responses (Chawla *et al.*, 2001; Honkakoski *et al.*, 2003).

Our understanding of the molecular genetics of induction in insects will benefit from the power of the *Drosophila* model. Phenobarbital (see below) is a known inducer of many P450 activities in *Drosophila* (Hallstrom and Grafstrom, 1981; Bigelow *et al.*, 1985; Fuchs *et al.*, 1994; Amichot *et al.*, 1998). Other inducers of one or more marker activities in *Drosophila* include: β -naphthoflavone or polychlorinated biphenyls (Hallstrom and Grafstrom, 1981), but not benzo[a]anthracene or dioxin (TCDD) (Bigelow *et al.*, 1985); butylated hydroxytoluene and rifampicin (Zijlstra *et al.*, 1984); *trans*-stilbene oxide, triphenyldioxane, benzo[a]pyrene, but not TCPOBOP, a potent inducer of *CYP2B* genes in mice but not in rats (Fuchs *et al.*, 1994); prochloraz, aminopyrine and clofibrate (Amichot *et al.*, 1998). Thus, the major types of inducers known in the vertebrate toxicology literature, if not each specific chemical, have shown to be active as inducers in *Drosophila*. Specific genes induced are shown in Table 8. Biochemical techniques for the measurement of an Ah receptor in *Drosophila* have detected characteristic TCDD binding to a cytosolic fraction (Bigelow *et al.*, 1985, but see Hahn, 2002). The *Drosophila* genome carries 12 paralogs of the vertebrate Ah receptor gene and a probable ortholog, *spineless* (Hahn, 2002). The mechanism of aryl hydrocarbon induction in insects remains uncertain.

13.5.2.3. Phenobarbital-inducible genes Phenobarbital-like inducers include a variety of chemicals with widely divergent physicochemical properties. In mammals, these inducers are mostly tumor promoters (phenobarbital, polychlorinated biphenyls, chlordane) but they are not genotoxic. They must affect some important homeostatic process in cells, as phenobarbital induction is observed in plants,

nematodes, insects and mammals, and some bacteria. Thus phenobarbital may not be an ecologically relevant inducer of environmental response genes *sensu* Berenbaum (2002), but a physiologically relevant inducer *sensu* Nebert (1991). It highlights a pathway shared by many chemically unrelated compounds.

In vitro studies with vertebrate phenobarbital-inducible genes have identified a phenobarbital-responsive enhancer module (PBREM) in the distal regulatory element of *CYP2B* genes. Heterodimers of the nuclear receptor CAR and of the retinoid X receptor activate the PBREM of the mouse *Cyp2b10* gene (Honkakoski and Negishi, 2000; Wei *et al.*, 2000). Both the CAR and the PXR nuclear receptors are involved in the induction of drug metabolizing enzymes in vertebrates. In the case of phenobarbital induction in *Drosophila*, the definition of *cis*- and *trans*-regulatory elements has begun. Transfection experiments with *Cyp6a2*/luciferase reporter constructs showed that dose-dependent phenobarbital induction can be studied in this system. Promoter elements sufficient for directing both basal and phenobarbital-inducible expression are located within 428 bp of 5' of the start codon (Dunkov *et al.*, 1997). Elements further upstream (984 and 1328 bp) are needed for higher basal activity. *In vivo* experiments with transgenic flies showed that (pheno)barbital induction was functional with 1331 or 985 bp of 5' upstream DNA, whereas 129 bp upstream of the start codon, while conferring a low level of basal expression, does not support induction (Dombrowski *et al.*, 1998). Further experiments with transgenic flies carrying the luciferase reporter gene driven by promoter sequences of the *Cyp6a8* gene showed low basal activity of a -11/-199 bp construct, with higher basal activities seen with 761 and 3100 bp of upstream DNA (Maitra *et al.*, 2002). Phenobarbital inducibility was apparent with the three types of constructs, the highest level of induced activity being achieved with the -11/-761 bp promoter region. These studies thus consistently identify a small upstream region of the promoter that appears necessary and sufficient for phenobarbital inducibility, and all point to the presence of barbie box-like sequences in the promoter region of these inducible genes (Dunkov *et al.*, 1997; Dombrowski *et al.*, 1998; Maitra *et al.*, 2002). Barbie box sequences are difficult to define structurally in insects as the only functionally verified sequences are bacterial (Shaw and Fulco, 1993). However, their distribution is not random, and has been observed upstream of phenobarbital-inducible P450 genes of other species

Table 8 Inducers of P450 genes in insects

<i>Inducers</i>	<i>P450 induced</i>	<i>Reference</i>
Hormones		
HTH	<i>CYP4C1</i>	Bradfield <i>et al.</i> (1991); Lu <i>et al.</i> (1995, 1999)
Ecdysteroids	<i>Cyp6a2, Cyp18</i>	Hurban and Thummel (1993); Spiegelman <i>et al.</i> (1997)
Ovarian hormone ^a	<i>CYP4C7</i>	Sutherland <i>et al.</i> (2000)
Alkaloids		
Nicotine	<i>CYP4M1, CYP4M3</i>	Snyder <i>et al.</i> (1995a)
Senita/saguaro cactus alkaloids	<i>CYP28A1, A2, A3, CYP4D10</i>	Danielson <i>et al.</i> (1997, 1998); Fogleman <i>et al.</i> (1998)
Terpenoids		
Monoterpenes (peppermint oil)	<i>CYP6B2</i>	Ranasinghe <i>et al.</i> (1997)
α -Pinene	<i>CYP6B2, CYP6B7</i>	Ranasinghe <i>et al.</i> (1997); Ranasinghe and Hobbs (1999b)
Menthol	<i>CYP6B2</i>	Ranasinghe <i>et al.</i> (1997)
Gossypol	<i>CYP6B27</i>	Li <i>et al.</i> (2002c)
Derived from phenylpropanoid pathway		
Salicylic acid	<i>CYP6B8, B9, B27, B28</i>	Li <i>et al.</i> (2002d)
Chlorogenic acid	<i>CYP6B8, B9, B27, B28</i>	Li <i>et al.</i> (2002c)
Coumarins		
Coumarin	<i>CYP6B27</i>	Li <i>et al.</i> (2002c)
Xanthotoxin	<i>CYP6B1, B3, B4, B8, B9, B17, B27, B28, CYP9A2, A4, A5</i>	Cohen <i>et al.</i> (1992); Hung <i>et al.</i> (1995b); Stevens <i>et al.</i> (2000); Li <i>et al.</i> (2000a, 2001, 2002c)
Bergapten	<i>CYP6B3</i>	Hung <i>et al.</i> (1995a)
Angelicin	<i>CYP6B3</i>	Hung <i>et al.</i> (1995a)
Sphondin	<i>CYP6B3</i>	Hung <i>et al.</i> (1995a)
Flavonoids		
Flavone	<i>CYP6B8, B9, B27, B28</i>	Li <i>et al.</i> (2002c)
Quercetin	<i>CYP6B8</i>	Li <i>et al.</i> (2002c)
Rutin	<i>CYP6B8, B27, B28</i>	Li <i>et al.</i> (2002c)
Various natural products		
Ethanol	<i>CYP6A1</i>	Cariño <i>et al.</i> (1992)
2-tridecanone	<i>CYP4M3, CYP6A2</i>	Snyder <i>et al.</i> (1995); Stevens <i>et al.</i> (2000)
2-undecanone	<i>CYP4M1, M3, CYP9A2, A4, A5</i>	Snyder <i>et al.</i> (1995); Stevens <i>et al.</i> (2000)
Jasmonic acid	<i>CYP6B8, B9, B27, B28</i>	Li <i>et al.</i> (2002d)
Indole-3-carbinol	<i>CYP6B8, B9, B27, B28, CYP9A2</i>	Stevens <i>et al.</i> (2000); Li <i>et al.</i> (2002c)
Synthetic chemicals		
Phenobarbital	<i>CYP4D10, Cyp4e2, CYP4L2, CYP4M1, CYP4M3, CYP6A1, Cyp6a2, CYP6B7, CYP6B8, CYP6B9, CYP6B27, CYP6B28, CYP6D1, CYP6D3, CYP9A2, CYP12A1, CYP28A1, A2, A3</i>	Snyder <i>et al.</i> (1995); Brun <i>et al.</i> (1996); Dunkov <i>et al.</i> (1996); Danielson <i>et al.</i> (1997, 1998); Guzov <i>et al.</i> (1998); Ranasinghe and Hobbs (1999a); Li <i>et al.</i> (2000a, 2002c), Kasai and Scott (2001b)
Barbital	<i>Cyp6a2, Cyp6a8, Cyp6a9</i>	Maitra <i>et al.</i> (1996), Dombrowski <i>et al.</i> (1998)
DDT	<i>Cyp12d1/2</i>	Brandt <i>et al.</i> (2002)
Alkylbenzenes (pentamethyl benzene)	<i>CYP4</i>	Scharf <i>et al.</i> (2001)
Butylated hydroxyanisole	<i>CYP6B2</i>	Ranasinghe <i>et al.</i> (1997)
Piperonyl butoxide	<i>CYP6A1, CYP6B2</i>	Cariño <i>et al.</i> (1992); Ranasinghe <i>et al.</i> (1997)
Clofibrate	<i>CYP4M1, M3, CYP9A2, 4</i>	Snyder <i>et al.</i> (1995); Stevens <i>et al.</i> (2000)
<i>p</i> -Hydroxybenzoate	<i>CYP6B8, B9, B27, B28</i>	Li <i>et al.</i> (2002d)
Permethrin	<i>CYP6X1</i>	Zhu and Snodgrass (2003)
Fenvalerate	<i>CYP6B7</i>	Ranasinghe and Hobbs (1999b)
Cypermethrin	<i>CYP6B7, B27, B28</i>	Ranasinghe and Hobbs (1999b); Li <i>et al.</i> (2002c)

^aIdentity of ovarian hormone unknown.

such as *CYP6A1*, *CYP6D1* (Dunkov *et al.*, 1997; Scott *et al.*, 1999), and of *CYP6B* genes (Hung *et al.*, 1996), of which some are phenobarbital-inducible (Li *et al.*, 2002c). The role of these sequences in regulating insect P450 genes thus remain conjectural. Microarray experiments show that less than 10% of the *Drosophila* P450 genes are inducible by phenobarbital, and this approach will facilitate the rational study of P450 induction in this model species.

Inducibility is under genetic control (Hallstrom, 1987), but is not trivial to quantify. Several pioneers have noted that induction in insecticide-resistant strains was modified (e.g., Terriere and Yu, 1974), often lower (in fold induction) than in susceptible strains in the housefly and in *Drosophila*. Non-inducibility maps to the resistance gene (Hallstrom *et al.*, 1982). Resistant strains have thus been called “constitutive mutants” (Hallstrom, 1985). It has been claimed that phenobarbital induction of *CYP6D1* is *due* to a *trans* acting factor on chromosome 2 of the housefly (Liu and Scott, 1997b). However, this claim is based on genetic crosses between an inducible multimarker strain *aabys*, and the pyrethroid-resistant strain LPR. In that strain, *CYP6D1* transcript levels are higher than those achieved in phenobarbital-treated *aabys* flies, so that lack of further induction cannot be characterized as refractoriness to induction (a term which should be reserved for a low, basal level that is not changed by phenobarbital treatment – a volunteer is not refractory to the draft). That study therefore has no relevance to the genetic control of phenobarbital inducibility, but merely confirms that a specific interaction of LPR chromosomes 1 and 2 is needed for high expression of *CYP6D1v1* (Liu and Scott, 1995, 1996).

13.5.2.4. Furanocoumarin-inducible genes The inducibility of *CYP6B* genes by furanocoumarins has been studied extensively in *Papilio* species (see Section 13.4.3.3). The *CYP6B1* and *CYP6B3* genes are inducible by the linear furanocoumarin xanthotoxin, and variably induced by bergapten, angelicin, and sphondin (Prapaipong *et al.*, 1994; Hung *et al.*, 1995a, 1995b). This variability may be the result of individual polymorphism in inducibility. The 5' flanking sequence of the *CYP6B1v3* gene comprising nt –838 to +22 (relative to the transcription start site) was fused to the reporter chloramphenicol acetyl transferase (CAT) gene and this construct was transfected into Sf9 cells (Prapaipong *et al.*, 1994). In this system, the promoter region of the *CYP6B3* gene had a low basal activity, and this was maximally induced (about twofold) by 2 µg xanthotoxin/ml culture. Thus, at least some of the sequences

required for induction are present in this upstream region, and the xanthotoxin signaling cascade is present in these cells from the generalist herbivore *Spodoptera frugiperda*. Analysis of the 5'-upstream region of the *CYP6B1*, *B3*, *B4*, and *B5* genes from *P. polyxenes* and *P. glaucus* indicated a high degree of similarity, with several putative regulatory elements being noted (Hung *et al.*, 1996). These include sequences similar to the XRE (xenobiotic response element) of the Ah receptor of vertebrates, the barbie box, or the ARE (antioxidant RE) of rodent GST genes. With the cloning of additional *CYP6B* genes, a number of additional sequences, conserved among *CYP6B* genes and with similarities to known regulatory sequences of other genes, have been recorded (Petersen *et al.*, 2001; Li *et al.*, 2002a; Petersen *et al.*, 2003). A region (–136 to –119 of the *CYP6B1* gene) named XRE-xan (for XRE-xanthotoxin) has reportedly been identified as a being required for basal transcription and xanthotoxin inducibility and is conserved among *CYP6B* genes (results quoted by Hung *et al.*, 1996; Berenbaum, 2002). Evidence for the function of this XRE-xan was presented in an extensive series of experiments with the promoter region of the *CYP6B1v3* gene driving expression of CAT in Sf9 cells (Petersen *et al.*, 2003). Serial deletion experiments from –5 kb to –97 indicated the presence of a negative regulatory element necessary for basal transcription between nt –228 and –146 and a strong positive element between –146 and +22. Xanthotoxin inducibility was similarly mapped to a region between –146 and –97. This region includes the CAAT box basal promoter element. Substitution mutagenesis of tracts of DNA within this region identified nt –136 to –119 (TGACTGGCAATTTTTTTT) as the element called XRE-xan. Sequences similar to the ecdysone RE (EcRE) and an ARE overlap on the 5' end of the XRE-xan. A region –37 to +22 was found to be necessary for both basal and induced activity and thus forms part of the core promoter of the *CYP6B1* gene. Mutagenesis of the EcRE portion 5' of the XRE-xan element slightly diminished basal and induced expression, but mutagenesis of the TGAC sequence common to the EcRE, ARE, and XRE-xan abolished basal and induced expression. However, this sequence is not conserved in the same region of the xanthotoxin-inducible *CYP6B3* gene. The induction pattern of the *CYP6B1v3* promoter fragment (–380 to +22) driving CAT expression in Sf9 cells revealed a significant induction by flavone, a coumarin, and angular furanocoumarins, in contrast to the pattern seen for the *CYP6B1* gene *in vivo* (Petersen *et al.*, 2003). This lack of fidelity may be caused by a different specificity of the receptor(s)

expressed in Sf9 cells from that of the *P. polyxenes* midgut receptor(s). It may also be due to the absence of additional regulatory elements upstream of -380 in the test system. The definition of xenobiotic-responsive elements of well-characterized P450 genes may facilitate their detection in other inducible genes (e.g., lepidopteran *CYP9A* genes; Stevens *et al.*, 2000), and will help identify the DNA-binding proteins that bind to them.

13.6. Conclusion and Prospects

Some examples of the multiple functions of insect P450 enzymes, of their complex biochemistry and of their toxicological and physiological importance have been presented. Is there a common thread and does the hopeless chaos of this chapter simply reflect our fragmentary knowledge? With evolution as the guide, we can now summarize the relative positions of the insect P450s that have been discussed on the phylogenetic tree of the P450 superfamily. Insect P450 sequences can be distinguished in four major clades, which fall into four subclasses of Gotoh's classification of P450 families (Gotoh, 1993) and this is schematically illustrated in **Figure 27**:

1. A large group of insect P450s comprising the CYP6, CYP9, CYP28 families as well as the CYP308-310 and CYP321 families is most closely related to vertebrate CYP3 and CYP5 families. Because of the initial "lumping" of CYP6B1 into the CYP6 family and later "splitting" of the CYP300s, this group is rather heterogeneous with regard to CYP families and subfamilies. Few of the CYP6 enzymes have been characterized, the CYP6A1 and 2 of Diptera are capable of metabolizing xenobiotics, and several CYP6B of Lepidoptera are known to metabolize a variety of furanocoumarins. Genes from this group appear to share the characteristics of "environmental response genes" as defined by Berenbaum (2002), specifically: (1) very high diversity; (2) proliferation by duplication events; (3) rapid rates of evolution; (4) occurrence in gene clusters; and (5) tissue- or temporal-specific expression. Of course these characteristics are not independent of each other, and they are difficult to measure objectively. Nonetheless, the multiple paralogs from these families can be dated to no more than 150-200 MYA and this gives a good idea of the dynamic nature of this clade's evolution.
2. Another clade includes the large CYP4 family that has members from vertebrates and insects, but also several P450 families from *C. elegans*,

as well as the insect CYP311, 312, 313, 316, and 325 families initially discovered with the sequence of the *Drosophila* and *Anopheles* genomes. This group of sequences is highly diversified, perhaps even more so than the CYP6/9/28 group, and some CYP4 genes are clearly inducible by xenobiotics. However, specialized physiological functions are recognized (Sutherland *et al.*, 1998) or proposed (Bradfield *et al.*, 1991) for some enzymes of this group.

3. The mitochondrial P450s of vertebrates and insects (as well as CYP10 of *Lymnea stagnalis* and CYP44A1 of *C. elegans*, both presumed mitochondrial P450 sequences) are monophyletic. Within the mitochondrial clade, the CYP12 family appears to behave like the CYP6/9/28 clade, as a rapidly evolving group of paralogous genes. Some of the other families of mitochondrial P450s are now clearly linked to the ecdysteroid metabolism pathway. Mitochondrial P450s of insects thus evolved differently from the vertebrate mitochondrial P450s and subcellular localization in mitochondria can no longer be considered as evidence for a role in endocrine physiology. These sequences are all derived from an ancestral microsomal P450, and are only distantly related to soluble bacterial P450s that rely on a similar tandem of redox partners (i.e., adrenodoxin and adrenodoxin reductase and their paralogs). It is likely that mitochondrial P450s evolved as a result of the mistargeting of a microsomal P450 after mutations affected the N-terminal sequence. Drastic changes in the redox partner interactions were probably not necessary as rabbit CYP2B4 activity is supported by the bacterial ferredoxin and ferredoxin reductases of the P450cam and P450lin systems (Bernhardt and Gunsalus, 1992). Also, truncated CYP1A1 targeted to mitochondria interacts productively with either adrenodoxin, P450 reductase, or bacterial flavodoxin (Anandatheerthavarada *et al.*, 2001).
4. In fact, mitochondrial sequences are more closely related to a group of sequences that include vertebrate microsomal CYP1, CYP2, CYP17, and CYP21 as well as insect CYP15, CYP18, and the CYP303-307 series. Several of these insect sequences represent enzymes involved in essential physiological functions (e.g., CYP15), as are CYP17 and CYP21 of vertebrates. The CYP2, however, are widely considered as "environmental response genes" in mammalian species.

No clear pattern emerges from this current knowledge of insect P450 evolution. There is not one

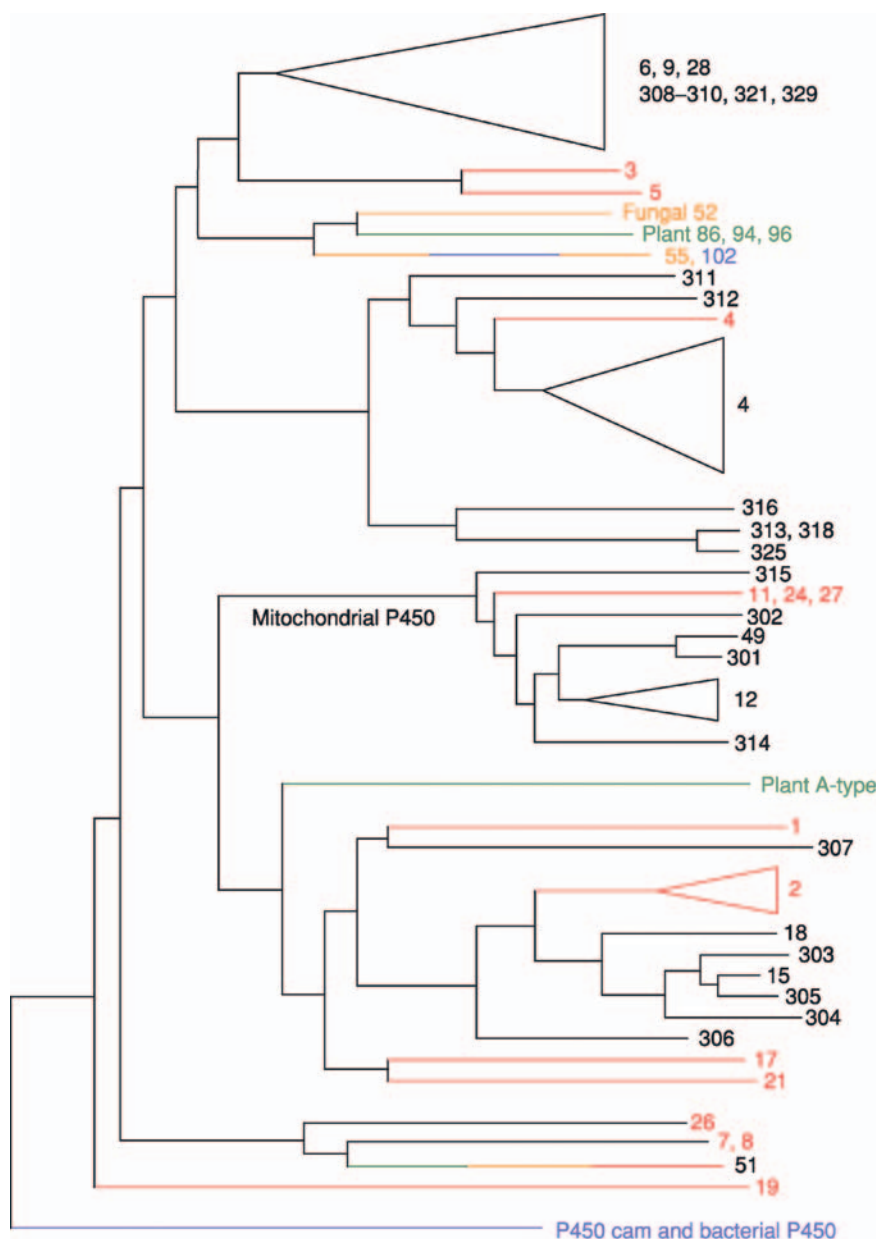


Figure 27 Schematic phylogeny of insect P450 genes and relationships with major P450 families from other organisms. The color code is: black, insects; red, vertebrates; brown, fungi; green, plants; blue, bacteria. Triangles represent families with large numbers of genes. Plant A-type P450 genes are found only in plants. CYP55 and CYP102 are P450foxy and P450BM3, fatty acid hydroxylases fused with a P450 reductase domain. CYP51 is the sterol 14 α -demethylase, the only P450 ortholog found in different phyla. The phylogeny is not drawn to scale. The deep branch topology may be revised and there are two main sources of bias: most P450 sequences are from Diptera, and the molecular clock is not constant. This is seen for instance in the deep branching of the steroid aromatase (CYP19).

class of P450s involved in physiological processes and another distinct class involved mostly in xenobiotic metabolism. Physiological functions are not restricted to one branch of the P450 evolutionary tree, and the ramifications that seem typical of “environmental response genes” are found in both microsomal and mitochondrial P450 encoding genes. Will this fuzzy image clear up with the availability

of more insect genome sequences, or will it be clouded even more by the discovery of dispersed physiological functions throughout the phylogeny?

There are many physiological functions for P450s that are not known or even suspected. In humans, mutations in the *CYP1B1* gene were linked to congenital glaucoma (Stoilov *et al.*, 1997), a clear physiological effect, still unexplained by what is known

of CYP1B1 biochemistry. In insects, CYP4C7 was shown to be selectively expressed in the corpora allata and to metabolize JH and its precursors to new metabolites (Sutherland *et al.*, 1998). Both the presence of this P450 in corpora allata and the existence of these metabolites were unexpected findings. Consequently, the search for new functions of P450 enzymes should involve broad, rational screens, and the technology is now available to perform such functional screens.

When significantly more data are obtained on the catalytic competence of a wide variety of insect P450 enzymes, it will become easier to understand the way in which insects maintain a wide repertoire of P450 genes. If positive selection of a few P450 genes can lead to specialized enzymes in oligophagous species (Li *et al.*, 2003), is this an evolutionary dead-end? Do the P450 enzymes with "broad and overlapping" specificity serve as a perpetual reservoir where some genes, because of their pattern of expression or inducibility or catalytic competence, can then serve as templates for the evolution of a new branch of specialized enzymes? Does this "primordial soup" perpetuate itself simply by a neutral process of intense gene duplication or are new chemical insults of the environment frequent enough to positively select for a minimal number of "jack-of-all-trades" P450 enzymes? How do P450 genes get recruited into physiological networks and biosynthetic pathways? The gap between "endogenous" and "xenobiotic" is being filled with new data and insights from P450 research (Nebert, 1991). Every 20 years or so researchers are discovering and re-discovering that treatments with inducers and inhibitors that cause major imbalances in P450 levels are deleterious to fitness in insects (Mitlin and Konecky, 1955; Yu and Terriere, 1974; Darvas *et al.*, 1992; Fuchs *et al.*, 1993). It is unlikely that these effects result solely from an interference with the handful of P450 enzymes involved in ecdysteroid and JH metabolism. Other regulatory pathways and other signal molecules remain to be discovered through a better understanding of insect P450 enzymes.

At the time of the first edition of this series, it seemed difficult enough to fully comprehend the function and role, the catalytic competence and the regulation of a single insect P450 enzyme. In the era of genomics, this task has now been multiplied by a hundred. It is hoped that interest in insect P450s will also grow by as much, and that new knowledge on insect P450 will continue to contribute to all branches of entomology, from toxicology to physiology and ecology.

References

- Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., *et al.*, 2000. The genome sequence of *Drosophila melanogaster*. *Science* 287, 2185–2195.
- Agosin, M., 1985. Role of microsomal oxidations in insecticide degradation. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 12. Pergamon, Oxford, pp. 647–712.
- Agrawal, A.A., Vala, F., Sabelis, M.W., 2002. Induction of preference and performance after acclimation to novel hosts in a phytophagous spider mite: adaptive plasticity? *Am. Nat.* 159, 553–565.
- Ahmad, S., 1986. Enzymatic adaptations of herbivorous insects and mites to phytochemicals. *J. Chem. Ecol.* 12, 533–560.
- Ahmad, S., Brattsten, L.B., Mullin, C.A., Yu, S.J., 1986. Enzymes involved in the metabolism of plant allelochemicals. In: Brattsten, L.B., Ahmad, S. (Eds.), *Molecular Aspects of Insect–Plant Interactions*. Plenum, New York, pp. 73–151.
- Ahmad, S., Kirkland, K.E., Blomquist, G.J., 1987. Evidence for a sex pheromone metabolizing cytochrome P-450 mono-oxygenase in the housefly. *Arch. Insect Biochem. Physiol.* 6, 21–40.
- Amichot, M., Brun, A., Cuany, A., Helvig, C., Salaun, J.P., *et al.*, 1994. Expression study of CYP genes in *Drosophila* strains resistant or sensitive to insecticides. In: Lechner, M.C. (Ed.), *Cytochrome P450*. John Libbey Eurotext, Paris, pp. 689–692.
- Amichot, M., Brun, A., Cuany, A., Souza, G.D., Mouel, T.L., *et al.*, 1998. Induction of cytochrome P450 activities in *Drosophila melanogaster* strains susceptible or resistant to insecticides. *Comp. Biochem. Physiol.* 121C, 311–319.
- Amichot, M., Tares, S., Brun-Barale, A., Arthaud, L., Bride, J.M., *et al.*, 2004. Point mutations associated with insecticide resistance in the *Drosophila* cytochrome P450 CYP6A2 enable DDT metabolism. *Eur. J. Biochem.* 271, 1250–1257.
- Anandatheerthavarada, H.K., Amuthan, G., Biswas, G., Robin, M.A., Murali, R., *et al.*, 2001. Evolutionary divergent electron donor proteins interact with P450MT2 through the same helical domain but different contact points. *EMBO J.* 20, 2394–2403.
- Anandatheerthavarada, H.K., Vijayasathya, C., Bhagwat, S.V., Biswas, G., Mullick, J., *et al.*, 1999. Physiological role of the N-terminal processed P4501A1 targeted to mitochondria in erythromycin metabolism and reversal of erythromycin-mediated inhibition of mitochondrial protein synthesis. *J. Biol. Chem.* 274, 6617–6625.
- Andersen, J.F., Ceruso, M., Unnithan, G.C., Kuwano, E., Prestwich, G.D., *et al.*, 1995. Photoaffinity labeling of methyl farnesoate epoxidase in cockroach corpora allata. *Insect Biochem. Mol. Biol.* 25, 713–719.
- Andersen, J.F., Utermohlen, J.G., Feyereisen, R., 1994. Expression of housefly CYP6A1 and NADPH-cytochrome P450 reductase in *Escherichia coli* and

- reconstitution of an insecticide-metabolizing P450 system. *Biochemistry* 33, 2171–2177.
- Andersen, J.F., Walding, J.K., Evans, P.H., Bowers, W.S., Feyereisen, R., 1997. Substrate specificity for the epoxidation of terpenoids and active site topology of housefly cytochrome P450 6A1. *Chem. Res. Toxicol.* 10, 156–164.
- Appel, H.M., Martin, M., 1992. Significance of metabolic load in the evolution of host specificity of *Manduca sexta*. *Ecology* 73, 216–228.
- Aragon, S., Claudinot, S., Blais, C., Maibeche, M., Dauphin-Villemant, C., 2002. Molting cycle-dependent expression of CYP4C15, a cytochrome P450 enzyme putatively involved in ecdysteroidogenesis in the crayfish, *Orconectes limosus*. *Insect Biochem. Mol. Biol.* 32, 153–159.
- Arbeitman, M.N., Furlong, E.E., Imam, F., Johnson, E., Null, B.H., et al., 2002. Gene expression during the life cycle of *Drosophila melanogaster*. *Science* 297, 2270–2275.
- Baldrige, G.D., Feyereisen, R., 1986. Blood meal and cytochrome P-450 monooxygenases in the northern house mosquito, *Culex pipiens*. *Pestic. Biochem. Physiol.* 25, 407–413.
- Barnes, H.J., Arlotto, M.P., Waterman, M.R., 1991. Expression and enzymatic activity of recombinant cytochrome P450 17 alpha-hydroxylase in *Escherichia coli*. *Proc. Natl Acad. Sci. USA* 88, 5597–5601.
- Bassett, M.H., McCarthy, J.L., Waterman, M.R., Sliter, T.J., 1997. Sequence and developmental expression of Cyp18, a member of a new cytochrome P450 family from *Drosophila*. *Mol. Cell. Endocrinol.* 131, 39–49.
- Berenbaum, M.R., 1990. Evolution of specialization in insect-umbellifer associations. *Annu. Rev. Entomol.* 35, 319–343.
- Berenbaum, M.R., 1995. The chemistry of defense: theory and practice. *Proc. Natl Acad. Sci. USA* 92, 2–8.
- Berenbaum, M.R., 1999. Animal–plant warfare: molecular basis for cytochrome P450-mediated natural adaptation. In: Puga, A., Wallace, K.B. (Eds.), *Molecular Biology of the Toxic Response*. Taylor & Francis, Philadelphia, pp. 553–571.
- Berenbaum, M.R., 2002. Postgenomic chemical ecology: from genetic code to ecological interactions. *J. Chem. Ecol.* 28, 873–896.
- Berenbaum, M.R., Cohen, M.B., Schuler, M.A., 1992. Cytochrome P450 monooxygenase genes in oligophagous lepidoptera. *ACS Symp. Ser.* 505, 114–124.
- Berenbaum, M.R., Favret, C., Schuler, M.A., 1996. On defining key innovations in an adaptive radiation – cytochrome P450s and Papilionidae. *Am. Nat.* 148, S139–S155.
- Berenbaum, M.R., Feeny, P., 1981. Toxicity of angular furanocoumarins to swallowtails: escalation in the coevolutionary arms race. *Science* 212, 927–929.
- Berenbaum, M.R., Neal, J.J., 1985. Synergism between myristicin and xanthotoxin, a naturally co-occurring plant toxicant. *J. Chem. Ecol.* 11, 1349–1358.
- Berenbaum, M.R., Neal, J.J., 1987. Interactions among allelochemicals and insect resistance in crop plants. *ACS Symp. Ser.* 330, 416–430.
- Bergé, J.B., Feyereisen, R., Amichot, M., 1998. Cytochrome P450 monooxygenases and insecticide resistance in insects. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 353, 1701–1705.
- Bernard, C.B., Philogene, B.J., 1993. Insecticide synergists: role, importance, and perspectives. *J. Toxicol. Environ. Health* 38, 199–223.
- Bernays, E., Graham, M., 1988. On the evolution of host specificity in phytophagous arthropods. *Ecology* 69, 886–892.
- Bernhardt, R., Gunsalus, I.C., 1992. Reconstitution of cytochrome P450B4 (LM2) activity with camphor and linalool monooxygenase electron donors. *Biochem. Biophys. Res. Commun.* 187, 310–317.
- Bertok, B., Pap, L., Arvai, G., Bakonyvari, I., Kuruczne Ribai, Z., 2003. Structure-activity relationship study of alkynyl ether insecticide synergists and the development of MB-599 (verbutin). *Pestic. Manag. Sci.* 59, 377–392.
- Bhagwat, S.V., Biswas, G., Anandatheerthavarada, H.K., Addya, S., Pandak, W., et al., 1999. Dual targeting property of the N-terminal signal sequence of P4501A1. Targeting of heterologous proteins to endoplasmic reticulum and mitochondria. *J. Biol. Chem.* 274, 24014–24022.
- Bigelow, S.W., Zijlstra, J.A., Vogel, E.W., Nebert, D.W., 1985. Measurements of the cytosolic Ah receptor among four stains of *Drosophila melanogaster*. *Arch. Toxicol.* 56, 219–225.
- Black, B.C., Hollingworth, R.M., Ahammadsahib, K.I., Kukel, C.D., Donovan, S., 1994. Insecticidal action and mitochondrial uncoupling activity of AC303,630 and related halogenated pyrroles. *Pestic. Biochem. Physiol.* 50, 115–128.
- Blais, C., Lafont, R., 1986. Ecdysone 20-hydroxylation in imaginal wing discs of *Pieris brassicae* (Lepidoptera): correlations with ecdysone and 20-hydroxyecdysone titers in pupae. *Arch. Insect Biochem. Physiol.* 3, 501–512.
- Blomquist, G.J., Dillwith, J.W., Pomonis, J.G., 1984. Sex pheromone of the housefly. Metabolism of (Z)-9-tricosene to (Z)-9,10-epoxytricosane and (Z)-14-tricosen-10-one. *Insect Biochem.* 14, 279–284.
- Bollenbacher, W.E., Smith, S.L., Wielgus, J.J., Gilbert, L.I., 1977. Evidence for an α -ecdysone cytochrome P-450 mixed function oxidase in insect fat body mitochondria. *Nature* 268, 660–663.
- Borras, T., Morozova, T.V., Heinsohn, S.L., Lyman, R.F., Mackay, T.F., et al., 2003. Transcription profiling in *Drosophila* eyes that overexpress the human glaucoma-associated trabecular meshwork-inductible glucocorticoid response protein/myocilin (TIGR/MYOC). *Genetics* 163, 637–645.
- Bowers, W.S., Ohta, T., Cleere, J.S., Marsella, P.A., 1976. Discovery of insect anti-juvenile hormones in plants. *Science* 193, 542–547.

- Bradfield, J.Y., Lee, Y.H., Keeley, L.L., 1991. Cytochrome P450 family 4 in a cockroach: molecular cloning and regulation by hypertrehalosemic hormone. *Proc. Natl Acad. Sci. USA* 88, 4558–4562.
- Brandt, A., Scharf, M., Pedra, J.H., Holmes, G., Dean, A., et al., 2002. Differential expression and induction of two *Drosophila* cytochrome P450 genes near the Rst(2)DDT locus. *Insect Mol. Biol.* 11, 337–341.
- Brattsten, L.B., 1979a. Ecological significance of mixed-function oxidations. *Drug Metab. Rev.* 10, 35–58.
- Brattsten, L.B., 1979b. Biochemical defense mechanisms in herbivores against plant allelochemicals. In: Rosenthal, G.A., Janzen, D.H. (Eds.), *Herbivores, their Interaction with Secondary Plant Metabolites*. Academic Press, New York, pp. 199–270.
- Brattsten, L.B., 1983. Cytochrome P-450 involvement in the interactions between plant terpenes and insect herbivores. In: Hedin, P.A. (Ed.), *Plant Resistance to Insects*. American Chemical Society, pp. 173–195.
- Brattsten, L.B., 1987. Sublethal virus infection depresses cytochrome P-450 in an insect. *Experientia* 43, 451–454.
- Brattsten, L.B., Metcalf, R.L., 1970. The synergistic ratio of carbaryl with piperonyl butoxide as an indicator of the distribution of multifunction oxidases in the insecta. *J. Econ. Entomol.* 63, 101–104.
- Brattsten, L.B., Wilkinson, C.F., 1977. Insecticide solvents: interference with insecticidal action. *Science* 196, 1211–1213.
- Brattsten, L.B., Wilkinson, C.F., Eisner, T., 1977. Herbivore-plant interactions: mixed-function oxidases and secondary plant substances. *Science* 196, 1349–1352.
- Bride, J.M., Cuany, A., Amichot, M., Brun, A., Babault, M., et al., 1997. Cytochrome P-450 field insecticide tolerance and development of laboratory resistance in grape vine populations of *Drosophila melanogaster* (Diptera: Drosophilidae). *J. Econ. Entomol.* 90, 1514–1520.
- Brindley, W.A., 1977. Synergist differences as an alternate interpretation of carbaryl-piperonyl butoxide toxicity data. *Environ. Entomol.* 6, 885–888.
- Brogdon, W.G., 1997. Heme peroxidase activity measured in single mosquitoes identifies individuals expressing an elevated oxidase for insecticide resistance. *J. Am. Mosq. Control Assoc.* 13, 233–237.
- Brooks, G.T., 1979. The metabolism of xenobiotics in insects. In: Bridges, J.W., Chasseaud, L. (Eds.), *Progress in Drug Metabolism*. Wiley, London, pp. 151–214.
- Brooks, G.T., Pratt, G.E., Mace, D.W., Cocks, J.A., 1985. Inhibitors of juvenile hormone biosynthesis in *corpura allata* of the cockroach *Periplaneta americana* (L.) *in vitro*. *Pestic. Sci.* 16, 132–142.
- Brown, T.M., Bryson, P.K., Payne, G.T., 1996. Synergism by propynyl aryl ethers in permethrin-resistant tobacco budworm larvae. *Pestic. Sci.* 46, 323–331.
- Brun, A., Cuany, A., Le Mouel, T., Berge, J., Amichot, M., 1996. Inducibility of the *Drosophila melanogaster* cytochrome P450 gene, CYP6A2, by phenobarbital in insecticide susceptible or resistant strains. *Insect Biochem. Mol. Biol.* 26, 697–703.
- Bull, D.L., Ivie, G.W., Beier, R.C., Pryor, N.W., 1986. In vitro metabolism of a linear furanocoumarin (8-methoxy-psoralen, xanthotoxin) by mixed-function oxidases of larvae of black swallowtail butterfly and fall armyworm. *J. Chem. Ecol.* 12, 885–892.
- Campbell, P.M., Newcomb, R.D., Russell, R.J., Oakeshott, J.G., 1998. Two different amino acid substitutions in the ali-esterase, E3, confer alternative types of organophosphorus insecticide resistance in the sheep blowfly, *Lucilia cuprina*. *Insect Biochem. Mol. Biol.* 28, 139–150.
- Capdevila, J.H., Harris, R.C., Falck, J.R., 2002. Microsomal cytochrome P450 and eicosanoid metabolism. *Cell. Mol. Life Sci.* 59, 780–789.
- Cariño, F., Koener, J.F., Plapp, F.W., Jr., Feyereisen, R., 1992. Expression of the cytochrome P450 gene CYP6A1 in the housefly, *Musca domestica*. *ACS Symp. Ser.* 505, 31–40.
- Cariño, F.A., Koener, J.F., Plapp, F.W., Jr., Feyereisen, R., 1994. Constitutive overexpression of the cytochrome P450 gene CYP6A1 in a housefly strain with metabolic resistance to insecticides. *Insect Biochem. Mol. Biol.* 24, 411–418.
- Casida, J.E., 1970. Mixed-function oxidase involvement in the biochemistry of insecticide synergists. *J. Agric. Food Chem.* 18, 753–771.
- Celniker, S.E., Wheeler, D.A., Kronmiller, B., Carlson, J.W., Halpern, A., et al., 2002. Finishing a whole-genome shotgun: Release 3 of the *Drosophila melanogaster* euchromatic genome sequence. *Genome Biol.* 3, 79.1–79.14.
- Chavez, V.M., Marques, G., Delbecque, J.P., Kobayashi, K., Hollingsworth, M., et al., 2000. The *Drosophila* disembodied gene controls late embryonic morphogenesis and codes for a cytochrome P450 enzyme that regulates embryonic ecdysone levels. *Development* 127, 4115–4126.
- Chawla, A., Repa, J.J., Evans, R.M., Mangelsdorf, D.J., 2001. Nuclear receptors and lipid physiology: opening the X-files. *Science* 294, 1866–1870.
- Chen, J.S., Berenbaum, M.R., Schuler, M.A., 2002. Amino acids in SRS1 and SRS6 are critical for furanocoumarin metabolism by CYP6B1v1, a cytochrome P450 monooxygenase. *Insect Mol. Biol.* 11, 175–186.
- Chen, C.D., Doray, B., Kemper, B., 1998. A conserved proline-rich sequence between the N-terminal signal-anchor and catalytic domains is required for assembly of functional cytochrome P450 2C2. *Arch. Biochem. Biophys.* 350, 233–238.
- Chen, J.H., Hara, T., Fisher, M.J., Rees, H.H., 1994. Immunological analysis of developmental changes in ecdysone 20-monooxygenase expression in the cotton leafworm, *Spodoptera littoralis*. *Biochem. J.* 299, 711–717.
- Cianfrogna, J.A., Zangerl, A.R., Berenbaum, M.R., 2002. Dietary and developmental influences on induced

- detoxification in an oligophage. *J. Chem. Ecol.* 28, 1349–1364.
- Cilek, J.E., Dahlman, D.L., Knapp, F.W., 1995. Possible mechanism of diazinon negative cross-resistance in pyrethroid-resistant horn flies (Diptera: Muscidae). *J. Econ. Entomol.* 88, 520–524.
- Claridge-Chang, A., Wijnen, H., Naef, F., Boothroyd, C., Rajewsky, N., *et al.*, 2001. Circadian regulation of gene expression systems in the *Drosophila* head. *Neuron* 32, 657–671.
- Clarke, S.E., Brealey, C.J., Gibson, G.G., 1989. Cytochrome P-450 in the housefly: induction, substrate specificity and comparison to three rat hepatic isoenzymes. *Xenobiotica* 19, 1175–1180.
- Claudianos, C., Crone, E., Coppin, C., Russell, R., Oakeshott, J.G., 2001. A genomics perspective on mutant aliesterases and metabolic resistance to organophosphates. In: Clark, J.M., Yamaguchi, I. (Eds.), *Agrochemical Resistance: Extent, Mechanism and Detection*. Oxford Press, Washington, pp. 90–101.
- Claudianos, C., Russell, R.J., Oakeshott, J.G., 1999. The same amino acid substitution in orthologous esterases confers organophosphate resistance on the housefly and a blowfly. *Insect Biochem. Mol. Biol.* 29, 675–686.
- Cohen, M.B., Berenbaum, M.R., Schuler, M.A., 1989. Induction of cytochrome P450-mediated detoxification of xanthotoxin in the black swallowtail. *J. Chem. Ecol.* 15, 2347–2355.
- Cohen, M.B., Feyereisen, R., 1995. A cluster of cytochrome P450 genes of the CYP6 family in the housefly. *DNA Cell Biol.* 14, 73–82.
- Cohen, M.B., Koener, J.F., Feyereisen, R., 1994. Structure and chromosomal localization of CYP6A1, a cytochrome P450-encoding gene from the housefly. *Gene* 146, 267–272.
- Cohen, M.B., Schuler, M.A., Berenbaum, M.R., 1992. A host-inducible cytochrome P-450 from a host-specific caterpillar: molecular cloning and evolution. *Proc. Natl Acad. Sci. USA* 89, 10920–10924.
- Couillaud, F., Debernard, S., Darrouzet, E., Rossignol, F., 1996. Hidden face of juvenile hormone metabolism in the African locust. *Arch. Insect Biochem. Physiol.* 32, 387–397.
- Crampton, A.L., Baxter, G.D., Barker, S.C., 1999. Identification and characterisation of a cytochrome P450 gene and processed pseudogene from an arachnid: the cattle tick, *Boophilus microplus*. *Insect Biochem. Mol. Biol.* 29, 377–384.
- Crankshaw, D.L., Hetnarski, K., Wilkinson, C.F., 1981. The functional role of NADPH-cytochrome *c* reductase in southern armyworm (*Spodoptera eridania*) midgut microsomes. *Insect Biochem.* 11, 515–522.
- Cuany, A., Helvig, C., Amichot, M., Pflieger, P., Mioskowski, C., *et al.*, 1995. Fate of a terminal olefin with *Drosophila* microsomes and its inhibitory effects on some P-450 dependent activities. *Arch. Insect Biochem. Physiol.* 28, 325–338.
- Cuany, A., Pralavorio, M., Pauron, D., Berge, J.B., Fournier, D., *et al.*, 1990. Characterization of microsomal oxidative activities in a wild-type and in a DDT resistant strain of *Drosophila melanogaster*. *Pestic. Biochem. Physiol.* 37, 293–302.
- Daborn, P., Boundy, S., Yen, J., Pittendrigh, B., ffrench-Constant, R., 2001. DDT resistance in *Drosophila* correlates with *Cyp6g1* over-expression and confers cross-resistance to the neonicotinoid imidacloprid. *Mol. Genet. Genomics* 266, 556–563.
- Daborn, P.J., Yen, J.L., Bogwitz, M.R., Le Goff, G., Feil, E., *et al.*, 2002. A single P450 allele associated with insecticide resistance in *Drosophila*. *Science* 297, 2253–2256.
- Danielson, P.B., Fogleman, J.C., 1997. Isolation and sequence analysis of cytochrome P450 12B1: the first mitochondrial insect P450 with homology to 1 alpha, 25 dihydroxy-D3 24-hydroxylase. *Insect Biochem. Mol. Biol.* 27, 595–604.
- Danielson, P.B., Foster, J.L., Cooper, S.K., Fogleman, J.C., 1999. Diversity of expressed cytochrome P450 genes in the adult Mediterranean fruit fly, *Ceratitis capitata*. *Insect Mol. Biol.* 8, 149–159.
- Danielson, P.B., Foster, J.L., McMahill, M.M., Smith, M.K., Fogleman, J.C., 1998. Induction by alkaloids and phenobarbital of Family 4 Cytochrome P450s in *Drosophila*: evidence for involvement in host plant utilization. *Mol. Gen. Genet.* 259, 54–59.
- Danielson, P.B., Letman, J.A., Fogleman, J.C., 1995. Alkaloid metabolism by cytochrome P-450 enzymes in *Drosophila melanogaster*. *Comp. Biochem. Physiol.* 110B, 683–688.
- Danielson, P.B., MacIntyre, R.J., Fogleman, J.C., 1997. Molecular cloning of a family of xenobiotic-inducible drosophilid cytochrome P450s: evidence for involvement in host-plant allelochemical resistance. *Proc. Natl Acad. Sci. USA* 94, 10797–10802.
- Dapkus, D., 1992. Genetic localization of DDT resistance in *Drosophila melanogaster* (Diptera: Drosophilidae). *J. Econ. Entomol.* 85, 340–347.
- Darrouzet, E., Mauchamp, B., Prestwich, G.D., Kerhoas, L., Ujvary, I., *et al.*, 1997. Hydroxy juvenile hormones: new putative juvenile hormones biosynthesized by locust corpora allata *in vitro*. *Biochem. Biophys. Res. Commun.* 240, 752–758.
- Darvas, B., Rees, H.H., Hoggard, N., 1993. Ecdysone 20-monoxygenase systems in flesh-flies (Diptera: Sarcophagidae), *Neobellieria bullata* and *Parasarcophaga argyrostoma*. *Comp. Biochem. Physiol.* 105B, 765–773.
- Darvas, B., Rees, H.H., Hoggard, N., Tag El-Din, M.H., Kuwano, E., *et al.*, 1992. Cytochrome P-450 inducers and inhibitors interfering with ecdysone 20-monoxygenases and their activities during postembryonic development of *Neobellieria bullata* Parker. *Pestic. Sci.* 36, 135–142.
- David, J.P., Rey, D., Cuany, A., Amichot, M., Meyran, J.C., 2000. Comparative ability to detoxify alder leaf

- litter in field larval mosquito collections. *Arch. Insect Biochem. Physiol.* 44, 143–150.
- David, J.P., Rey, D., Cuany, A., Bride, J.M., Meyran, J.C., 2002. Larvicidal properties of decomposed leaf litter in the subalpine mosquito breeding sites. *Environ. Toxicol. Chem.* 21, 62–66.
- Davis, R.H., Nahrstedt, A., 1987. Biosynthesis of cyanogenic glucosides in butterflies and moths. *Insect Biochem.* 17, 689–693.
- De Gregorio, E., Spellman, P.T., Rubin, G.M., Lemaitre, B., 2001. Genome-wide analysis of the *Drosophila* immune response by using oligonucleotide microarrays. *Proc. Natl Acad. Sci. USA* 98, 12590–12595.
- De Gregorio, E., Spellman, P.T., Tzou, P., Rubin, G.M., Lemaitre, B., 2002. The Toll and Imd pathways are the major regulators of the immune response in *Drosophila*. *EMBO J.* 21, 2568–2579.
- Delpuech, J.M., Aquadro, C.F., Roush, R.T., 1993. Non-involvement of the long terminal repeat of transposable element 17.6 in insecticide resistance in *Drosophila*. *Proc. Natl Acad. Sci. USA* 90, 5643–5647.
- De Mot, R., Parret, A.H.A., 2002. A novel class of self-sufficient cytochrome P450 monooxygenases in prokaryotes. *Trends Microbiol.* 10, 502–508.
- Denison, M.S., Nagy, S.R., 2003. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Ann. Rev. Pharmacol. Toxicol.* 43, 309–334.
- de Sousa, G., Cuany, A., Brun, A., Amichot, M., Rahmani, R., et al., 1995. A microfluorometric method for measuring ethoxycoumarin-O-deethylase activity on individual *Drosophila melanogaster* abdomens: interest for screening resistance in insect populations. *Anal. Biochem.* 229, 86–91.
- Dethier, V.G., 1954. Evolution of feeding preferences in phytophagous insects. *Evolution* 8, 33–54.
- Domanski, T.L., Halpert, J.R., 2001. Analysis of mammalian cytochrome P450 structure and function by site-directed mutagenesis. *Curr. Drug Metab.* 2, 117–137.
- Dombrowski, S.M., Krishnan, R., Witte, M., Maitra, S., Diesing, C., et al., 1998. Constitutive and barbital-induced expression of the *Cyp6a2* allele of a high producer strain of CYP6A2 in the genetic background of a low producer strain. *Gene* 221, 69–77.
- Dowd, P.F., Smith, C.M., Sparks, T.C., 1983. Detoxification of plant toxins by insects. *Insect Biochem.* 13, 453–468.
- Drabek, J., Neumann, R., 1985. Proinsecticides. In: Hutson, D.H., Roberts, T.R. (Eds.), *Insecticides*. Wiley, London, pp. 35–86.
- Dunkov, B.C., Guzov, V.M., Mocelin, G., Shotkoski, F., Brun, A., et al., 1997. The *Drosophila* cytochrome P450 gene *Cyp6a2*: structure, localization, heterologous expression, and induction by phenobarbital. *DNA Cell Biol.* 16, 1345–1356.
- Dunkov, B.C., Rodriguez-Arnaiz, R., Pittendigh, B., French-Constant, R.H., Feyerisen, R., 1996. Cytochrome P450 gene clusters in *Drosophila melanogaster*. *Mol. Gen. Genet.* 251, 290–297.
- Durham, E.W., Siegfried, B.D., Scharf, M.E., 2002. *In vivo* and *in vitro* metabolism of fipronil by larvae of the European corn borer *Ostrinia nubilalis*. *Pest Manag. Sci.* 58, 799–804.
- Ehrlich, P.R., Raven, P.H., 1964. Butterflies and plants: a study in coevolution. *Evolution* 18, 586–608.
- Estabrook, R.W., 1996. The remarkable P450s: a historical overview of these versatile heme protein catalysts. *FASEB J.* 10, 202–204.
- Feng, R., Houseman, J.G., Downe, A.E.R., 1992. Effect of ingested meridic diet and corn leaves on midgut detoxification processes in the European corn borer, *Ostrinia nubilalis*. *Pestic. Biochem. Physiol.* 42, 203–210.
- Feyereisen, R., 1977. Cytochrome P-450 et hydroxylation de l'ecdysone en ecdysterone chez *Locusta migratoria*. *C.R. Acad. Sci. Paris* 284, 1831–1834.
- Feyereisen, R., 1983. Polysubstrate monooxygenases (cytochrome P-450) in larvae of susceptible and resistant strains of house flies. *Pestic. Biochem. Physiol.* 19, 262–269.
- Feyereisen, R., 1999. Insect P450 enzymes. *Annu. Rev. Entomol.* 44, 507–533.
- Feyereisen, R., Baldrige, G.D., Farnsworth, D.E., 1985. A rapid method for preparing insect microsomes. *Comp. Biochem. Physiol.* 82B, 559–562.
- Feyereisen, R., Durst, F., 1978. Ecdysterone biosynthesis: a microsomal cytochrome-P-450-linked ecdysone 20-monooxygenase from tissues of the African migratory locust. *Eur. J. Biochem.* 88, 37–47.
- Feyereisen, R., Durst, F., 1980. Development of microsomal cytochrome P-450 monooxygenases during the last larval instar of the locust, *Locusta migratoria*: correlation with the hemolymph 20-hydroxyecdysone titer. *Mol. Cell. Endocrinol.* 20, 157–169.
- Feyereisen, R., Farnsworth, D.E., 1985. Developmental changes of microsomal cytochrome P-450 monooxygenases in larval and adult *Diptera punctata*. *Insect Biochem.* 15, 755–761.
- Feyereisen, R., Koener, J.F., Cariño, F.A., Daggett, A.S., 1990. Biochemistry and molecular biology of insect cytochrome P450. In: Hagedorn, H.H., Hildebrand, J.G., Kidwell, M.G., Law, J.H. (Eds.), *Molecular Insect Science*. Plenum, New York, pp. 263–272.
- Feyereisen, R., Koener, J.F., Farnsworth, D.E., Nebert, D.W., 1989. Isolation and sequence of cDNA encoding a cytochrome P-450 from an insecticide-resistant strain of the housefly, *Musca domestica*. *Proc. Natl Acad. Sci. USA* 86, 1465–1469.
- Feyereisen, R., Langry, K.C., Ortiz de Montellano, P.R., 1984. Self-catalyzed destruction of insect cytochrome P-450. *Insect Biochem.* 14, 19–26.
- Feyereisen, R., Pratt, G.E., Hamnett, A.F., 1981. Enzymic synthesis of juvenile hormone in locust corpora allata: evidence for a microsomal cytochrome P-450 linked methyl farnesoate epoxidase. *Eur. J. Biochem.* 118, 231–238.
- Feyereisen, R., Vincent, D.R., 1984. Characterization of antibodies to housefly NADPH-cytochrome P-450 reductase. *Insect Biochem.* 14, 163–168.

- french-Constant, R.H., Park, Y., Feyereisen, R., 1999. Molecular biology of insecticide resistance. In: Puga, A., Wallace, K.B. (Eds.), *Molecular Biology of the Toxic Response*. Taylor & Francis, Philadelphia, pp. 533–551.
- Fisher, T., Crane, M., Callaghan, A., 2003. Induction of cytochrome P450 activity in individual *Chironomus riparius* Meigen larvae exposed to xenobiotics. *Ecotoxicol. Environ. Saf.* 54, 1–6.
- Fogleman, J.C., 2000. Response of *Drosophila melanogaster* to selection for P450-mediated resistance to isoquinoline alkaloids. *Chem. Biol. Interact.* 125, 93–105.
- Fogleman, J.C., Danielson, P.B., 2000. Analysis of fragment homology among DNA sequences from cytochrome P450 families 4 and 6. *Genetica* 110, 257–265.
- Fogleman, J.C., Danielson, P.B., MacLntyre, R.J., 1998. The molecular basis of adaptation in *Drosophila*: the role of cytochrome P450s. In: Hecht, M., MacLntyre, R., Clegg, M. (Eds.), *Evolutionary Biology*, vol. 30. Plenum, New York, pp. 15–77.
- Fraenkel, G.S., 1959. The raison d'être of secondary plant substances. *Science* 129, 1466–1470.
- Frank, M.R., Fogleman, J.C., 1992. Involvement of cytochrome P450 in host-plant utilization by Sonoran Desert *Drosophila*. *Proc. Natl Acad. Sci. USA* 89, 11998–12002.
- Freeman, M.R., Dobritsa, A., Gaines, P., Segraves, W.A., Carlson, J.R., 1999. The dare gene: steroid hormone production, olfactory behavior, and neural degeneration in *Drosophila*. *Development* 126, 4591–4602.
- Frolov, M.V., Alatorsev, V.E., 1994. Cluster of cytochrome P450 genes on the X chromosome of *Drosophila melanogaster*. *DNA Cell Biol.* 13, 663–668.
- Fuchs, S., Spiegelman, V.S., Belitsky, G.A., 1993. The effect of the cytochrome P-450 system inducers on the development of *Drosophila melanogaster*. *J. Biochem. Toxicol.* 8, 83–88.
- Fuchs, S.Y., Spiegelman, V.S., Belitsky, G.A., 1994. Inducibility of various cytochrome P450 isozymes by phenobarbital and some other xenobiotics in *Drosophila melanogaster*. *Biochem. Pharmacol.* 47, 1867–1873.
- Fujii, S., Amrein, H., 2002. Genes expressed in the *Drosophila* head reveal a role for fat cells in sex-specific physiology. *EMBO J.* 21, 5353–5363.
- Fuji-Kuriyama, Y., Mizukami, Y., Kawajiri, K., Sogawa, K., Muramatsu, M., 1982. Primary structure of a cytochrome P-450: coding nucleotide sequence of phenobarbital-inducible cytochrome P-450 cDNA from rat liver. *Proc. Natl. Acad. Sci. USA* 79, 2793–2797.
- Furlong, E.E., Andersen, E.C., Null, B., White, K.P., Scott, M.P., 2001. Patterns of gene expression during *Drosophila* mesoderm development. *Science* 293, 1629–1633.
- Gandhi, R., Varak, E., Goldberg, M.L., 1992. Molecular analysis of a cytochrome P450 gene of family 4 on the *Drosophila* X chromosome. *DNA Cell Biol.* 11, 397–404.
- Glendinning, J.I., Slansky, F., Jr., 1995. Consumption of a toxic food by caterpillars increases with dietary exposure: support for a role of induced detoxification enzymes. *J. Comp. Physiol. A* 176, 337–345.
- Gordon, H.T., 1961. Nutritional factors in insect resistance to chemicals. *Annu. Rev. Entomol.* 6, 27–54.
- Gotoh, O., 1992. Substrate recognition sites in cytochrome P450 family 2 (CYP2) proteins inferred from comparative analyses of amino acid and coding nucleotide sequences. *J. Biol. Chem.* 267, 83–90.
- Gotoh, O., 1993. Evolution and differentiation of P-450 genes. In: Omura, T., Ishimura, Y., Fuji-Kuriyama, Y. (Eds.), *Cytochrome P-450*, 2nd edn. Kodansha, Tokyo, pp. 255–272.
- Gotoh, O., 1998. Divergent structures of *Caenorhabditis elegans* cytochrome P450 genes suggest the frequent loss and gain of introns during the evolution of nematodes. *Mol. Biol. Evol.* 15, 1447–1459.
- Gould, F., 1984. Mixed function oxidases and herbivore polyphagy: the devil's advocate position. *Ecol. Entomol.* 9, 29–34.
- Greenwood, D.R., Rees, H.H., 1984. Ecdysone 20-monooxygenase in the desert locust, *Schistocerca gregaria*. *Biochem. J.* 223, 837–847.
- Grieneisen, M.L., Warren, J.T., Gilbert, L.I., 1993. Early steps in ecdysteroid biosynthesis: evidence for the involvement of cytochrome P-450 enzymes. *Insect Biochem. Mol. Biol.* 23, 13–23.
- Gunderson, C.A., Brattsten, L.B., Fleming, J.T., 1986. Microsomal oxidase and glutathione transferase as factors influencing the effects of pulegone in southern and fall armyworm larvae. *Pestic. Biochem. Physiol.* 26, 238–249.
- Guzov, V.M., Houston, H.L., Murataliev, M.B., Walker, F.A., Feyereisen, R., 1996. Molecular cloning, overexpression in *Escherichia coli*, structural and functional characterization of housefly cytochrome *b*₅. *J. Biol. Chem.* 271, 26637–26645.
- Guzov, V.M., Unnithan, G.C., Chernogolov, A.A., Feyereisen, R., 1998. CYP12A1, a mitochondrial cytochrome P450 from the housefly. *Arch. Biochem. Biophys.* 359, 231–240.
- Hahn, M.E., 2002. Aryl hydrocarbon receptors: diversity and evolution. *Chem. Biol. Interact.* 141, 131–160.
- Hainzl, D., Casida, J.E., 1996. Fipronil insecticide: novel photochemical desulfinylation with retention of neurotoxicity. *Proc. Natl Acad. Sci. USA* 93, 12764–12767.
- Hainzl, D., Cole, L.M., Casida, J.E., 1998. Mechanisms for selective toxicity of fipronil insecticide and its sulfone metabolite and desulfinyl photoproduct. *Chem. Res. Toxicol.* 11, 1529–1535.
- Halliday, W.R., Farnsworth, D.E., Feyereisen, R., 1986. Hemolymph ecdysteroid titer and midgut ecdysone 20-monooxygenase activity during the last larval stage of *Diploptera punctata*. *Insect Biochem.* 16, 627–634.
- Hallstrom, I., 1985. Genetic regulation of the cytochrome P-450 system in *Drosophila melanogaster*: II. Localization of some genes regulating cytochrome P-450 activity. *Chem. Biol. Interact.* 56, 173–184.

- Hallstrom, I., 1987. Genetic variation in cytochrome P-450-dependent demethylation in *Drosophila melanogaster*. *Biochem. Pharmacol.* 36, 2279–2282.
- Hallstrom, I., Blanck, A., 1985. Genetic regulation of the cytochrome P-450 system in *Drosophila melanogaster*: I. Chromosomal determination of some cytochrome P-450-dependent reactions. *Chem. Biol. Interact.* 56, 157–171.
- Hallstrom, I., Grafstrom, R., 1981. The metabolism of drugs and carcinogens in isolated subcellular fractions of *Drosophila melanogaster*. II. Enzyme induction and metabolism of benzo[a]pyrene. *Chem. Biol. Interact.* 34, 145–159.
- Hallstrom, I., Magnusson, J., Ramel, C., 1982. Relation between the somatic toxicity of dimethylnitrosamine and a genetically determined variation in the level and induction of cytochrome P450 in *Drosophila melanogaster*. *Mutat. Res.* 92, 161–168.
- Halpern, M.E., Morton, R.A., 1987. Reproductive and developmental defects in a malathion-resistant laboratory-selected population of *Drosophila melanogaster*. *Pestic. Biochem. Physiol.* 28, 44–56.
- Hammock, B.D., 1975. NADPH dependent epoxidation of methyl farnesoate to juvenile hormone in the cockroach *Blaberus giganteus* L. *Life Sci.* 17, 323–328.
- Hammock, B.D., 1985. Regulation of juvenile hormone titer: degradation. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 7. Pergamon, Oxford, pp. 431–472.
- Hammock, B.D., Mumby, S.M., 1978. Inhibition of epoxidation of methyl farnesoate to juvenile hormone III by cockroach corpus allatum homogenates. *Pestic. Biochem. Physiol.* 9, 39–47.
- Harrison, P.M., Milburn, D., Zhang, Z., Bertone, P., Gerstein, M., 2003. Identification of pseudogenes in the *Drosophila melanogaster* genome. *Nucleic Acids Res.* 31, 1033–1037.
- Harrison, T.L., Zangerl, A.R., Schuler, M.A., Berenbaum, M.R., 2001. Developmental variation in cytochrome P450 expression in *Papilio polyxenes* in response to xanthotoxin, a hostplant allelochemical. *Arch. Insect Biochem. Physiol.* 48, 179–189.
- Harwood, S.H., Moldenke, A.F., Berry, R.E., 1990. Toxicity of peppermint monoterpenes to the variegated cutworm, *Peridroma saucia* Hubner (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 83, 1761–1767.
- Hasemann, C.A., Kurumbail, R.G., Boddupalli, S.S., Peterson, J.A., Deisenhofer, J., 1995. Structure and function of cytochromes P450: a comparative analysis of three crystal structures. *Structure* 3, 41–62.
- Hatano, R., Scott, J.G., 1993. Anti-P450_{lpr} antiserum inhibits the activation of chlorpyrifos to chlorpyrifos oxon in housefly microsomes. *Pestic. Biochem. Physiol.* 45, 228–233.
- Hatano, R., Scott, J.G., Dennehy, T.J., 1992. Enhanced activation is the mechanism of negative cross-resistance to chlorpyrifos in the Dicotol-IR strain of *Tetranychus urticae* (Acari: Tetranychidae). *J. Econ. Entomol.* 85, 1088–1091.
- He, H., Chen, A.C., Davey, R.B., Ivie, G.W., 2002. Molecular cloning and nucleotide sequence of a new P450 gene, CYP319A1, from the cattle tick, *Boophilus microplus*. *Insect Biochem. Mol. Biol.* 32, 303–309.
- Hehn, A., Morant, M., Werck-Reichhart, D., 2002. Partial recoding of P450 and P450 reductase cDNAs for improved expression in yeast and plants. *Methods in Enzymol.* 357, 343–351.
- Helvig, C., Koener, J.F., Unnithan, G.C., Feyereisen, R., 2004. CYP15A1, the cytochrome P450 that catalyzes epoxidation of methyl farnesoate to juvenile hormone III in cockroach corpora allata. *Proc. Natl Acad. Sci. USA* 101, 4024–4029.
- Hickey, D.A., Bally-Cuif, L., Abukashawa, S., Patyant, V., Benkel, B.F., 1991. Concerted evolution of duplicated protein coding genes in *Drosophila*. *Proc. Natl Acad. Sci. USA* 88, 1611–1615.
- Hodgson, E., 1985. Microsomal monooxygenases. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 11. Pergamon, Oxford, pp. 225–331.
- Hoggard, N., Fisher, M.J., Rees, H.H., 1989. Possible role for covalent modification in the reversible activation of ecdysone 20-monooxygenase activity. *Arch. Insect Biochem. Physiol.* 10, 241–253.
- Hoggard, N., Rees, H.H., 1988. Reversible activation-inactivation of mitochondrial ecdysone 20-mono-oxygenase: a possible role for phosphorylation-dephosphorylation. *J. Insect Physiol.* 34, 647–653.
- Hold, K.M., Sirisoma, N.S., Casida, J.E., 2001. Detoxification of alpha- and beta-Thujones (the active ingredients of absinthe): site specificity and species differences in cytochrome P450 oxidation in vitro and in vivo. *Chem. Res. Toxicol.* 14, 589–595.
- Holt, R.A., Subramanian, G.M., Halpern, A., Sutton, G.G., Charlab, R., et al., 2002. The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science* 298, 129–149.
- Holzkamp, G., Nahrstedt, A., 1994. Biosynthesis of cyanogenic glucosides in the lepidoptera. Incorporation of [U-14C]-2-methylpropanealdoxime, 2S-[U-14C]-nethylbutanealdoxime and -[U-14C]-N-hydroxyisoleucine into linamarin and lotaustralin by the larvae of *Zygaena trifolii*. *Insect Biochem. Mol. Biol.* 24, 161–165.
- Honkakoski, P., Negishi, M., 2000. Regulation of cytochrome P450 (CYP) genes by nuclear receptors. *Biochem. J.* 347, 321–337.
- Honkakoski, P., Sueyoshi, T., Negishi, M., 2003. Drug-activated nuclear receptors CAR and PXR. *Ann. Med.* 35, 172–182.
- Horike, N., Sonobe, H., 1999. Ecdysone 20-monooxygenase in eggs of the silkworm, *Bombyx mori*: enzymatic properties and developmental changes. *Arch. Insect Biochem. Physiol.* 41, 9–17.
- Horike, N., Takemori, H., Nonaka, Y., Sonobe, H., Okamoto, M., 2000. Molecular cloning of

- NADPH-cytochrome P450 oxidoreductase from silkworm eggs. Its involvement in 20-hydroxyecdysone biosynthesis during embryonic development. *Eur. J. Biochem.* 267, 6914–6920.
- Houpt, D.R., Pursey, J.C., Morton, R.A., 1988. Genes controlling malathion resistance in a laboratory-selected population of *Drosophila melanogaster*. *Genome* 30, 844–853.
- Hovemann, B.T., Sehlmeier, F., Malz, J., 1997. *Drosophila melanogaster* NADPH-cytochrome P450 oxidoreductase: pronounced expression in antennae may be related to odorant clearance. *Gene* 189, 213–219.
- Hughes, P.B., Devonshire, A.L., 1982. The biochemical basis of resistance to organophosphorus insecticides in the sheep blowfly, *Lucilia cuprina*. *Pestic. Biochem. Physiol.* 18, 289–297.
- Hung, C.F., Berenbaum, M.R., Schuler, M.A., 1997. Isolation and characterization of CYP6B4, a furanocoumarin-inducible cytochrome P450 from a polyphagous caterpillar (Lepidoptera:papilionidae). *Insect Biochem. Mol. Biol.* 27, 377–385.
- Hung, C.F., Harrison, T.L., Berenbaum, M.R., Schuler, M.A., 1995a. CYP6B3: a second furanocoumarin-inducible cytochrome P450 expressed in *Papilio polyxenes*. *Insect Mol. Biol.* 4, 149–160.
- Hung, C.F., Holzmacher, R., Connolly, E., Berenbaum, M.R., Schuler, M.A., 1996. Conserved promoter elements in the CYP6B gene family suggest common ancestry for cytochrome P450 monooxygenases mediating furanocoumarin detoxification. *Proc. Natl Acad. Sci. USA* 93, 12200–12205.
- Hung, C.F., Prapaipong, H., Berenbaum, M.R., Schuler, M.A., 1995b. Differential induction of cytochrome P-450 transcripts in *Papilio polyxenes* by linear and angular furanocoumarins. *Insect Biochem. Mol. Biol.* 25, 89–99.
- Hunt, D.W.A., Smirle, M.J., 1988. Partial inhibition of pheromone production in *Dendroctonus ponderosae* (Coleoptera: Scolytidae) by polysubstrate monooxygenase inhibitors. *J. Chem. Ecol.* 14, 529–536.
- Hurban, P., Thummel, C.S., 1993. Isolation and characterization of fifteen ecdysone-inducible *Drosophila* genes reveal unexpected complexities in ecdysone regulation. *Mol. Cell Biol.* 13, 7101–7111.
- Ikezawa, N., Morisaki, M., Fujimoto, Y., 1993. Sterol metabolism in insects: dealkylation of phytosterol to cholesterol. *Acc. Chem. Res.* 26, 139–146.
- Ikezawa, N., Tanaka, M., Nagayoshi, M., Shinkyō, R., Sakaki, T., et al., 2003. Molecular cloning and characterization of CYP719, a methylenedioxy bridge-forming enzyme that belongs to a novel P450 family, from cultured *Coptis japonica* cells. *J. Biol. Chem.* 278, 38557–38565.
- Issemann, I., Green, S., 1990. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature* 347, 645–650.
- Ivie, G.W., Bull, D.L., Beier, R.C., Pryor, N.W., Oertli, E.H., 1983. Metabolic detoxification: mechanism of insect resistance to plant psoralens. *Science* 221, 374–376.
- Iyengar, S., Arnason, J.T., Philogene, B.J.R., Werstiuk, N.H., Morand, P., 1990. Comparative metabolism of the phototoxic allelochemical α -terthienyl in three species of Lepidoptera. *Pestic. Biochem. Physiol.* 37, 154–164.
- Jansson, I., 1993. Posttranslational modification of cytochrome P450. In: Schenkman, J.B., Greim, H. (Eds.), *Cytochrome P450*. Springer, Berlin, pp. 561–580.
- Jefcoate, C.F., 1978. Measurement of substrate and inhibitor binding to microsomal cytochrome P-450 by optical-difference spectroscopy. *Methods Enzymol.* 52, 258–279.
- Jermey, T., 1984. Evolution of insect/host plant relationships. *Am. Naturalist.* 124, 609–630.
- Jin, W., Riley, R.M., Wolfinger, R.D., White, K.P., Passador-Gurgel, G., et al., 2001. The contributions of sex, genotype and age to transcriptional variance in *Drosophila melanogaster*. *Nat. Genet.* 29, 389–395.
- Johnson, P., Rees, H.H., 1977. The mechanism of C-20 hydroxylation of α -ecdysone in the desert locust, *Schistocerca gregaria*. *Biochem. J.* 168, 513–520.
- Jowett, T., Wajidi, M.F., Oxtoby, E., Wolf, C.R., 1991. Mammalian genes expressed in *Drosophila*: a transgenic model for the study of mechanisms of chemical mutagenesis and metabolism. *EMBO Journal* 10, 1075–1081.
- Kabbouh, M., Kappler, C., Hetru, C., Durst, F., 1987. Further characterization of the 2-deoxyecdysone C-2 hydroxylase from *Locusta migratoria*. *Insect Biochem.* 17, 1155–1161.
- Kahn, R.A., Bak, S., Svendsen, I., Halkier, B.A., Moller, B.L., 1997. Isolation and reconstitution of cytochrome P450ox and in vitro reconstitution of the entire biosynthetic pathway of the cyanogenic glucosidedhurrin from sorghum. *Plant Physiol.* 115, 1661–1670.
- Kamatani, T., Neal, R.A., 1976. Metabolism of diethyl p-nitrophenyl phosphorothionate (parathion) by a reconstituted mixed-function oxidase enzyme system: studies of the covalent binding of the sulfur atom. *Mol. Pharmacol.* 12, 933–944.
- Kao, L.M., Wilkinson, C.F., Brattsten, L.B., 1995. In vivo effects of 2,4-D and atrazine on cytochrome P-450 and insecticide toxicity in southern armyworm (*Spodoptera eridania*) larvae. *Pestic. Sci.* 45, 331–334.
- Kappers, W.A., Edwards, R.J., Murray, S., Boobis, A.R., 2001. Diazinon is activated by CYP2C19 in human liver. *Toxicol. Appl. Pharmacol.* 177, 68–76.
- Kappler, C., Kabbouh, M., Durst, F., Hoffmann, J.A., 1986. Studies on the C-2 hydroxylation of 2-deoxyecdysone in *Locusta migratoria*. *Insect Biochem.* 16, 25–32.
- Kappler, C., Kabbouh, M., Hetru, C., Durst, F., Hoffmann, J.A., 1988. Characterization of three hydroxylases involved in the final steps of biosynthesis of the steroid hormone ecdysone in *Locusta migratoria* (Insecta, Orthoptera). *J. Steroid Biochem.* 31, 891–898.

- Kasai, S., Scott, J.G., 2000. Overexpression of cytochrome P450 CYP6D1 is associated with monooxygenase-mediated pyrethroid resistance in house flies from Georgia. *Pestic. Biochem. Physiol.* 68, 34–41.
- Kasai, S., Scott, J.G., 2001a. Cytochrome P450s CYP6D3 and CYP6D1 are part of a P450 gene cluster on autosome 1 in the housefly. *Insect Mol. Biol.* 10, 191–196.
- Kasai, S., Scott, J.G., 2001b. Expression and regulation of CYP6D3 in the housefly, *Musca domestica* (L.). *Insect Biochem. Mol. Biol.* 32, 1–8.
- Kasai, S., Shono, T., Yamakawa, M., 1998a. Molecular cloning and nucleotide sequence of a cytochrome P450 cDNA from a pyrethroid-resistant mosquito, *Culex quinquefasciatus* say. *Insect Mol. Biol.* 7, 185–190.
- Kasai, S., Tomita, T., 2003. Male specific expression of a cytochrome P450 (*Cyp312a1*) in *Drosophila melanogaster*. *Biochem. Biophys. Res. Commun.* 300, 894–900.
- Kasai, S., Weerasinghe, I.S., Shono, T., 1998b. P450 monooxygenases are an important mechanism of permethrin resistance in *Culex quinquefasciatus* Say larvae. *Arch. Insect Biochem. Physiol.* 37, 47–56.
- Kasai, S., Weerasinghe, I.S., Shono, T., Yamakawa, M., 2000. Molecular cloning, nucleotide sequence and gene expression of a cytochrome P450 (CYP6F1) from the pyrethroid-resistant mosquito, *Culex quinquefasciatus* Say. *Insect Biochem. Mol. Biol.* 30, 163–171.
- Kayser, H., Eilinger, P., 2001. Metabolism of diafenthiuron by microsomal oxidation: proicide activation and inactivation as mechanisms contributing to selectivity. *Pest Manag. Sci.* 57, 975–980.
- Kayser, H., Ertl, P., Eilinger, P., Spindler-Barth, M., Winkler, T., 2002. Diastereomeric ecdysteroids with a cyclic hemiacetal in the side chain produced by cytochrome P450 in hormonally resistant insect cells. *Arch. Biochem. Biophys.* 400, 180–187.
- Kayser, H., Winkler, T., Spindler-Barth, M., 1997. 26-Hydroxylation of ecdysteroids is catalyzed by a typical cytochrome P-450-dependent oxidase and related to ecdysteroid resistance in an insect cell line. *Eur. J. Biochem.* 248, 707–716.
- Kennaugh, L., Pearce, D., Daly, J.C., Hobbs, A.A., 1993. A piperonyl butoxide synergizable resistance to permethrin in *Helicoverpa armigera* which is not due to increased detoxification by cytochrome P450. *Pestic. Biochem. Physiol.* 45, 234–241.
- Kennedy, G.G., 1984. 2-Tridecanone, tomatoes and *Heliothis zea*: potential incompatibility of plant antibiosis with insecticidal control. *Entomol. Exp. Appl.* 35, 305–311.
- Kirby, M.L., Ottea, J.A., 1995. Multiple mechanisms for enhancement of glutathione S-transferase activities in *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *Insect Biochem. Mol. Biol.* 25, 347–353.
- Kirby, M.L., Young, R.J., Ottea, J.A., 1994. Mixed-function oxidase and glutathione S-transferase activities from field-collected larval and adult tobacco budworms, *Heliothis virescens* (F.). *Pestic. Biochem. Physiol.* 49, 24–36.
- Koener, J.F., Cariño, F.A., Feyereisen, R., 1993. The cDNA and deduced protein sequence of housefly NADPH-cytochrome P450 reductase. *Insect Biochem. Mol. Biol.* 23, 439–447.
- Komori, M., Kitamura, R., Fukuta, H., Inoue, H., Baba, H., et al., 1993. Transgenic *Drosophila* carrying mammalian cytochrome P-4501A1: an application to toxicology testing. *Carcinogenesis* 14, 1683–1688.
- Konno, T.E.H., Dauterman, W.C., 1989. Studies on methyl parathion resistance in *Heliothis virescens*. *Pestic. Biochem. Physiol.* 33, 189–199.
- Korytko, P.J., MacLntyre, R.J., Scott, J.G., 2000a. Expression and activity of a house-fly cytochrome P450, CYP6D1, in *Drosophila melanogaster*. *Insect Mol. Biol.* 9, 441–449.
- Korytko, P.J., Scott, J.G., 1998. CYP6D1 protects thoracic ganglia of houseflies from the neurotoxic insecticide cypermethrin. *Arch. Insect Biochem. Physiol.* 37, 57–63.
- Korytko, P.J., Quimby, F.W., Scott, J.G., 2000b. Metabolism of phenanthrene by housefly CYP6D1 and dog liver cytochrome P450. *J. Biochem. Mol. Toxicol.* 14, 20–25.
- Kotze, A.C., 1995. Induced insecticide tolerance in larvae of *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae) following dietary phenobarbital treatment. *J. Austral. Entomol. Soc.* 34, 205–209.
- Kotze, A.C., Sales, N., 1995. Elevated in vitro monooxygenase activity associated with insecticide resistances in field-strain larvae of the Australian sheep blowfly (Diptera: Calliphoridae). *J. Econ. Entomol.* 88, 782–787.
- Krieger, R.I., Feeny, P.P., Wilkinson, C.F., 1971. Detoxication enzymes in the guts of caterpillars: an evolutionary answer to plant defenses? *Science* 172, 579–581.
- Kula, M.E., Rozek, C.E., 2000. Expression and translocation of *Drosophila* nuclear encoded cytochrome b(5) proteins to mitochondria. *Insect Biochem. Mol. Biol.* 30, 927–935.
- Kula, M.E., Allay, E.R., Rozek, C.E., 1995. Evolutionary divergence of the cytochrome *b*₅ gene of *Drosophila*. *J. Mol. Evol.* 41, 430–439.
- Latli, B., Prestwich, G.D., 1991. Metabolically blocked analogs of housefly sex pheromone: I. Synthesis of alternative substrates for the cuticular monooxygenases. *J. Chem. Ecol.* 17, 1745–1768.
- Le Goff, G., Boundy, S., Daborn, P.J., Yen, J.L., Sofer, L., et al., 2003. Microarray analysis of cytochrome P450 mediated insecticide resistance in *Drosophila*. *Insect Biochem. Mol. Biol.* 33, 701–708.
- Li, X., Berenbaum, M.R., Schuler, M.A., 2000a. Molecular cloning and expression of CYP6B8: a xanthotoxin-inducible cytochrome P450 cDNA from *Helicoverpa zea*. *Insect Biochem. Mol. Biol.* 30, 75–84.
- Li, X., Berenbaum, M.R., Schuler, M.A., 2002c. Plant allelochemicals differentially regulate *Helicoverpa zea* cytochrome P450 genes. *Insect Mol. Biol.* 11, 343–351.
- Li, W., Berenbaum, M.R., Schuler, M.A., 2001. Molecular analysis of multiple CYP6B genes from polyphagous

- Papilio* species. *Insect Biochem. Mol. Biol.* 31, 999–1011.
- Li, X., Berenbaum, M.R., Schuler, M.A., 2002b. Cytochrome P450 and actin genes expressed in *Helicoverpa zea* and *Helicoverpa armigera*: paralogy/orthology identification, gene conversion and evolution. *Insect Biochem. Mol. Biol.* 32, 311–320.
- Li, W., Petersen, R.A., Schuler, M.A., Berenbaum, M.R., 2002a. CYP6B cytochrome P450 monooxygenases from *Papilio canadensis* and *Papilio glaucus*: potential contributions of sequence divergence to host plant associations. *Insect Mol. Biol.* 11, 543–551.
- Li, X., Schuler, M.A., Berenbaum, M.R., 2002d. Jasmonate and salicylate induce expression of herbivore cytochrome P450 genes. *Nature* 419, 712–715.
- Li, W., Schuler, M.A., Berenbaum, M.R., 2003. Diversification of furanocoumarin-metabolizing cytochrome P450 monooxygenases in two papilionids: specificity and substrate encounter rate. *Proc. Natl Acad. Sci. USA* 100, 14593–14598.
- Li, X., Zangerl, A.R., Schuler, M.A., Berenbaum, M., 2000b. Cross-resistance to α -cypermethrin after xanthotoxin ingestion in *Helicoverpa zea* (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 93, 18–25.
- Liebrich, W., Hoffmann, K.H., 1991. Ecdysone 20-monooxygenase in a cricket, *Gryllus bimaculatus* (Ensifera, Gryllidae): characterization of the microsomal midgut steroid hydroxylase in adult females. *J. Comp. Physiol. B* 161, 93–99.
- Lindigkeit, R., Biller, A., Buch, M., Schiebel, H.M., Boppre, M., et al., 1997. The two facies of pyrrolizidine alkaloids: the role of the tertiary amine and its N-oxide in chemical defense of insects with acquired plant alkaloids. *Eur. J. Biochem.* 245, 626–636.
- Lindroth, R.L., 1989. Host plant alteration of detoxication activity in *Papilio glaucus glaucus*. *Entomol. Exp. Appl.* 50, 29–35.
- Lindroth, R.L., 1991. Differential toxicity of plant allelochemicals to insects: roles of enzymatic detoxication systems. In: Bernays, E.A. (Ed.), *Insect-Plant Interactions*, vol. 3. CRC Press, Boca Raton, pp. 1–33.
- Lindsley, D.L., Zimm, G.G., 1992. *The Genome of Drosophila melanogaster*. Academic Press, San Diego.
- Liu, N., Scott, J.G., 1995. Genetics of resistance to pyrethroid insecticides in the housefly, *Musca domestica*. *Pestic. Biochem. Physiol.* 52, 116–124.
- Liu, N., Scott, J.G., 1996. Genetic analysis of factors controlling high-level expression of cytochrome P450, CYP6D1, cytochrome *b*₅, P450 reductase, and monooxygenase activities in LPR house flies, *Musca domestica*. *Biochem. Genet.* 34, 133–148.
- Liu, N., Scott, J.G., 1997a. Inheritance of CYP6D1-mediated pyrethroid resistance in housefly (Diptera: Muscidae). *J. Econ. Entomol.* 90, 1478–1481.
- Liu, N., Scott, J.G., 1997b. Phenobarbital induction of CYP6D1 is due to a trans acting factor on autosome 2 in house flies, *Musca domestica*. *Insect Mol. Biol.* 6, 77–81.
- Liu, N., Scott, J.G., 1998. Increased transcription of CYP6D1 causes cytochrome P450-mediated insecticide resistance in housefly. *Insect Biochem. Mol. Biol.* 28, 531–535.
- Liu, N., Tomita, T., Scott, J.G., 1995. Allele-specific PCR reveals that CYP6D1 is on chromosome 1 in the housefly, *Musca domestica*. *Experientia* 51, 164–167.
- Liu, N., Yue, X., 2001. Genetics of pyrethroid resistance in a strain (ALHF) of house flies (Diptera: Muscidae). *Pestic. Biochem. Physiol.* 70, 151–158.
- Liu, N., Zhang, L., 2002. Identification of two new cytochrome P450 genes and their 5'-flanking regions from the housefly, *Musca domestica*. *Insect Biochem. Mol. Biol.* 32, 755–764.
- Lockey, K.H., 1991. Insect hydrocarbon classes: implications for chemotaxonomy. *Insect Biochem.* 21, 91–97.
- Lu, K.H., Bradfield, J.Y., Keeley, L.L., 1995. Hypertrehalosemic hormone-regulated gene expression for cytochrome P4504C1 in the fat body of the Cockroach, *Blaberus discoidalis*. *Arch. Insect Biochem. Physiol.* 28, 79–90.
- Lu, K.H., Bradfield, J.Y., Keeley, L.L., 1999. Juvenile hormone inhibition of gene expression for cytochrome P4504C1 in adult females of the cockroach, *Blaberus discoidalis*. *Insect Biochem. Mol. Biol.* 29, 667–673.
- Luu, B., Werner, F., 1996. Sterols that modify moulting in insects. *Pestic. Sci.* 46, 49–53.
- Lynch, M., Conery, J.S., 2000. The evolutionary fate and consequences of duplicate genes. *Science* 290, 1151–1155.
- Ma, R., Cohen, M.B., Berenbaum, M.R., Schuler, M.A., 1994. Black swallowtail (*Papilio polyxenes*) alleles encode cytochrome P450s that selectively metabolize linear furanocoumarins. *Arch. Biochem. Biophys.* 310, 332–340.
- Maibeche-Coisne, M., Jacquin-Joly, E., Francois, M.C., Nagnan-Le Meillour, P., 2002. cDNA cloning of bio-transformation enzymes belonging to the cytochrome P450 family in the antennae of the noctuid moth *Mamestra brassicae*. *Insect Mol. Biol.* 11, 273–281.
- Maibeche-Coisne, M., Monti-Dedieu, L., Aragon, S., Dauphin-Villemant, C., 2000. A new cytochrome P450 from *Drosophila melanogaster*, CYP4G15, expressed in the nervous system. *Biochem. Biophys. Res. Commun.* 273, 1132–1137.
- Maitra, S., Dombrowski, S.M., Basu, M., Raustol, O., Waters, L.C., et al., 2000. Factors on the third chromosome affect the level of *Cyp6a2* and *Cyp6a8* expression in *Drosophila melanogaster*. *Gene* 248, 147–156.
- Maitra, S., Dombrowski, S.M., Waters, L.C., Ganguly, R., 1996. Three second chromosome-linked clustered *Cyp6* genes show differential constitutive and barbital-induced expression in DDT-resistant and susceptible strains of *Drosophila melanogaster*. *Gene* 180, 165–171.
- Maitra, S., Price, C., Ganguly, R., 2002. *Cyp6a8* of *Drosophila melanogaster*: gene structure, and sequence and

- functional analysis of the upstream DNA. *Insect Biochem. Mol. Biol.* 32, 859–870.
- Mansuy, D., 1998. The great diversity of reactions catalyzed by cytochrome P450. *Comp. Biochem. Physiol.* 121C, 5–14.
- Mansuy, D., Renaud, J.P., 1995. Heme-thiolate proteins different from cytochromes P450 catalyzing monooxygenations. In: Ortiz de Montellano, P.R. (Ed.), *Cytochrome P450*. Plenum, New York and London, pp. 537–574.
- Martin, T., Ochou, O.G., Vaissayre, M., Fournier, D., 2003. Oxidases responsible for resistance to pyrethroids sensitize *Helicoverpa armigera* (Hubner) to triazophos in West Africa. *Insect Biochem. Mol. Biol.* 33, 883–887.
- Matsunaga, E., Umeno, M., Gonzalez, F.J., 1990. The rat P450 IID subfamily: complete sequences of four closely linked genes and evidence that gene conversions maintained sequence homogeneity at the heme-binding region of the cytochrome P450 active site. *J. Mol. Evol.* 30, 155–169.
- Mauchamp, B., Darrouzet, E., Malosse, C., Couillaud, F., 1999. 4'-OH-JH-III: an additional hydroxylated juvenile hormone produced by locust corpora allata in vitro. *Insect Biochem. Mol. Biol.* 29, 475–480.
- McDonald, M.J., Rosbash, M., 2001. Microarray analysis and organization of circadian gene expression in *Drosophila*. *Cell* 107, 567–578.
- Megias, A., Saborido, A., Muncio, A.M., 1984. NADH-cytochrome *b*₅ reductase from the insect *Ceratitidis capitata*. Enzyme properties and membrane binding capacity. *Comp. Biochem. Physiol.* 77B, 679–685.
- Miota, F., Siegfried, B.D., Scharf, M.E., Lydy, M.J., 2000. Atrazine induction of cytochrome P450 in *Chironomus tentans* larvae. *Chemosphere* 40, 285–291.
- Misra, S., Crosby, M.A., Mungall, C.J., Matthews, B.B., Campbell, K.S., et al., 2002. Annotation of the *Drosophila melanogaster* euchromatic genome: a systematic review. *Genome Biol.* 3, 83.1–83.22.
- Mitlin, N., Konecky, M.S., 1955. The inhibition of development in the housefly by piperonyl butoxide. *J. Econ. Entomol.* 48, 93–94.
- Moldenke, A.F., Berry, R.E., Miller, J.C., Kelsey, R.G., Wernz, J.G., et al., 1992. Carbaryl susceptibility and detoxication enzymes in gypsy moth (Lepidoptera: Lymantriidae): influence of host plant. *J. Econ. Entomol.* 85, 1628–1635.
- Mpuru, S., Blomquist, G.J., Schal, C., Roux, M., Kuenzli, M., et al., 2001. Effect of age and sex on the production of internal and external hydrocarbons and pheromones in the housefly, *Musca domestica*. *Insect Biochem. Mol. Biol.* 31, 139–155.
- Mpuru, S., Reed, J.R., Reitz, R.C., Blomquist, G.J., 1996. Mechanism of hydrocarbon biosynthesis from aldehyde in selected insect species: requirement for O₂ and NADPH and carbonyl group released as CO₂. *Insect Biochem. Mol. Biol.* 26, 203–208.
- Mullin, C.A., 1986. Adaptive divergence of chewing and sucking arthropods to plant allelochemicals. In: Brattsten, L.B., Ahmad, S. (Eds.), *Molecular Aspects of Insect-Plant Interactions*. Plenum, New York, pp. 175–209.
- Mullin, C.A., Croft, B.A., Strickler, K., Matsumura, F., Miller, J.R., 1982. Detoxification enzyme differences between a herbivorous and predatory mite. *Science* 217, 1270–1272.
- Murataliev, M.B., Ariño, A., Guzov, V.M., Feyereisen, R., 1999. Kinetic mechanism of cytochrome P450 reductase from the housefly (*Musca domestica*). *Insect Biochem. Mol. Biol.* 29, 233–242.
- Murataliev, M.B., Feyereisen, R., 1999. Mechanism of cytochrome P450 reductase from the housefly: evidence for an FMN semiquinone as electron donor. *FEBS Lett.* 453, 201–204.
- Murataliev, M.B., Feyereisen, R., 2000. Interaction of NADP(H) with oxidized and reduced P450 reductase during catalysis. Studies with nucleotide analogues. *Biochemistry* 39, 5066–5074.
- Murataliev, M.B., Feyereisen, R., Walker, F.A., 2004a. Electron transfer by diflavin reductases. *Biochem. Biophys. Acta* 1698, 1–26.
- Murataliev, M.B., Trinh, L.N., Moser, L.V., Bates, R.B., Feyereisen, R., et al., 2004b. Chimeragenesis of the fatty acid binding site of cytochrome P450BM3. Replacement of residues 73–84 with the homologous residues from the insect cytochrome P450 CYP4C7. *Biochemistry* 43, 1771–1780.
- Mutch, E., Daly, A.K., Leathart, J.B., Blain, P.G., Williams, F.M., 2003. Do multiple cytochrome P450 isoforms contribute to parathion metabolism in man? *Arch. Toxicol.* 77, 313–320.
- Nahri, L.O., Fulco, A.J., 1986. Characterization of a catalytically self-sufficient 119,000-dalton cytochrome P450 monooxygenase induced by barbiturates in *Bacillus megaterium*. *J. Biol. Chem.* 261, 7160–7169.
- Nahrstedt, A., 1988. Cyanogenesis and the role of cyanogenic compounds in insects. *Ciba Found. Symp.* 140, 131–150.
- Nakayama, N., Takemae, A., Shoun, H., 1996. Cytochrome P450foxy, a catalytically self-sufficient fatty acid hydroxylase of the fungus *Fusarium oxysporum*. *J. Biochem.* 119, 435–440.
- Naumann, C., Hartmann, T., Ober, D., 2002. Evolutionary recruitment of a flavin-dependent monooxygenase for the detoxication of host plant-acquired pyrrolizidine alkaloid-defended arctiid moth *Tyria Jacobaeae*. *Proc. Natl Acad. Sci. USA* 99, 6085–6090.
- Neal, J.J., 1987. Metabolic costs of mixed-function oxidase induction in *Heliothis zea*. *Entomol. Exp. Appl.* 43, 175–179.
- Neal, J.J., Berenbaum, M.R., 1989. Decreased sensitivity of mixed-function oxidases from *Papilio polyxenes* to inhibitors in host plants. *J. Chem. Ecol.* 15, 439–446.
- Neal, J.J., Reuveni, M., 1992. Separation of cytochrome P450 containing vesicles from the midgut microsomal fraction of *Manduca sexta*. *Comp. Biochem. Physiol.* 102C, 77–82.

- Neal, J.J., Wu, D., 1994. Inhibition of insect cytochromes P450 by furanocoumarins. *Pestic. Biochem. Physiol.* 50, 43–50.
- Nebert, D.W., 1991. Proposed role of drug-metabolizing enzymes: regulation of steady state levels of the ligands that effect growth, homeostasis, differentiation, and neuroendocrine functions. *Mol. Endocrinol.* 5, 1203–1214.
- Nebert, D.W., Adesnik, M., Coon, M.J., Estabrook, R.W., Gonzalez, F.J., *et al.*, 1987. The P450 gene superfamily: recommended nomenclature. *DNA* 6, 1–11.
- Nebert, D.W., Nelson, D.R., Coon, M.J., Estabrook, R.W., Feyereisen, R., *et al.*, 1991. The P450 superfamily: update on new sequences, gene mapping, and recommended nomenclature [published erratum appears in *DNA Cell Biol.* 1991 Jun;10(5):397–398]. *DNA Cell Biol.* 10, 1–14.
- Nelson, D.R., 1998. Metazoan cytochrome P450 evolution. *Comp. Biochem. Physiol.* 121C, 15–22.
- Nelson, D.R., Kamataki, T., Waxman, D.J., Guengerich, F.P., Estabrook, R.W., *et al.*, 1993. The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol.* 12, 1–51.
- Nelson, D.R., Koymans, L., Kamataki, T., Stegeman, J.J., Feyereisen, R., *et al.*, 1996. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* 6, 1–42.
- Newcomb, R.D., Campbell, P.M., Ollis, D.L., Cheah, E., Russell, R.J., *et al.*, 1997. A single amino acid substitution converts a carboxylesterase to an organophosphorus hydrolase and confers insecticide resistance on a blowfly. *Proc. Natl Acad. Sci. USA* 94, 7464–7468.
- Newcomb, M., Hollenberg, P.F., Coon, M.J., 2003. Multiple mechanisms and multiple oxidants in P450-catalyzed hydroxylations. *Arch. Biochem. Biophys.* 409, 72–79.
- Nikou, D., Ranson, H., Hemingway, J., 2003. An adult-specific CYP6 P450 gene is overexpressed in a pyrethroid-resistant strain of the malaria vector, *Anopheles gambiae*. *Gene* 318, 91–102.
- Nitao, J.K., 1989. Enzymatic adaptation in a specialist herbivore for feeding on furanocoumarin-containing plants. *Ecology* 70, 629–635.
- Nitao, J.K., 1990. Metabolism and excretion of the furanocoumarin xanthotoxin by parsnip webworm, *Depressaria pastinacella*. *J. Chem. Ecol.* 16, 417–428.
- Nitao, J.K., Berhow, M., Duval, S.M., Weisleder, D., Vaughn, S.F., *et al.*, 2003. Characterization of furanocoumarin metabolites in parsnip webworm, *Depressaria pastinacella*. *J. Chem. Ecol.* 29, 671–682.
- Oakeshott, J.G., Horne, I., Sutherland, T.D., Russell, R.J., 2003. The genomics of insecticide resistance. *Genome Biol.* 4, 202.
- Oesch-Bartlomowicz, B., Oesch, F., 2003. Cytochrome P450 phosphorylation as a functional switch. *Arch. Biochem. Biophys.* 409, 228–234.
- Oi, M., Dauterman, W.C., Motoyama, N., 1990. Biochemical factors responsible for an extremely high level of diazinon resistance in a housefly strain. *J. Pestic. Sci.* 15, 217–224.
- Omura, T., 1993. History of cytochrome P450. In: Omura, T., Ishimura, Y., Fujii-Kuriyama, Y. (Eds.), *Cytochrome P-450*. Kodansha, Tokyo, pp. 1–15.
- Omura, T., Ishimura, Y., Fujii-Kuriyama, Y. (Eds.), 1993. *Cytochrome P-450*. Kodansha, Tokyo.
- Omura, T., Sato, R., 1964. The carbon monoxide-binding pigment of liver microsomes I. Evidence for its hemo-protein nature. *J. Biol. Chem.* 239, 2370–2378.
- Ortiz de Montellano, P.R., 1995a. Oxygen activation and reactivity. In: Ortiz de Montellano, P.R. (Ed.), *Cytochrome P450*. Plenum, New York and London, pp. 245–303.
- Ortiz de Montellano, P.R. (Ed.), 1995b. *Cytochrome P450, Structure, Mechanism, and Biochemistry*. Plenum, New York and London.
- Ortiz de Montellano, P.R., Correia, M.A., 1995. Inhibition of cytochrome P450 enzymes. In: Ortiz de Montellano, P.R. (Ed.), *Cytochrome P450*. Plenum, New York and London, pp. 305–364.
- Ortiz de Montellano, P.R., Graham-Lorence, S.E., 1993. Structure of cytochrome P450: heme-binding site and heme reactivity. In: Schenkman, J.B., Greim, H. (Eds.), *Cytochrome P450*. Springer, Berlin, pp. 169–181.
- Paquette, S.M., Bak, S., Feyereisen, R., 2000. Intron-exon organization and phylogeny in a large superfamily, the paralogous cytochrome P450 genes of *Arabidopsis thaliana*. *DNA Cell Biol.* 19, 307–317.
- Pare, P.W., Alborn, H.T., Tumlinson, J.H., 1998. Concerted biosynthesis of an insect elicitor of plant volatiles. *Proc. Natl Acad. Sci. USA* 95, 13971–13975.
- Pauli, D., Tonka, C.H., 1987. A *Drosophila* heat shock gene from locus 67B is expressed during embryogenesis and pupation. *J. Mol. Biol.* 198, 235–240.
- Petersen, R.A., Niamsup, H., Berenbaum, M.R., Schuler, M.A., 2003. Transcriptional response elements in the promoter of CYP6B1, an insect P450 gene regulated by plant chemicals. *Biochimica et Biophysica Acta (BBA) – General Subjects* 1619, 269–282.
- Petersen, R.A., Zangerl, A.R., Berenbaum, M.R., Schuler, M.A., 2001. Expression of CYP6B1 and CYP6B3 cytochrome P450 monooxygenases and furanocoumarin metabolism in different tissues of *Papilio polyxenes* (Lepidoptera: Papilionidae). *Insect Biochem. Mol. Biol.* 31, 679–690.
- Petryk, A., Warren, J.T., Marques, G., Jarcho, M.P., Gilbert, L.I., *et al.*, 2003. Shade is the *Drosophila* P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. *Proc. Natl Acad. Sci. USA* 100, 13773–13778.
- Pikuleva, I.A., Cao, C., Waterman, M.R., 1999. An additional electrostatic interaction between adrenodoxin and P450c27 (CYP27A1) results in tighter binding than between adrenodoxin and P450scc (CYP11A1). *J. Biol. Chem.* 274, 2045–2052.
- Pikuleva, I.A., Mackman, R.L., Kagawa, N., Waterman, M.R., Ortiz de Montellano, P.R., 1995. Active-site

- topology of bovine cholesterol side-chain cleavage cytochrome P450 (P450_{scc}) and evidence for interaction of tyrosine 94 with the side chain of cholesterol. *Arch. Biochem. Biophys.* 322, 189–197.
- Pimprale, S.S., Besco, C.L., Bryson, P.K., Brown, T.M., 1997. Increased susceptibility of pyrethroid-resistant tobacco budworm (Lepidoptera: Noctuidae) to chlorfenvinpyr. *J. Econ. Entomol.* 90, 49–54.
- Pittendrigh, B., Aronstein, K., Zinkovsky, E., Andreev, O., Campbell, B., *et al.*, 1997. Cytochrome P450 genes from *Helicoverpa armigera*: expression in a pyrethroid-susceptible and resistant strain. *Insect Biochem. Mol. Biol.* 27, 507–512.
- Plapp, F.W., Jr., 1984. The genetic basis of insecticide resistance in the housefly: evidence that a single locus plays a major role in metabolic resistance to insecticides. *Pestic. Biochem. Physiol.* 22, 94–201.
- Plettner, E., Slessor, K.N., Winston, M.L., 1998. Biosynthesis of mandibular acids in honey bees (*Apis mellifera*): De novo synthesis, route of fatty acid hydroxylation and caste selective β -oxidation. *Insect Biochem. Mol. Biol.* 28, 31–42.
- Pompon, D., Louerat, B., Bronine, A., Urban, P., 1996. Yeast expression of animal and plant P450s in optimized redox environments. *Methods Enzymol.* 272, 51–64.
- Porter, T.D., 2002. The roles of cytochrome *b*₅ in cytochrome P450 reactions. *J. Biochem. Mol. Toxicol.* 16, 311–316.
- Porter, T.D., Kasper, C.B., 1986. NADPH-cytochrome P-450 oxidoreductase: flavin mononucleotide and flavin adenine dinucleotide domains evolved from different flavoproteins. *Biochemistry* 25, 1682–1687.
- Poulos, T.L., Cupp-Vickery, J., Li, H., 1995. Structural studies on prokaryotic cytochromes P450. In: Ortiz de Montellano, P.R. (Ed.), *Cytochrome P450*. Plenum, New York and London, pp. 125–150.
- Poulos, T.L., Finzel, B.C., Gunsalus, I.C., Wagner, G.C., Kraut, J., 1985. The 2.6-Å crystal structure of *Pseudomonas putida* cytochrome P-450. *J. Biol. Chem.* 260, 16122–16130.
- Prapaipong, H., Berenbaum, M.R., Schuler, M.A., 1994. Transcriptional regulation of the *Papilio polyxenes* CYP6B1 gene. *Nucleic Acids Res.* 22, 3210–3217.
- Pratt, G.E., Kuwano, E., Farnsworth, D.E., Feyereisen, R., 1990. Structure/activity studies on 1,5-disubstituted imidazoles as inhibitors of juvenile hormone biosynthesis in isolated corpora allata of the cockroach, *Diploptera punctata*. *Pestic. Biochem. Physiol.* 38, 223–230.
- Ranasinghe, C., Headlam, M., Hobbs, A.A., 1997. Induction of the mRNA for CYP6B2, a pyrethroid inducible cytochrome P450, in *Helicoverpa armigera* (Hubner) by dietary monoterpenes. *Arch. Insect Biochem. Physiol.* 34, 99–109.
- Ranasinghe, C., Hobbs, A.A., 1998. Isolation and characterization of two cytochrome P450 cDNA clones for CYP6B6 and CYP6B7 from *Helicoverpa armigera* (Hubner): possible involvement of CYP6B7 in pyrethroid resistance. *Insect Biochem. Mol. Biol.* 28, 571–580.
- Ranasinghe, C., Hobbs, A.A., 1999a. Isolation and characterization of a cytochrome *b*₅ cDNA clone from *Helicoverpa armigera* (Hubner): possible involvement of cytochrome *b*₅ in cytochrome P450 CYP6B7 activity towards pyrethroids. *Insect Biochem. Mol. Biol.* 29, 145–151.
- Ranasinghe, C., Hobbs, A.A., 1999b. Induction of cytochrome P450 CYP6B7 and cytochrome *b*₅ mRNAs from *Helicoverpa armigera* (Hubner) by pyrethroid insecticides in organ culture. *Insect Mol. Biol.* 8, 443–447.
- Ranson, H., Claudianos, C., Orтели, F., Abgrall, C., Hemingway, J., *et al.*, 2002a. Evolution of supergene families associated with insecticide resistance. *Science* 298, 179–181.
- Ranson, H., Nikou, D., Hutchinson, M., Wang, X., Roth, C.W., *et al.*, 2002b. Molecular analysis of multiple cytochrome P450 genes from the malaria vector, *Anopheles gambiae*. *Insect Mol. Biol.* 11, 409–418.
- Ravichandran, K.G., Boddupalli, S.S., Haseman, C.A., Peterson, J.A., Deisenhofer, J., 1993. Crystal structure of hemoprotein domain of P450BM-3, a prototype for microsomal P450's. *Science* 261, 731–736.
- Reed, J.R., Quilici, D.R., Blomquist, G.J., Reitz, R.C., 1995. Proposed mechanism for the cytochrome P450-catalyzed conversion of aldehydes to hydrocarbons in the housefly, *Musca domestica*. *Biochemistry* 34, 16221–16227.
- Reed, J.R., Vanderwel, D., Choi, S., Pomonis, J.G., Reitz, R.C., *et al.*, 1994. Unusual mechanism of hydrocarbon formation in the housefly: cytochrome P450 converts aldehyde to the sex pheromone component (Z)-9-tricosene and CO₂. *Proc. Natl Acad. Sci. USA* 91, 10000–10004.
- Rees, H.H., 1995. Ecdysteroid biosynthesis and inactivation in relation to function. *Eur. J. Entomol.* 92, 9–39.
- Ringo, J., Jona, G., Rockwell, R., Segal, D., Cohen, E., 1995. Genetic variation for resistance to chlorpyrifos in *Drosophila melanogaster* (Diptera: Drosophilidae) infesting grapes in Israel. *J. Econ. Entomol.* 88, 1158–1163.
- Riskallah, M.R., Dauterman, W.C., Hodgson, E., 1986a. Nutritional effects on the induction of cytochrome P-450 and glutathione transferase in larvae of the tobacco budworm, *Heliothis virescens* (F.). *Insect Biochem.* 16, 491–499.
- Riskallah, M.R., Dauterman, W.C., Hodgson, E., 1986b. Host plant induction of microsomal monooxygenases activity in relation to diazinon metabolism and toxicity in larvae of the tobacco budworm *Heliothis virescens* (F.). *Pestic. Biochem. Physiol.* 25, 233–247.
- Robertson, H.M., Martos, R., Sears, C.R., Todres, E.Z., Walden, K.K., *et al.*, 1999. Diversity of odourant binding proteins revealed by an expressed sequence tag project on male *Manduca sexta* moth antennae. *Insect Mol. Biol.* 8, 501–518.

- Ronis, M.J.J., Hodgson, E., Dauterman, W.C., 1988. Characterization of multiple forms of cytochrome P-450 from an insecticide resistant strain of housefly (*Musca domestica*). *Pestic. Biochem. Physiol.* 32, 74–90.
- Rose, H.A., 1985. The relationship between feeding specialization and host plants to aldrin epoxidase activities of midgut homogenates in larval Lepidoptera. *Ecol. Entomol.* 10, 455–467.
- Rose, R.L., Barbhuiya, L., Roe, R.M., Rock, G.C., Hodgson, E., 1995. Cytochrome P450-associated insecticide resistance and the development of biochemical diagnostic assays in *Heliothis virescens*. *Pestic. Biochem. Physiol.* 51, 178–191.
- Rose, R.L., Goh, D., Thompson, D.M., Verma, K.D., Heckel, D.G., et al., 1997. Cytochrome P450 (CYP)9A1 in *Heliothis virescens*: the first member of a new CYP family. *Insect Biochem. Mol. Biol.* 27, 605–615.
- Rose, R.L., Gould, F., Levi, P.E., Hodgson, E., 1991. Differences in cytochrome P450 activities in tobacco budworm larvae as influenced by resistance to host plant allelochemicals and induction. *Comp. Biochem. Physiol.* 99B, 535–540.
- Sabourault, C., Guzov, V.M., Koener, J.F., Claudianos, C., Plapp, F.W., Jr., et al., 2001. Overproduction of a P450 that metabolizes diazinon is linked to a loss-of-function in the chromosome 2 ali-esterase (Md α E7) gene in resistant house flies. *Insect Mol. Biol.* 10, 609–618.
- Salzemann, A., Nagnan, P., Tellier, F., Jaffe, K., 1992. Leaf-cutting ant *Atta laevigata* (Formicidae: Attini) marks its territory with colony-specific Dufour gland secretion. *J. Chem. Ecol.* 18, 183.
- Saner, C., Weibel, B., Wurgler, F.E., Sengstag, C., 1996. Metabolism of promutagens catalyzed by *Drosophila melanogaster* CYP6A2 enzyme in *Saccharomyces cerevisiae*. *Environ. Mol. Mutagen.* 27, 46–58.
- Scharf, M.E., Lee, C.Y., Neal, J.J., Bennett, G.W., 1999. Cytochrome P450 MA expression in insecticide-resistant German cockroaches (Dictyoptera: Blattellidae). *J. Econ. Entomol.* 92, 788–793.
- Scharf, M.E., Neal, J.J., Marcus, C.B., Bennett, G.W., 1998. Cytochrome P450 purification and immunological detection in an insecticide resistant strain of German cockroach (*Blattella germanica*, L.). *Insect Biochem. Mol. Biol.* 28, 1–9.
- Scharf, M.E., Parimi, S., Meinke, L.J., Chandler, L.D., Siegfried, B.D., 2001. Expression and induction of three family 4 cytochrome P450 (CYP4)* genes identified from insecticide-resistant and susceptible western corn rootworms, *Diabrotica virgifera virgifera*. *Insect Mol. Biol.* 10, 139–146.
- Scharf, M., Siegfried, B.D., Meinke, L.J., Chandler, L.D., 2000. Fipronil metabolism, oxidative sulfone formation and toxicity among organophosphate- and carbamate-resistant and susceptible western corn rootworm populations. *Pest Manag. Sci.* 56, 757–766.
- Schenkman, J.B., Greim, H. (Eds.), 1993. Cytochrome P450. Springer, Berlin.
- Schenkman, J.B., Jansson, I., 2003. The many roles of cytochrome *b*₅. *Pharmacol. Ther.* 97, 139–152.
- Schlenke, T.A., Begun, D.J., 2004. Strong selective sweep associated with a transposon insertion in *Drosophila simulans*. *Proc. Natl Acad. Sci. USA* 101, 1626–1631.
- Schlichting, I., Berendzen, J., Chu, K., Stock, A.M., Maves, S.A., et al., 2000. The catalytic pathway of cytochrome P450cam at atomic resolution. *Science* 287, 1615–1622.
- Schonbrod, R.D., Khan, M.A.Q., Terriere, L.C., Plapp, F.W., Jr., 1968. Microsomal oxidases in the housefly: a survey of fourteen strains. *Life Sci.* 7, 681–688.
- Schrag, M.L., Wienkers, L.C., 2000. Topological alteration of the CYP3A4 active site by the divalent cation Mg⁽²⁺⁾. *Drug Metab. Disposition* 28, 1198–1201.
- Schuler, M.A., Werck-Reichhart, D., 2003. Functional genomics of P450s. *Ann. Rev. Plant Biol.* 54, 629–667.
- Scott, J.G., 1999. Cytochromes P450 and insecticide resistance. *Insect Biochem. Mol. Biol.* 29, 757–777.
- Scott, J.A., Collins, F.H., Feyerisen, R., 1994. Diversity of cytochrome P450 genes in the mosquito, *Anopheles albimanus*. *Biochem. Biophys. Res. Commun.* 205, 1452–1459.
- Scott, J.G., Foroozesh, M., Hopkins, N.E., Alefantis, T.G., Alworth, W.L., 2000. Inhibition of cytochrome P450 6D1 by alkynylarenes, methylenedioxyarenes, and other substituted aromatics. *Pestic. Biochem. Physiol.* 67, 63–71.
- Scott, J.G., Lee, S.S., 1993a. Tissue distribution of microsomal cytochrome P-450 monooxygenases and their inducibility by phenobarbital in the insecticide resistant LPR strain of housefly, *Musca domestica* L. *Insect Biochem. Mol. Biol.* 23, 729–738.
- Scott, J.G., Lee, S.S., 1993b. Purification and characterization of a cytochrome P-450 from insecticide susceptible and resistant strains of housefly, *Musca domestica* L., before and after phenobarbital exposure. *Arch. Insect Biochem. Physiol.* 24, 1–19.
- Scott, J.G., Liu, N., Wen, Z., Smith, F.F., Kasai, S., et al., 1999. House-fly cytochrome P450 CYP6D1: 5' flanking sequences and comparison of alleles. *Gene* 226, 347–353.
- Scott, J.G., Sridhar, P., Liu, N., 1996. Adult specific expression and induction of cytochrome P450lpr in house flies. *Arch. Insect Biochem. Physiol.* 31, 313–323.
- Scott, J.G., Zhang, L., 2003. The housefly aliesterase gene (Md α E7) is not associated with insecticide resistance or P450 expression in three strains of housefly. *Insect Biochem. Mol. Biol.* 33, 139–144.
- Scrimshaw, S., Kerfoot, W.C., 1987. Chemical defenses of freshwater organisms: beetles and bugs. In: Kerfoot, W.C., Sih, A. (Eds.), *Predation: Direct and Indirect Impacts on Aquatic Communities*. University Press of New England, London, pp. 240–262.
- Seifert, J., Scott, J.G., 2002. The CYP6D1v1 allele is associated with pyrethroid resistance in the housefly, *Musca domestica*. *Pestic. Biochem. Physiol.* 72, 40–44.
- Shaw, G.C., Fulco, A.J., 1993. Inhibition by barbiturates of the binding of Bm3R1 repressor to its operator site

- on the barbiturate-inducible cytochrome P450BM-3 gene of *Bacillus megaterium*. *J. Biol. Chem.* 268, 2997–3004.
- Shen, B., Dong, H.Q., Tian, H.S., Ma, L., Li, X.L., *et al.*, 2003. Cytochrome P450 genes expressed in the delta-methrin-susceptible and -resistant strains of *Culex pipiens pallens*. *Pestic. Biochem. Physiol.* 75, 19–26.
- Sheppard, D.G., Joyce, J.A., 1998. Increased susceptibility of pyrethroid-resistant horn flies (Diptera: Muscidae) to chlorfenapyr. *J. Econ. Entomol.* 91, 398–400.
- Shergill, J.K., Cammack, R., Chen, J.H., Fisher, M.J., Madden, S., *et al.*, 1995. EPR spectroscopic characterization of the iron-sulphur proteins and cytochrome P-450 in mitochondria from the insect *Spodoptera littoralis* (cotton leafworm). *Biochem. J.* 307, 719–728.
- Shono, T., Kasai, S., Kamiya, E., Kono, Y., Scott, J.G., 2002. Genetics and mechanisms of permethrin resistance in the YPER strain of housefly. *Pestic. Biochem. Physiol.* 73, 27–36.
- Smith, S.L., 1985. Regulation of ecdysteroid titer: synthesis. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 7. Pergamon, Oxford, pp. 295–341.
- Smith, S.L., Bollenbacher, W.E., Cooper, D.Y., Schleyer, H., Wielgus, J.J., *et al.*, 1979. Ecdysone 20-monooxygenase: characterization of an insect cytochrome P-450 dependent steroid hydroxylase. *Mol. Cell. Endocrinol.* 15, 111–133.
- Smith, F.F., Scott, J.G., 1997. Functional expression of housefly (*Musca domestica*) cytochrome P450 CYP6D1 in yeast (*Saccharomyces cerevisiae*). *Insect Biochem. Mol. Biol.* 27, 999–1006.
- Snyder, M.J., Glendinning, J.I., 1996. Causal connection between detoxification enzyme activity and consumption of a toxic plant compound. *J. Comp. Physiol. A* 179, 255–261.
- Snyder, M.J., Hsu, E.-L., Feyereisen, R., 1993. Induction of cytochrome P450 activities by nicotine in the tobacco hornworm, *Manduca sexta*. *J. Chem. Ecol.* 19, 2903–2916.
- Snyder, M.J., Scott, J.A., Andersen, J.F., Feyereisen, R., 1996. Sampling P450 diversity by cloning polymerase chain reaction products obtained with degenerate primers. *Methods Enzymol.* 272, 304–312.
- Snyder, M.J., Stevens, J.L., Andersen, J.F., Feyereisen, R., 1995. Expression of cytochrome P450 genes of the CYP4 family in midgut and fat body of the tobacco hornworm, *Manduca sexta*. *Arch. Biochem. Biophys.* 321, 13–20.
- Snyder, M.J., Walding, J.K., Feyereisen, R., 1994. Metabolic fate of the allelochemical nicotine in the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol.* 24, 837–846.
- Spellman, P.T., Rubin, G.M., 2002. Evidence for large domains of similarly expressed genes in the *Drosophila* genome. *J. Biol.* 1, 5.
- Spiegelman, V.S., Fuchs, S.Y., Belitsky, G.A., 1997. The expression of insecticide resistance-related cytochrome P450 forms is regulated by molting hormone in *Drosophila melanogaster*. *Biochem. Biophys. Res. Commun.* 232, 304–307.
- Stathopoulos, A., Van Drenth, M., Erives, A., Markstein, M., Levine, M., 2002. Whole-genome analysis of dorsal-ventral patterning in the *Drosophila* embryo. *Cell* 111, 687–701.
- Stevens, J.L., Snyder, M.J., Koener, J.F., Feyereisen, R., 2000. Inducible P450s of the CYP9 family from larval *Manduca sexta* midgut. *Insect Biochem. Mol. Biol.* 30, 559–568.
- Stoilov, I., Akarsu, A.N., Sarfarazi, M., 1997. Identification of three different truncating mutations in cytochrome P4501B1 (CYP1B1) as the principal cause of primary congenital glaucoma (Buphthalmos) in families linked to the GLC3A locus on chromosome 2p21. *Hum. Mol. Genet.* 6, 641–647.
- Sundseth, S.S., Nix, C.E., Waters, L.C., 1990. Isolation of insecticide resistance-related forms of cytochrome P-450 from *Drosophila melanogaster*. *Biochem. J.* 265, 213–217.
- Sutherland, T.D., Unnithan, G.C., Andersen, J.F., Evans, P.H., Murataliev, M.B., *et al.*, 1998. A cytochrome P450 terpenoid hydroxylase linked to the suppression of insect juvenile hormone synthesis. *Proc. Natl Acad. Sci. USA* 95, 12884–12889.
- Sutherland, T.D., Unnithan, G.C., Feyereisen, R., 2000. Terpenoid omega-hydroxylase (CYP4C7) messenger RNA levels in the corpora allata: a marker for ovarian control of juvenile hormone synthesis in *Diploptera punctata*. *J. Insect Physiol.* 46, 1219–1227.
- Suwanchaichinda, C., Brattsten, L.B., 2002. Induction of microsomal cytochrome P450s by tire-leachate compounds, habitat components of *Aedes albopictus* mosquito larvae. *Arch. Insect Biochem. Physiol.* 49, 71–79.
- Tamura, T., Thibert, C., Royer, C., Kanda, T., Abraham, E., *et al.*, 2000. Germline transformation of the silkworm *Bombyx mori* L. using a piggyBac transposon-derived vector. *Nat. Biotechnol.* 18, 81–84.
- Tares, S., Berge, J., Amichot, M., 2000. Cloning and expression of cytochrome P450 genes belonging to the CYP4 family and to a novel family, CYP48, in two hymenopteran insects, *Trichogramma cacoeciae* and *Apis mellifera*. *Biochem. Biophys. Res. Commun.* 268, 677–682.
- Taylor, M., Feyereisen, R., 1996. Molecular biology and evolution of resistance of toxicants. *Mol. Biol. Evol.* 13, 719–734.
- Terriere, L.C., Yu, S.J., 1974. The induction of detoxifying enzymes in insects. *J. Agric. Food Chem.* 22, 366–373.
- Thomas, J.D., Ottea, J.A., Boethel, D.J., Ibrahim, S., 1996. Factors influencing pyrethroid resistance in a permethrin-resistant strain of the soybean looper, *Pseudoplusia includens* (Walker). *Pestic. Biochem. Physiol.* 55, 1–9.
- Thompson, M., Svoboda, J., Lusby, W., Rees, H., Oliver, J., *et al.*, 1985. Biosynthesis of a C21 steroid conjugate in an insect. The conversion of [¹⁴C]cholesterol

- to 5-[¹⁴C]pregnen-3 beta, 20 beta-diol glucoside in the tobacco hornworm, *Manduca sexta*. *J. Biol. Chem.* 260, 15410–15412.
- Tijet, N., Helvig, C., Feyereisen, R., 2001. The cytochrome P450 gene superfamily in *Drosophila melanogaster*: annotation, intron-exon organization and phylogeny. *Gene* 262, 189–198.
- Tomancak, P., Beaton, A., Weiszmam, R., Kwan, E., Shu, S., *et al.*, 2002. Systematic determination of patterns of gene expression during *Drosophila* embryogenesis. *Genome Biol.* 3, 88.1–88.14.
- Tomita, T., Liu, N., Smith, F.F., Sridhar, P., Scott, J.G., 1995. Molecular mechanisms involved in increased expression of a cytochrome P450 responsible for pyrethroid resistance in the housefly, *Musca domestica*. *Insect Mol. Biol.* 4, 135–140.
- Tomita, T., Scott, J.G., 1995. cDNA and deduced protein sequence of CYP6D1: the putative gene for a cytochrome P450 responsible for pyrethroid resistance in housefly. *Insect Biochem. Mol. Biol.* 25, 275–283.
- Ueda, H.R., Matsumoto, A., Kawamura, M., Iino, M., Tanimura, T., *et al.*, 2002. Genome-wide transcriptional orchestration of circadian rhythms in *Drosophila*. *J. Biol. Chem.* 277, 14048–14052.
- Ugaki, M., Shono, T., Fukami, J.I., 1985. Metabolism of fenitrothion by organophosphorus-resistant and susceptible house flies, *Musca domestica* L. *Pestic. Biochem. Physiol.* 23, 33–40.
- Unnithan, G.C., Andersen, J.F., Hisano, T., Kuwano, E., Feyereisen, R., 1995. Inhibition of juvenile hormone biosynthesis and methyl farnesoate epoxidase activity by 1,5-disubstituted imidazoles in the cockroach, *Diploptera punctata*. *Pestic. Sci.* 43, 13–19.
- Valles, S.M., Koehler, P.G., Brenner, R.J., 1997. Antagonism of fipronil toxicity by piperonyl butoxide and S,S,S-tributyl phosphorotrithioate in the German cockroach (Dictyoptera: Blattellidae). *J. Econ. Entomol.* 90, 1254–1258.
- Wada, A., Waterman, M.R., 1992. Identification by site-directed mutagenesis of two lysine residues in cholesterol side chain cleavage cytochrome P450 that are essential for adrenodoxin binding. *J. Biol. Chem.* 267, 22877–22882.
- Walsh, J.B., 1987. Sequence-dependent gene conversion: can duplicated genes diverge fast enough to escape conversion? *Genetics* 117, 543–557.
- Wang, L., Bieber Urbauer, R.J., Urbauer, J.L., Benson, D.R., 2003. Housefly cytochrome *b*₅ exhibits kinetically trapped hemin and selectivity in hemin binding. *Biochem. Biophys. Res. Commun.* 305, 840–845.
- Wang, Q., Hasan, G., Pikielny, C.W., 1999. Preferential expression of biotransformation enzymes in the olfactory organs of *Drosophila melanogaster*, the antennae. *J. Biol. Chem.* 274, 10309–10315.
- Wang, X.P., Hobbs, A.A., 1995. Isolation and sequence analysis of a cDNA clone for a pyrethroid inducible cytochrome P450 from *Helicoverpa armigera*. *Insect Biochem. Mol. Biol.* 25, 1001–1009.
- Wang, M., Roberts, D.L., Paschke, R., Shea, T.M., Masters, B.S., *et al.*, 1997. Three-dimensional structure of NADPH-cytochrome P450 reductase: prototype for FMN- and FAD-containing enzymes. *Proc. Natl Acad. Sci. USA* 94, 8411–8416.
- Warren, J.T., Petryk, A., Marques, G., Jarcho, M., Parvy, J.P., *et al.*, 2002. Molecular and biochemical characterization of two P450 enzymes in the ecdysteroidogenic pathway of *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA* 99, 11043–11048.
- Warren, J.T., Wismar, J., Subrahmanyam, B., Gilbert, L.I., 2001. Woc (without children) gene control of ecdysone biosynthesis in *Drosophila melanogaster*. *Mol. Cell. Endocrinol.* 181, 1–14.
- Waters, L.C., Nix, C.E., 1988. Regulation of insecticide resistance-related cytochrome P-450 expression in *Drosophila melanogaster*. *Pestic. Biochem. Physiol.* 30, 214–227.
- Waters, L.C., Zelhof, A.C., Shaw, B.J., Ch'ang, L.Y., 1992. Possible involvement of the long terminal repeat of transposable element 17.6 in regulating expression of an insecticide resistance-associated P450 gene in *Drosophila*. [published erratum appears in Proc. Natl Acad. Sci. USA 1992, 89, 12209]. *Proc. Natl Acad. Sci. USA* 89, 4855–4859.
- Wei, P., Zhang, J., Egan-Hafley, M., Liang, S., Moore, D.D., 2000. The nuclear receptor CAR mediates specific xenobiotic induction of drug metabolism. *Nature* 407, 920–923.
- Weirich, G.F., Williams, V.P., Feldlaufer, M.F., 1996. Ecdysone 20-hydroxylation in *Manduca sexta* midgut: kinetic parameters of mitochondrial and microsomal ecdysone 20-monooxygenases. *Arch. Insect Biochem. Physiol.* 31, 305–312.
- Weller, G.L., Foster, G.G., 1993. Genetic maps of the sheep blowfly *Lucilia cuprina*: linkage-group correlations with other dipteran genera. *Genome* 36, 495–506.
- Wen, Z., Horak, C.E., Scott, J.G., 2001. CYP9E2, CYP4C21 and related pseudogenes from German cockroaches, *Blattella germanica*: implications for molecular evolution, expression studies and nomenclature of P450s. *Gene* 272, 257–266.
- Wen, Z., Pan, L., Berenbaum, M.B., Schuler, M.A., 2003. Metabolism of linear and angular furanocoumarins by *Papilio polyxenes* CYP6B1 co-expressed with NADPH cytochrome P450 reductase. *Insect Biochem. Mol. Biol.* 33, 937–947.
- Wen, Z., Scott, J.G., 2001a. Cytochrome P450 CYP6L1 is specifically expressed in the reproductive tissues of adult male German cockroaches, *Blattella germanica* (L.). *Insect Biochem. Mol. Biol.* 31, 179–187.
- Wen, Z., Scott, J.G., 2001b. Cloning of two novel P450 cDNAs from German cockroaches, *Blattella germanica* (L.): CYP6K1 and CYP6J1. *Insect Mol. Biol.* 10, 131–137.
- Werck-Reichhart, D., Feyereisen, R., 2000. Cytochromes P450: a success story. *Genome Biol.* 1, 3003.1–3003.9.
- Wheeler, G.S., Slansky, F., Jr., Yu, S.J., 1993. Fall armyworm sensitivity to flavone: limited role of constitutive

- and induced detoxifying enzyme activity. *J. Chem. Ecol.* 19, 645–667.
- Wheelock, G.D., Scott, J.G., 1989. Simultaneous purification of a cytochrome P-450 and cytochrome *b*₅ from the housefly, *Musca domestica* L. *Insect Biochem.* 19, 481–488.
- Wheelock, G.D., Scott, J.G., 1990. Immunological detection of cytochrome P450 from insecticide resistant and susceptible house flies (*Musca domestica*). *Pestic. Biochem. Physiol.* 38, 130–139.
- Wheelock, G.D., Scott, J.G., 1992a. Anti-P450lpr antiserum inhibits specific monooxygenase activities in LPR housefly microsomes. *J. Exp. Zool.* 264, 153–158.
- Wheelock, G.D., Scott, J.G., 1992b. The role of cytochrome P450 in deltamethrin metabolism by pyrethroid-resistant and susceptible strains of house flies. *Pestic. Biochem. Physiol.* 43, 67–77.
- White, J.R.A., Franklin, R.T., Agosin, M., 1979. Conversion of α -pinene to α -pinene oxide by rat liver and the bark beetle *Dendroctonus terebrans* microsomal fractions. *Pestic. Biochem. Physiol.* 10, 233–242.
- White, K.P., Rifkin, S.A., Hurban, P., Hogness, D.S., 1999. Microarray analysis of *Drosophila* development during metamorphosis. *Science* 286, 2179–2184.
- Whittaker, R.H., Feeny, P.P., 1971. Allelochemicals: chemical interactions between species. *Science* 171, 757–770.
- Wilkinson, C.F., 1979. The use of insect subcellular components for studying the metabolism of xenobiotics. *ACS Symp. Ser.* 97, 249–284.
- Wilkinson, C.F., Brattsten, L.B., 1972. Microsomal drug metabolizing enzymes in insects. *Drug Metab. Rev.* 1, 153–228.
- Williams, D.R., Chen, J.H., Fisher, M.J., Rees, H.H., 1997. Induction of enzymes involved in molting hormone (ecdysteroid) inactivation by ecdysteroids and an agonist, 1,2-dibenzoyl-1-*tert*-butylhydrazine (RH-5849). *J. Biol. Chem.* 272, 8427–8432.
- Williams, P.A., Cosme, J., Sridhar, V., Johnson, E.F., McRee, D.E., 2000a. Mammalian microsomal cytochrome P450 monooxygenase: structural adaptations for membrane binding and functional diversity. *Mol. Cell* 5, 121–131.
- Williams, D.R., Fisher, M.J., Rees, H.H., 2000b. Characterization of ecdysteroid 26-hydroxylase: an enzyme involved in molting hormone inactivation. *Arch. Biochem. Biophys.* 376, 389–398.
- Wilson, T.G., 2001. Resistance of *Drosophila* to toxins. *Annu. Rev. Entomol.* 46, 545–571.
- Winter, J., Bilbe, G., Richener, H., Sehringer, B., Kayser, H., 1999. Cloning of a cDNA encoding a novel cytochrome P450 from the insect *Locusta migratoria*: CYP6H1, a putative ecdysone 20-hydroxylase. *Biochem. Biophys. Res. Commun.* 259, 305–310.
- Winter, J., Eckerskorn, C., Waditschatka, R., Kayser, H., 2001. A microsomal ecdysone-binding cytochrome P450 from the insect *Locusta migratoria* purified by sequential use of type-II and type-I ligands. *Biol. Chem.* 382, 1541–1549.
- Wojtasek, H., Leal, W.S., 1999. Degradation of an alkaloid pheromone from the pale-brown chafer, *Phyllolpertha diversa* (Coleoptera: Scarabaeidae), by an insect olfactory cytochrome P450. *FEBS Lett.* 458, 333–336.
- Xu, W., Bak, S., Decker, A., Paquette, S.M., Feyereisen, R., et al., 2001. Microarray-based analysis of gene expression in very large gene families: the cytochrome P450 gene superfamily of *Arabidopsis thaliana*. *Gene* 272, 61–74.
- Yamazaki, S., Sato, K., Suhara, K., Sakaguchi, M., Mihara, K., et al., 1993. Importance of the proline-rich region following signal-anchor sequence in the formation of correct conformation of microsomal cytochrome P-450s. *J. Biochem. (Tokyo)* 114, 652–657.
- Yu, S.J., 1984. Interactions of allelochemicals with detoxication enzymes of insecticide-susceptible and resistant fall armyworms. *Pestic. Biochem. Physiol.* 22, 60–68.
- Yu, S.J., 1986. Consequences of induction of foreign compound-metabolizing enzymes in insects. In: Brattsten, L.B., Ahmad, S. (Eds.), *Molecular Aspects of Insect-Plant Interactions*. Plenum, New York, pp. 211–255.
- Yu, S.J., 1987. Microsomal oxidation of allelochemicals in generalist (*Spodoptera frugiperda*) and semispecialist (*Anticarsia gemmatalis*) insects. *J. Chem. Ecol.* 13, 423–436.
- Yu, S.J., 1991. Insecticide resistance in the fall armyworm, *Spodoptera frugiperda* (J.E. Smith). *Pestic. Biochem. Physiol.* 39, 84–91.
- Yu, S.J., 1992. Detection and biochemical characterization of insecticide resistance in fall armyworm (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 85, 675–682.
- Yu, S.J., 1995. Allelochemical stimulation of ecdysone 20-monooxygenase in fall armyworm larvae. *Arch. Insect Biochem. Physiol.* 28, 365–375.
- Yu, S.J., Terriere, L.C., 1974. A possible role for microsomal oxidases in metamorphosis and reproduction in the housefly. *J. Insect Physiol.* 20, 1901–1912.
- Zdobnov, E.M., von Mering, C., Letunic, I., Torrents, D., Suyama, M., et al., 2002. Comparative genome and proteome analysis of *Anopheles gambiae* and *Drosophila melanogaster*. *Science* 298, 149–159.
- Zhang, L., Harada, K., Shono, T., 1997. Genetic analysis of pyriproxifen resistance in the housefly, *Musca domestica* L. *Appl. Ent. Zool.* 32, 217–226.
- Zhang, L., Kasai, S., Shono, T., 1998. In vitro metabolism of pyriproxifen by microsomes from susceptible and resistant housefly larvae. *Arch. Insect Biochem. Physiol.* 37, 215–224.
- Zhang, M., Scott, J.G., 1994. Cytochrome *b*₅ involvement in cytochrome P450 monooxygenase activities in housefly microsomes. *Arch. Insect Biochem. Physiol.* 27, 205–216.
- Zhang, M., Scott, J.G., 1996a. Purification and characterization of cytochrome *b*₅ reductase from the housefly, *Musca domestica*. *Comp. Biochem. Physiol.* 113B, 175–183.

- Zhang, M., Scott, J.G., 1996b. Cytochrome *b*₅ is essential for cytochrome P450 6D1-mediated cypermethrin resistance in LPR house flies. *Pestic. Biochem. Physiol.* 55, 150–156.
- Zhou, S., Gao, Y., Jiang, W., Huang, M., Xu, A., *et al.*, 2003. Interactions of herbs with cytochrome P450. *Drug Metab. Rev.* 35, 35–98.
- Zhu, Y.C., Snodgrass, G.L., 2003. Cytochrome P450 CYP6X1 cDNAs and mRNA expression levels in three strains of the tarnished plant bug *Lygus lineolaris* (Heteroptera: Miridae) having different susceptibilities to pyrethroid insecticide. *Insect Mol. Biol.* 12, 39–49.
- Ziegler, D.M., 2002. An overview of the mechanism, substrate specificities, and structure of FMOs. *Drug Metab. Rev.* 34, 503–511.
- Zijlstra, J.A., Vogel, E.W., Breimer, D.D., 1984. Strain-differences and inducibility of microsomal oxidative enzymes in *Drosophila melanogaster* flies. *Chem. Biol. Interact.* 48, 317–338.
- Zinke, I., Schutz, C.S., Katzenberger, J.D., Bauer, M., Pankratz, M.J., 2002. Nutrient control of gene expression in *Drosophila*: microarray analysis of starvation and sugar-dependent response. *EMBO J.* 21, 6162–6173.
- Zou, S., Meadows, S., Sharp, L., Yan, L.Y., Jan, Y.N., 2000. Genome-wide study of aging and oxidative stress response in *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA* 97, 13726–13731.
- Zuber, M.X., Simpson, E.R., Waterman, M.R., 1986. Expression of bovine 17 α -hydroxylase cytochrome P450 cDNA in non-steroidogenic (COS1) cells. *Science* 234, 1258–1261.
- Zumwalt, J.G., Neal, J.J., 1993. Cytochromes P450 from *Papilio polyxenes*: adaptations to host plant allelochemicals. *Comp. Biochem. Physiol.* 106C, 111–118.

Relevant Websites

- <http://www.imm.ki.se> – Human cytochrome P450 (CYP) allele nomenclature committee. A complete referenced and annotated list of human P450 alleles.
- <http://P450.antibes.inra.fr> – The insect P450 site at INRA. A searchable annotation of insect cytochrome P450 sequences.
- <http://dinelson.utm.edu> – David Nelson's cytochrome P450 homepage. Listing of P450 genes from a variety of organisms.

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14 Insect G Protein-Coupled Receptors: Recent Discoveries and Implications

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14.1. Introduction

G protein-coupled receptors (GPCRs) are integral membrane proteins that sense and transduce extracellular signals into cellular responses. The binding of small ligands to the extracellular domains of these proteins elicits a cascade of intracellular responses through activation of heterotrimeric G proteins. In insects, many signaling molecules and their corresponding biological functions have been identified, but knowledge of the signal transduction subsequent to receptor binding has been limited by the relative paucity of information about GPCR structure and function.

It is clear that remarkably diverse physiological processes are mediated by GPCRs. Some of these include sensing of environmental signals such as light, odors, and taste. Others sense extracellular signaling molecules such as calcium, nucleotides, neurotransmitters, biogenic amines, and neuroendocrine peptides. Evolution of GPCRs for specific functions in a vast array of organisms is one interesting area of study, for which insects in many ways are ideal for comparative studies. In addition to their value for basic science, GPCRs mediate vital physiological events in insects that may provide promising targets for development of pest control measures

as well as therapeutic targets for promotion of human health.

The advent of the postgenomics era is providing excellent opportunities to explore insect GPCRs and their biological functions. Genomic surveys of GPCRs in the fruit fly *Drosophila melanogaster* and in the malaria mosquito *Anopheles gambiae* have identified at least ~200 and 276 GPCRs, respectively (Brody and Cravchik, 2000; Hill *et al.*, 2002a). Sequence analysis reveals an appreciable degree of groupwise homology relationships between insect and vertebrate GPCRs, suggesting many GPCRs have common ancestry dating more than 600 million years ago (Benton and Ayala, 2003). Data mining combined with functional studies of insect GPCRs has begun to reveal specific ligand–receptor interactions.

Deorphaning insect GPCRs by finding cognate ligands in *Drosophila* is now being followed by expansion of physiological studies in other species of insects that are more amenable to physiological experiments. The use of genomics as a springboard for understanding physiological mechanisms is complemented by advances in molecular probes, genetics, and imaging technologies, leading to a new era of “molecular physiology.” This review will cover

recent progress in the molecular and functional characterization of insect GPCRs.

14.2. Structure–Function Relationships for GPCRs

GPCRs are characterized by conserved core structures, consisting of an extracellular N-terminus, seven transmembrane (TM) α -helices each connected by alternating extracellular and intracellular loops (e1, e2, e3, i1, i2, and i3), and an intracellular C-terminus. The structure of the seven transmembrane protein bovine rhodopsin revealed by X-ray crystallography epitomizes the generalized bauplan predicted for GPCRs (Palczewski *et al.*, 2000; Teller *et al.*, 2001; review: Filipek *et al.*, 2003) (Figure 1). A number of important general structural features emerging from studies of vertebrate GPCRs are worth summarizing here as a prelude to discussions of insect GPCRs. Of course, caution is necessary in extrapolating knowledge from these works directly to other groups of phylogenetically diversified GPCRs.

The location of ligand binding sites varies considerably between different families of GPCRs. Indeed, GPCRs can be broadly categorized according to ligand size and location of the binding site. Such information is critical in the design of candidate drugs with either agonist or antagonist properties. For instance, GPCRs for small ligands such as

retinoic acid, odorants, and biogenic amines appear to have a binding pocket surrounded by the TM bundle (Gotzes and Baumann, 1996; Vaidehi *et al.*, 2002). However, ligand binding to other groups of GPCRs involves extracellular epitopes of the protein, including the N-terminus and extracellular loops (Bockaert and Pin, 1999). In the case of the metabotropic glutamate and γ -aminobutyric acid B (GABA_B) receptors, the large N-terminus is composed of two lobes arranged for trapping ligands (Hermans and Challiss, 2001). In *Drosophila*, the N-terminal ectodomain of methuselah, an orphan GPCR associated with extended lifespan, consists of three β structure regions, which meet to form a shallow interdomain groove. This region characterized by an exposed tryptophan residue forms a putative ligand binding site by analogy with ectodomains of other GPCRs (West *et al.*, 2001).

The seven TM helices of GPCRs are arranged in an anticlockwise sequential manner when viewed from the extracellular aspect (Figure 1). Ligand binding induces changes in core structure, and in particular TM3 is thought to have a major role in receptor activation by altering the relative orientation of TM6 (Bockaert and Pin, 1999). Likewise, the conserved tripeptide signature of the TM3 cytoplasmic interface ([D,E]R[Y,W]) is considered to be important in receptor activation. The TM helices often are interrupted by distortions of the α -helix backbone, which may serve as hinge points

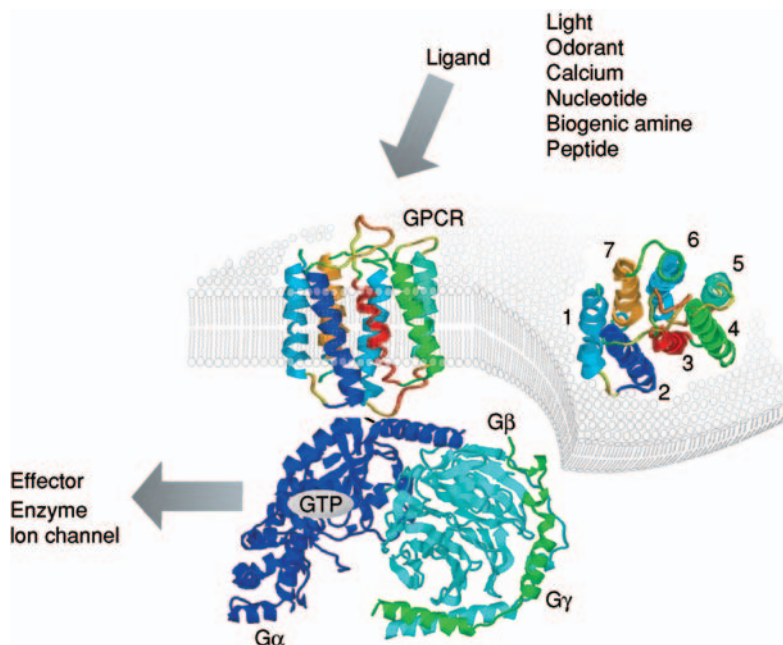


Figure 1 A diagram summarizing G protein-coupled receptor (GPCR) signaling. An array of seven transmembrane segments is shown according to the bovine rhodopsin structure. The trimeric G protein consisting of G α , G β , and G γ is coupled to the GPCR. Activation of the GPCR by ligands binding to the extracellular cell surface is amplified by intracellular effector proteins.

underlying conformational changes induced by ligand binding.

The cytoplasmic face of GPCRs interacts with heterotrimeric G-proteins, which dissociate upon receptor activation to trigger specific signal transduction pathways (Figure 1). Specificity for particular subtypes of heterotrimeric G-proteins is an important factor in determining downstream pathways. The *Drosophila* genome presents at least seven genes encoding $G\alpha$, six for $G\beta$, and two for $G\gamma$ subunits. The subunit $G\alpha$ is in direct contact with the cytoplasmic interface of GPCRs (Bourne, 1997; Wess, 1998; Horn *et al.*, 2000; Moller *et al.*, 2001). Prediction of G protein subtype coupling specificity has been attempted in vertebrate GPCRs through identification of characteristic cytoplasm-facing sequence motifs (Bourne, 1997; Wess, 1998; Horn *et al.*, 2000; Moller *et al.*, 2001). This is a nontrivial exercise, considering reports showing GPCRs coupled with multiple G proteins as well as agonist-dependent changes in GPCR coupling with different G proteins (Robb *et al.*, 1994; Reale *et al.*, 1997; Cordeaux *et al.*, 2001; Cordeaux and Hill, 2002).

Proper functioning of GPCRs often may depend on oligomerization with other proteins (Gomes *et al.*, 2001; Angers *et al.*, 2002). Such interactions may serve to localize receptors for effective coupling with downstream pathways and/or to influence desensitization, which regulates receptor levels on the cell surface and temporal parameters of the biological response (Pierce and Lefkowitz, 2001; Brady and Limbird, 2002; Kohout and Lefkowitz, 2003).

14.3. Evolution of Insect GPCRs

In a pioneering study, Hewes and Taghert (2001) performed a comprehensive phylogenetic analysis of *Drosophila* peptide GPCRs covering the entire array of annotated genes in the *Drosophila* genome. The analysis revealed 42 putative peptide GPCRs arranged within 15 monophyletic subgroups together with vertebrate peptide GPCRs. The relationships established in this study also appear to be valid generally for the *Anopheles* mosquito (Hill *et al.*, 2002a). Evolutionary relationships between *Drosophila* and vertebrate peptide GPCRs have provided impetus for formulation of hypotheses to guide the process of deorphaning insect GPCRs. The success of this approach has been enhanced by many instances of previously established vertebrate ligand–receptor relationships. As this process advances, coevolutionary relationships between peptide GPCRs and their ligands have been brought into clearer focus.

Ligand–receptor coevolution has occurred as a consequence of reciprocal evolutionary influences between two interacting proteins – ligand and receptor – while the original definition of coevolution emphasized interactions between organisms (see Ridley, 1993). Assuming GPCRs share a common ancestral gene, diverse GPCRs must have arisen through multiple steps of gene duplication types of evolutionary processes (Chothia *et al.*, 2003). In phylogenetic analyses, closely related GPCRs have cognate ligands also related each other, indicating that an ancestral ligand–receptor partnership has effectively rooted new sets of ligand–receptors with a novel function. The ligand–receptor partners appear to be maintained during evolution without punctuated changes of their relationships; thus coevolution may be a common phenomenon.

In the coevolution of ligand–receptor partners, it has been proposed that divergence of the receptor precedes the evolution of its cognate novel neuropeptide ligand (Darlison and Richter, 1999). Such reasoning is based on the frequent occurrence of multiple receptors for a single peptide. This hypothesis, however, may be questioned because many peptide encoding genes have multiple repeats of isopeptides and processing sites. A good example is the tetrapeptide FMRFamide, for which eight homologous repeats appear in the *Drosophila* propeptide gene (Schneider and Taghert, 1988, 1990). There also appears to be a significant degree of flexibility in the system, evidenced first by the presence of multiple receptors for a given ligand that are coupled to different G proteins (Darlison and Richter, 1999; Park *et al.*, 2003) and second by differential selectivity of receptors to a number of isopeptides (Darlison and Richter, 1999). Further details regarding processes of ligand–receptor coevolution can be found in a number of recent publications (van Kesteren *et al.*, 1996; Darlison and Richter, 1999; Goh and Cohen, 2002).

While discerning evolutionary relationships among GPCRs is challenging, establishing such relationships for signaling peptides is a much more daunting task. The main problem lies in the fact that only a small number of functionally important, conserved amino acids within peptide sequences defines their evolutionary relationships. A valuable search tool in bioinformatics, the position-specific iterated BLAST (PSI-BLAST; Altschul *et al.*, 1997), provides a powerful means of using small short, conserved sequence motifs for searching distantly related peptides. The method uses a score matrix generated from multiple alignments of the highest scoring hits in each round of the search. Conservation of consensus amino acids in the multiple

sequence alignment implies functionally and evolutionarily important amino acid residues common for a group of signaling peptides.

A good example of ligand–receptor coevolution was found for a group of neuropeptides having a C-terminal PRXamide motif (Pro-Arg-XXX with C-terminal amide). This motif is common to an array of peptides occurring in both insects and vertebrates, including neuromedin U (NMU), cardioacceleratory peptide 2b (CAP2b), pyrokinins, and ecdysis triggering hormone (ETH). This group of peptides activates a cluster of GPCRs related to vertebrate receptors for NMU and thyrotropin releasing hormone (Park *et al.*, 2002) (Figure 3). While phylogenetic relationships among receptors are established almost exclusively on the basis of amino acid sequence, the patterns of pharmacological cross-reactivity for these GPCRs to cognate PRXamide peptides provide a new and “functional” criterion for inferring evolutionary relationships for ligand–receptor pairs.

Despite the frequent correspondence of insect and vertebrate ligand–receptor relationships, a number of *Drosophila* GPCRs are found to be relatively unique (Hewes and Taghert, 2001), such as *Drosophila* GPCRs having bootstrap values of less than 300 in 1000 pseudosamples. For example, closely related GPCRs within the group CG13229, CG13803, and CG8985 are found to have no significant relationship with other vertebrate GPCRs. Recently, two of these receptors CG13803 and CG8985 were found to be activated by Dromyosuppressin (Johnson *et al.*, 2003a; Egerod *et al.*, 2003a) having the C-terminal motif FLRLamide, a member of FMRFamide-related peptides (FaRPs) containing a C-terminal RFamide. Such unique GPCRs could arise either through rapidly evolving branches of GPCRs, or through recent loss of the related GPCRs in the vertebrates.

14.4. Insect GPCRs

14.4.1. Classification of GPCRs

Classification of the GPCR superfamily by families and groups has been made based on a combination of natural ligand categories and receptor sequence homology (Kolakowski, 1994; Horn *et al.*, 2000; Hewes and Taghert, 2001). For the following discussions, these classifications are used to cover comprehensive categories of GPCRs in all organisms. Accordingly, insect GPCRs are classified into the following families: family A, rhodopsin and various peptide receptors; family B, secretin-like peptide receptors and methuselah (mth); family C, metabotropic glutamate receptors, atypical GPCRs such as

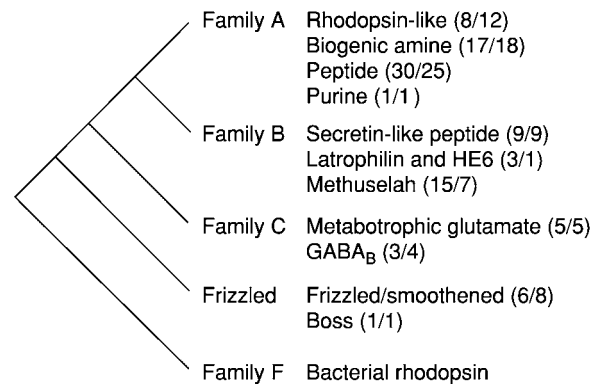


Figure 2 A conceptual phylogenetic relationship of the GPCR superfamily. The numbers of annotated genes for *Drosophila* (left) and *Anopheles* (right) are in parentheses. The conceptual tree is still not congruent and reconstructed based on Josefsson (1999).

frizzled/smoothened and bride of sevenless (boss), and odorant receptors (Figure 2).

Assuming all groups of GPCRs (or 7-TM proteins) are ancestrally related, a conceptual tree depicting evolutionary relationships among them can be drawn based on combined results from previous studies (Figure 2), while construction of a congruent tree is still controversial because of its high sequence divergency (Josefsson, 1999). The number of *Drosophila* genes encoding putative GPCRs as a fraction of the total in each group is shown in parenthesis for each category (Figure 2). In rooting the tree on family F, which includes bacterial rhodopsin, the group frizzled appears to be the most ancestral form. Family A with ~90 GPCRs is the largest group, and includes biogenic amines, peptides, rhodopsin, and ~30 orphan receptors (Hill *et al.*, 2002a). Given the excellent phylogenetic analysis for *Drosophila* peptide receptors in the previous study (Hewes and Taghert, 2001). We provide Tables 1–3 summarizing the GPCRs that have been cloned so far. This table includes *Drosophila* data and GPCRs from other insects, but not the GPCRs of *A. gambiae* where experimental data is unavailable.

14.4.2. Family A: Biogenic Amine, Rhodopsin, and Neuropeptide Receptors

14.4.2.1. Biogenic amine receptors Most small signaling molecules used as transmitters, modulators, and hormones are common to vertebrates and invertebrates, including insects. The most prominent are acetylcholine, glutamate, GABA, and the biogenic amines serotonin, dopamine, and histamine (Table 1). Norepinephrine and epinephrine, abundant in the vertebrates, are absent or present only in very low amounts in insects (Maxwell *et al.*,

Table 1 Insect G protein-coupled receptors for biogenic amines

Name	Synonym (GenBank accession number)	Species	Cellular response (expression system)	Expression pattern	Reference
<i>Serotonin receptor</i>					
5-HT2	CG1056, 5HT-dro1, 5HT2dro, Dm5HT2 (X81835)	<i>Drosophila melanogaster</i>		Coexpression with pair-rule gene <i>ftz</i> in embryo	Colas <i>et al.</i> (1999, 1995)
5HT7	CG12073, 5HT-dro1, 5HT7dro, Dm5HTdro1, 5HT-R1 (M55533)	<i>Drosophila melanogaster</i>	Increased cAMP (Sf9, NIH 3T3)	Adult head, larval ventral ganglion	Witz <i>et al.</i> (1990), Saudou <i>et al.</i> (1992), Obosi <i>et al.</i> (1996)
5-HT1A	CG16720, 5HT-dro2A (Z11489)	<i>Drosophila melanogaster</i>	Inhibition cAMP (COS-7)	Ventral unpaired median neurons	Saudou <i>et al.</i> (1992)
5-HT1B	CG15113, 5HT-dro2B (Z11490)	<i>Drosophila melanogaster</i>	Inhibition cAMP (COS-7)	Larval ventral ganglion	Saudou <i>et al.</i> (1992), Obosi <i>et al.</i> (1996)
CG8007	(AE003679, AAN13390)	<i>Drosophila melanogaster</i>			Brody and Cravchik (2000)
Aedes 5-HT7	(AF296125)	<i>Aedes aegypti</i>		Malpighian tubules, tracheolar cells	Pietrantonio <i>et al.</i> (2001)
Bm5HT	B150 <i>Bombyx</i> (X95604)	<i>Bombyx mori</i>			von Nickisch-Rosenegk <i>et al.</i> (1996)
Hv5HT	K15 <i>Heliothis</i> (X95605)	<i>Heliothis virescens</i>			von Nickisch-Rosenegk <i>et al.</i> (1996)
<i>Dopamine receptor</i>					
DopR1	CG9652, dDA1, DmDop1 (X77234)	<i>Drosophila melanogaster</i>	Increased cAMP (HEK-293)	Somata of optic lobe	Gotzes <i>et al.</i> (1994), Gotzes and Baumann (1996)
DopR2	CG7569, CG18741, DopR99B, DAMB (U34383)	<i>Drosophila melanogaster</i>	Ca ²⁺ mobilization (<i>Xenopus</i> oocyte), Increased cAMP (<i>Xenopus</i> oocyte, HEK-292, S2)	Mushroom body	Feng <i>et al.</i> (1996), Han <i>et al.</i> (1996), Reale <i>et al.</i> (1997)
DD2R	CG17004 (AY150862)	<i>Drosophila melanogaster</i>	Increased cAMP (CRE-luciferase assay in HEK-293)	Head and body	Hearn <i>et al.</i> (2002)
AmDop1	(CAA73841)	<i>Apis mellifera</i>	Increased cAMP (HEK-293)		Blenau <i>et al.</i> (1998)
AmBAR6	D2 (AAM19330)	<i>Apis mellifera</i>		Mushroom body	Ebert <i>et al.</i> (1998), Blenau and Baumann (2001)
<i>Muscarinic acetylcholine receptor</i>					
AchR-60C	CG4356, mAChR-1, DM1 (M23412)	<i>Drosophila melanogaster</i>	Increased IP3 metabolism (mouse Y1)	Mushroom body, optic lobe, antennal lobe	Shapiro <i>et al.</i> (1989), Blake <i>et al.</i> (1993), Hannan and Hall (1996)
CG7918	(AE003677, AAF54188)	<i>Drosophila melanogaster</i>			Brody and Cravchik (2000)
<i>Octopamine/tyramine receptor</i>					
OAMB	CG3856, CG15698, DmOCT1B (AF065443)	<i>Drosophila melanogaster</i>	Increased cAMP (S2), increased cAMP (CHO-K1), Ca ²⁺ mobilization (CHO-K1)	Mushroom body	Arakawa <i>et al.</i> (1990), Robb <i>et al.</i> (1994), Han <i>et al.</i> (1998)
BmOcr	B96 <i>Bombyx</i> (X95607)	<i>Bombyx mori</i>			von Nickisch-Rosenegk <i>et al.</i> (1996)

Continued

Table 1 Continued

Name	Synonym (GenBank accession number)	Species	Cellular response (expression system)	Expression pattern	Reference
HvOcr	K50 <i>Heliothis</i> (X95606)	<i>Heliothis virescens</i>	Inhibition cAMP (LLC-PK1)	Antennae	von Nickisch-Rosenegk <i>et al.</i> (1996)
TyrR	CG7485, Tyr-dro, honoka (X54794, AB073914)	<i>Drosophila melanogaster</i>	Inhibition cAMP, Ca ²⁺ mobilization (NIH 3T3)	Antennae, CNS	Saudou <i>et al.</i> (1990), Kutsukake <i>et al.</i> (2000)
Amyr1	(AJ245824)	<i>Apis mellifera</i>	Inhibition cAMP, Ca ²⁺ mobilization (HEK-293)	Mushroom body, CNS	Blenau <i>et al.</i> (2000)
TyrLoc	Tyr-Loc (X69520)	<i>Locusta migratoria</i>	Inhibition cAMP, Ca ²⁺ mobilization (murine erythroleukemia cell (MEL-C88L, S2)		Vanden Broeck <i>et al.</i> (1995), Poels <i>et al.</i> (2001)
MabOcR	(AAK14402)	<i>Mamestra brassicae</i>			

1978; Evans, 1980). Prominent amines in insects and crustaceans are octopamine and tyramine (Evans, 1980). Octopamine is thought to play a modulatory role in arousal, paralleling that of noradrenaline in the sympathetic system of vertebrates (Evans, 1993; Blenau and Baumann, 2001). Octopamine and tyramine, while only present as “trace amines” in the vertebrates, may nevertheless play functional roles (Borowsky *et al.*, 2001). Among the biogenic amines known to be signaling molecules in insects, histamine is so far the only one for which GPCRs are unknown; electrophysiological data provide conclusive evidence for gating of ionotropic receptors by histamine. This contrasts with histamine receptors in the vertebrates, where both ionotropic and GPCR receptors are known (Roeder, 2003).

The evolutionary patterns of biogenic amine receptors among vertebrates and invertebrates are generally in accordance with monophyletic ancestries (Roeder, 2002), although recent gene duplication events have led to rapid expansions of certain vertebrate receptor groups. Furthermore, there are apparent shifts in ligand specificity in a number of receptor phylogenies, for which the ligands are structurally similar. This phenomenon is particularly apparent for *Bombyx* and *Heliothis* octopamine receptors, which occur in a monophyletic clade with tyramine receptors (Blenau *et al.*, 1998; Ebert *et al.*, 1998; Roeder, 2002).

Numerous functions of biogenic amines in insects are described at the behavioral and physiological levels, including modulation of sensory input and motor output, learning and memory, development,

and elevated states of arousal (Blenau and Baumann, 2001; Roeder, 2002). Modulation of olfactory and visual stimuli is indicated by expression patterns of amines in sensory lobes, and by physiological experiments. For example, sensitization of pheromone receptor cells by octopamine and serotonin is indicated in *Bombyx mori* and *Manduca sexta* (Pophof, 2000; Dolzer *et al.*, 2001), and a possible role for serotonin in feedback from higher centers in the brain is indicated from morphological studies (Hill *et al.*, 2002b). Octopamine plays a major hormonal role in energy metabolism during locust flight by acting directly on the fat body and indirectly through release of adipokinetic hormone (Orchard *et al.*, 1993). Setting the day state in circadian cycling by serotonin is suggested for a cricket (*Gryllus bimaculatus*) (Saifullah and Tomioka, 2002), and dopamine receptors are thought to be targets of circadian modulation (Andretic and Hirsh, 2000). Egg diapause in *B. mori* appears to be controlled by dopaminergic pathways in the mother (Noguchi and Hayakawa, 2001).

In a growing number of cases, biogenic amine receptor-specific functions are reported. The *honoka* mutant in *Drosophila* carries a tyramine receptor defect and shows reduced responses to repellent odors. In addition, this mutant exhibited reduced tyramine modulation at the neuromuscular junction, while normal modulation by octopamine was observed (Kutsukake *et al.*, 2000). A role for the serotonin receptor subtype 5-HT₂ in embryonic development was shown by a *Drosophila* mutant (Colas *et al.*, 1995, 1999). In these studies, serotonin signaling was found to be necessary for increased

Table 2 Insect G protein-coupled receptors for neuropeptides: family A group III and group V, and family B

Name	Synonym (GenBank accession no.)	Species	Active ligand ^a	Vertebrate homology group	Evidence/functional assay (expression system) ^b	Expression pattern	Reference
<i>Family A group III</i>							
CCKLR-17D3	CG6857 (AE003509, AAF48857)	<i>Drosophila melanogaster</i>		Cholecystokinin/gastrin R	Homology to CCKLR-17D1		McBride <i>et al.</i> (2001), Kubiak <i>et al.</i> (2002)
CCKLR-17D1	CG6881, CG6894, CG32540, dsk-r1 (AE003510, AAF48879)	<i>Drosophila melanogaster</i>	Sulfakinin	Cholecystokinin/gastrin R	Ca ²⁺ mobilization (HEK-293, CHO-K1, SH-EP)		McBride <i>et al.</i> (2001), Kubiak <i>et al.</i> (2002)
NepYr	CG5811 (M81490)	<i>Drosophila melanogaster</i>		Neurokinin R	Inward current (<i>Xenopus</i> oocyte)		Li <i>et al.</i> (1992)
Leucokinin receptor	CG10626 (AE003566, NM139711, AY070926)	<i>Drosophila melanogaster</i>	Leucokinin	Neurokinin R	Ca ²⁺ mobilization (S2)	Malpighian tubules, CNS	Radford <i>et al.</i> (2002)
Myokinin receptor	(AF228521)	<i>Boophilus microplus</i>		Neurokinin R	Homology cloning		Holmes <i>et al.</i> (2000)
Takr86C	CG6515, NKD (M77168)	<i>Drosophila melanogaster</i>	Tachykinin	Neurokinin R	Increased IP3 (mouse NIH-3T3), translocation of β -arrestin2	CNS	Monnier <i>et al.</i> (1992), Johnson <i>et al.</i> (2003b)
Takr99D	CG7887, DTKD (X62711)	<i>Drosophila melanogaster</i>	Tachykinin	Neurokinin R	Inward current (<i>Xenopus</i> oocyte), translocation of β -arrestin2	CNS	Li <i>et al.</i> (1991), Johnson <i>et al.</i> (2003b)
STKR	<i>Stomoxys</i> tachykinin receptor	<i>Stomoxys calcitrans</i>	Tachykinin-like	Neurokinin R	Ca ²⁺ mobilization (S2)		Guerrero (1997), Torfs <i>et al.</i> (2001, 2002a)
LTKR	<i>Leucophaea</i> tachykinin receptor (AJ310390)	<i>Leucophaea maderae</i>		Neurokinin R	Homology cloning	Brain	Johard <i>et al.</i> (2001)
SNPF-R	CG7395, Drm-sNPF-R, NPFR76F, GPCR60 (NP_524176)	<i>Drosophila melanogaster</i>	Short neuropeptide F	Neurokinin R	Ca ²⁺ mobilization (CHO-K1/G α_{16}), inward current (<i>Xenopus</i> oocyte)		Mertens <i>et al.</i> (2002), Feng <i>et al.</i> (2003)
NPFR1	CG1147 (AF364400)	<i>Drosophila melanogaster</i>	Neuropeptide F	Neurokinin R	Ca ²⁺ mobilization (CHO-K1/G α_{16}), ¹²⁵ I-NPF binding (CHO), translocation of β -arrestin2	CNS, midgut	Garczynski <i>et al.</i> (2002), Johnson <i>et al.</i> (2003b)

Continued

Table 2 Continued

Name	Synonym (GenBank accession no.)	Species	Active ligand ^a	Vertebrate homology group	Evidence/functional assay (expression system) ^b	Expression pattern	Reference
FMRFamide receptor	CG2114 (NM139501)	<i>Drosophila melanogaster</i>	FMRFamide	Neurokinin R	Ca ²⁺ mobilization (CHO-K1/G α_{16}), translocation of β -arrestin2	Trachea, gut, fat body, Malpighian tubules, ovary	Cazzamali and Grimmelikhuijzen (2002), Meeusen <i>et al.</i> (2002), Johnson <i>et al.</i> (2003b)
Allatostatin B receptor	CG14484, CG18192 (NP611241)	<i>Drosophila melanogaster</i>	Allatostatin B	Bombesin R	translocation of β -arrestin2		Johnson <i>et al.</i> (2003a)
PRXa receptor 1	CG9918 (AF522191)	<i>Drosophila melanogaster</i>	Cardioacceleratory peptide CAP2b	Neuromedin U R	Inward current (<i>Xenopus</i> oocyte)		Park <i>et al.</i> (2002)
PRXa receptor 2	CG8784 (AF522189)	<i>Drosophila melanogaster</i>	Hugin	Neuromedin U R	Inward current (<i>Xenopus</i> oocyte), Ca ²⁺ mobilization (CHO-K1/G α_{16})		Park <i>et al.</i> (2002), Rosenkilde <i>et al.</i> (2003)
PRXa receptor 3	CG8795 (AF522190)	<i>Drosophila melanogaster</i>	Hugin	Neuromedin U R	Inward current (<i>Xenopus</i> oocyte), Ca ²⁺ mobilization (CHO-K1/G α_{16})		Park <i>et al.</i> (2002), Rosenkilde <i>et al.</i> (2003)
HezPBAN R	AY319852	<i>Helicoverpa zea</i>	HezPBAN	Neuromedin U R	Ca ²⁺ mobilization (Sf9)		Choi <i>et al.</i> (2003)
CAP2b receptor	CG14575 (AF522193)	<i>Drosophila melanogaster</i>	Cardioacceleratory peptide (CAP2b)	Neuromedin U R	Inward current (<i>Xenopus</i> oocyte), Ca ²⁺ mobilization (CHO-K1/G α_{16})		Iversen <i>et al.</i> (2002), Park <i>et al.</i> (2002)
ETH receptor a	CG5911a (AY220741)	<i>Drosophila melanogaster</i>	Ecdysis triggering hormone (ETH)	Neuromedin UR / thyrotropin releasing hormone R	Ca ²⁺ mobilization (CHO-K1/G α_{16})		Iversen <i>et al.</i> (2002), Park <i>et al.</i> (2003)
ETH receptor b	CG5911b (AY220742)	<i>Drosophila melanogaster</i>	Ecdysis triggering hormone (ETH)	Neuromedin U / thyrotropin releasing hormone R	Ca ²⁺ mobilization (CHO-K1/G α_{16})		Iversen <i>et al.</i> (2002), Park <i>et al.</i> (2003)
Myosuppressin receptor 1	CG8985 (AF545042)	<i>Drosophila melanogaster</i>	Myosuppressin	Neuromedin U / thyrotropin releasing hormone R	Ca ²⁺ mobilization (CHO-K1/G α_{16}), translocation of β -arrestin2, inhibition cAMP (HEK-292)		Egerod <i>et al.</i> (2003a), Johnson <i>et al.</i> (2003a)

Myosuppressin receptor 2	CG13803 (AF544244)	<i>Drosophila melanogaster</i>	Myosuppressin	Neuromedin U/ thyrotropin releasing hormone R	Ca ²⁺ mobilization (CHO-K1/Gα ₁₆), translocation of β-arrestin2, inhibition cAMP (HEK-292), GTPγS binding (HEK-292)		Egerod <i>et al.</i> (2003a), Johnson <i>et al.</i> (2003a)
Proctolin receptor	CG6986 (NM167020)	<i>Drosophila melanogaster</i>	Proctolin	Neuromedin U/ thyrotropin releasing hormone R	Ca ²⁺ mobilization (HEK-293), translocation of β-arrestin2	Brain, medulla, hindgut, pericardial cell, heart	Egerod <i>et al.</i> (2003b), Johnson <i>et al.</i> (2003b)
<i>Family A group V</i> Corazonin receptor	CG10698 (AF522192, AF373862)	<i>Drosophila melanogaster</i>	Corazonin	Vasopressin R	Inward current (<i>Xenopus</i> oocyte), Ca ²⁺ mobilization (CHO-K1/Gα ₁₆), translocation of β-arrestin2		Cazzamali <i>et al.</i> (2002), Park <i>et al.</i> (2002), Johnson <i>et al.</i> (2003b)
<i>Bombyx</i> AKH receptor	BAKHR (AF403542)	<i>Bombyx mori</i>	Adipokinetic hormone (AKH)	Gonadotropin releasing hormone R	Ca ²⁺ mobilization (CHO-K1/Gα ₁₆)		Staubli <i>et al.</i> (2002)
AKH receptor	CG11325, GRHR, DAKHR (AF522194, AAC61523)	<i>Drosophila melanogaster</i>	Adipokinetic hormone (AKH)	Gonadotropin releasing hormone R	Inward current (<i>Xenopus</i> oocyte), Ca ²⁺ mobilization (CHO-K1/Gα ₁₆)		Park <i>et al.</i> (2002), Staubli <i>et al.</i> (2002)
CCAP receptor	CG6111 (AF522188)	<i>Drosophila melanogaster</i>	Crustacean cardioactive peptide (CCAP)	Vasopressin R	Inward current (<i>Xenopus</i> oocyte), Ca ²⁺ mobilization (CHO-K1/Gα ₁₆)		Park <i>et al.</i> (2002), Staubli <i>et al.</i> (2002)
Drostar1	CG7285 (AAG54080, NM140783)	<i>Drosophila melanogaster</i>	Allatostatin C	Somatostatin R/ opioid R	GIRK current (<i>Xenopus</i> oocyte)	Corpora allata	Kreienkamp <i>et al.</i> (2002a), Johnson <i>et al.</i> (2003a)
Drostar 2	CG13702 (NM140782)	<i>Drosophila melanogaster</i>	Allatostatin C	Somatostatin R/ opioid R	GIRK current (<i>Xenopus</i> oocyte)	Optic lobe, corpora allata	Kreienkamp <i>et al.</i> (2002a), Johnson <i>et al.</i> (2003a)
DAR-1	AlstR, EG:121E7.2, CG2872 (AF163775, NM166982)	<i>Drosophila melanogaster</i>	Allatostatin A	Galanin	GIRK current (<i>Xenopus</i> oocyte), Ca ²⁺ mobilization (CHO-K1), GTPγS binding (CHO-K1)		Birgul <i>et al.</i> (1999), Larsen <i>et al.</i> (2001)
DAR-2	AR-2, CG10001	<i>Drosophila melanogaster</i>	Allatostatin A	Galanin	Ca ²⁺ mobilization (CHO-K1), GTPγS binding (CHO-K1)	Gut	Lenz <i>et al.</i> (2000, 2001), Larsen <i>et al.</i> (2001)

Continued

Table 2 Continued

Name	Synonym (GenBank accession no.)	Species	Active ligand ^a	Vertebrate homology group	Evidence/functional assay (expression system) ^b	Expression pattern	Reference
Pea-A1stR	<i>Periplaneta</i> A1stR (AF336364)	<i>Periplaneta americana</i>	Allatostatin A	Galanin	GIRK current (<i>Xenopus</i> oocyte)		Auerswald <i>et al.</i> (2001)
BAR	<i>Bombyx</i> A1stR (AH011256)	<i>Bombyx mori</i>	Allatostatin A	Galanin	Ca ²⁺ mobilization (CHO-K1/Gα ₁₆)	Gut	Secher <i>et al.</i> (2001)
LGR1	CG7665, Fsh (U47005)	<i>Drosophila melanogaster</i>	nd	FSH/LH/TRH	FSH receptor homology cloning		Hauser <i>et al.</i> (1997), Nishi <i>et al.</i> (2000)
LGR2	CG8930, Rickets (AF142343)	<i>Drosophila melanogaster</i>	Bursicon	FSH/LH/TRH	Mutant insensitive to bursicon-like factor	Embryo and pupae	Eriksen <i>et al.</i> (2000), Nishi <i>et al.</i> (2000), Baker and Truman (2002)
<i>Family B</i>							
Mas-DH-R	<i>Manduca</i> DH receptor (U03489)	<i>Manduca sexta</i>	Mas-diuretic hormone	Corticotropin releasing hormone R	[³ H]Mas-DH binding and cAMP (COS-7), [¹²⁵ I], Mas-DH binding and cAMP (Sf-9)		Reagan (1994, 1995)
AcD-DH-R	<i>Acheta</i> DH receptor (U15959)	<i>Acheta domesticus</i>	Acheta-diuretic hormone	Corticotropin releasing hormone R	[³ H]AcD-DH binding and cAMP (COS-7),		Reale <i>et al.</i> (1997)
Bm-DH-R	<i>Bombyx</i> DH receptor	<i>Bombyx mori</i>		Corticotropin releasing hormone R	Homology cloning		Ha <i>et al.</i> (2000)

^aThe most active or putative ligand suggested in the respective reference(s).

^bMethods used for cloning and assay, or other evidence provided.

Table 3 Family C: Metabotropic glutamate receptors and GABA_B receptors

Name	Synonym	Evidence	Expression	Reference
mGluRA	CG11144, metabotropic glutamate receptor A, DmGluRA,	Inhibition of AC (HEK 293), GIRK current (<i>Xenopus</i> oocyte)	CNS	Raymond <i>et al.</i> (1999)
mGluRB	CG30361, CG18447, CG8692, metabotropic glutamate receptor B,	Homology		Brody and Cravchik (2000)
GABA-B-R1	CG15274, D-GABA _B R1	GIRK current (<i>Xenopus</i> oocyte)	CNS	Mezler <i>et al.</i> (2001)
GABA-B-R2	CG6706, D-GABA _B R2, GH07312	GIRK current (<i>Xenopus</i> oocyte)	CNS	Mezler <i>et al.</i> (2001)
GABA-B-R3	CG3022, GABA _B R3	Homology	CNS	Mezler <i>et al.</i> (2001)

cell adhesiveness required for cell–cell intercalation during gastrulation. Expression patterns of the serotonin 5-HT₇-like receptor in *Aedes* Malpighian tubules and tracheolar cells are suggestive of possible functions in the control of diuresis and respiration (Pietrantonio *et al.*, 2001).

14.4.2.2. Rhodopsin Rhodopsins are a class of retinal-binding chromophores widespread in visual cells of animals. In each rhodopsin, the light-absorbing retinal binds to a pocket within the seven-transmembrane opsin protein. Incoming photons induce isomerization of retinal, which passes protons to its partner opsin, resulting in G protein activation. The eyes of insects are tuned to specific spectral ranges through diversity in genes encoding the opsin structure, whereby amino acid sequence variability occurs in the vicinity of the retinal binding pocket. The coupling of opsins to G-proteins leads to visual transduction through the opening or closing of cation channels in the visual cell. The evolution of genes encoding rhodopsins with different spectral properties have been described in various insect species (review: Briscoe and Chittka, 2001).

The insect compound eye is composed of cartridges called ommatidia, each organized as a set of eight photoreceptor retinula cells (R1–R8). In *Drosophila*, the compound eye expresses seven rhodopsin genes (*Rh1–Rh7*), which show unique expression patterns and spectral properties. A specialized portion of the plasma membrane in each retinula cell is convoluted via microvilli to form the rhodopsin-rich rhabdomere. The rhabdomere of each retinula cell faces the others within the cartridge to create the composite rhabdom, where light is absorbed. Rhodopsin Rh1, also called ninaE (neither inactivation nor after potential E), is expressed in retinula cells R1–R6 (O'Tousa *et al.*, 1985; Feiler *et al.*, 1988). Rh2 is expressed in ocelli (Cowman *et al.*, 1986), simple eyes located on the vertical side of the head, while Rh3 and Rh4 are expressed in R7 in nonoverlapping subsets of ommatidia (Fryxell and Meyerowitz, 1987). Rh5 and Rh6 are expressed

in R8 in nonoverlapping subsets of ommatidia (Chou *et al.*, 1996, 1999). Rh7 (CG5638) has been identified in the genome sequence, but has not been associated with a physiological function as yet (Brody and Cravchik, 2000).

Insect rhodopsins are classified generally into three categories according to their responsiveness to ultraviolet, blue, and green colors (Townson *et al.*, 1998). In some cases, rhodopsins sensitive to red color have been identified in the Lepidoptera and Hymenoptera (Briscoe and Chittka, 2001), where adaptive evolution appears to have taken place through replacement of amino acids adjacent to the retinal binding site and thus shifting the spectral sensitivity (Briscoe, 2002). Recent expansions of the rhodopsin gene in different species of insects are observed in the phylogenetic relationships among rhodopsins in the orders Hymenoptera, Diptera, and Lepidoptera (Montell, 1999; Salcedo *et al.*, 1999; Briscoe, 2001; Briscoe and Chittka, 2001; Hill *et al.*, 2002a).

The phototransduction cascade in invertebrates begins with light-absorbing rhodopsin coupled to G_q. This activates phosphoinositide turnover through activation of phospholipase C β (PLCβ), liberation of inositol trisphosphate (IP3), and the opening of transient receptor potential (TRP) channels, leading to inward cation (Na⁺ and Ca²⁺) flux and depolarizing potentials in retinula cells (Alvarez *et al.*, 1996; Montell, 1999; Bahner *et al.*, 2000; Hardie and Raghu, 2001). Invertebrate phototransduction contrasts with that of vertebrates, where at low light intensities a large “dark current” flows through cyclic guanine monophosphate (cGMP) nucleotide gated sodium channels to maintain a relatively depolarized state (approximately –40 mV). Under dark conditions, photoreceptor cells maintain high levels of cytosolic cGMP and hence a high number of open cyclic nucleotide gated sodium channels. Light absorbance by rhodopsin activates the G protein transducin (G_t), leading to elevation of phosphodiesterase, destruction of cGMP, and consequent closing of sodium channels (Shichida and Imai, 1998). Through this

mechanism, vertebrate photoreceptors hyperpolarize in response to light stimuli.

14.4.2.3. Neuropeptide receptors in family A
Neuropeptides are ubiquitous signaling molecules acting mainly through GPCRs for activation of cellular responses. Numerous signaling peptides in insects have been characterized by their abilities to activate visceral activity, heartbeat, or glandular secretion (reviews: Gäde *et al.*, 1997; Nassel, 2002). Studies of neuropeptide signaling have been accomplished mainly in large insects where biochemical studies and physiological assays are relatively easy. Subsequent findings of neuropeptides in various species show that amino acid sequences of neuropeptides are generally well conserved. With the opening of the postgenomic era, genomic sequences in *Drosophila* and *Anopheles* revealed more than 23 and 35 genes, respectively, encoding neuropeptides (Hewes and Taghert, 2001; Riehle *et al.*, 2002). Peptidomics of the central nervous system (CNS) of *Drosophila* (Baggerman *et al.*, 2002) identified 28 neuropeptides including eight novel peptides using capillary liquid chromatography in conjunction with tandem mass spectrometry.

Studies of neuropeptide receptors in vertebrates have identified a large number of GPCRs grouped as family A with rhodopsins and biogenic amine receptors (Kolakowski, 1994). Phylogenetic analysis of *Drosophila* and *Anopheles* genome sequences for putative GPCRs revealed ~40 GPCRs in family A in each species (Hewes and Taghert, 2001; Hill *et al.*, 2002a). Analysis of phylogenetic relationships revealed that many insect neuropeptide receptors occur in monophyletic groups with vertebrate receptors, implying evolution from common ancestors in each group (Hewes and Taghert, 2001; Hill *et al.*, 2002a). Family A is further classified into several subgroups, and insect peptide GPCRs currently are placed into group III or group V (Kolakowski, 1994; Hewes and Taghert, 2001).

Numerous insect GPCRs in family A group III and group V have been cloned and expressed, and functional assays have been key to identification of their ligands (Table 2). The earliest efforts to clone neuropeptide GPCRs started with cDNA library screenings that targeted *Drosophila* homologs of the vertebrate neuropeptide Y and tachykinin receptors (Li *et al.*, 1991, 1992; Monnier *et al.*, 1992). Following identification of these receptors in *Drosophila*, subsequent investigations located their homologs in the stable fly (*Stomoxys Calcitrans*) and cockroach (*Leucophaea maderae*) (Guerrero, 1997; Torfs *et al.*, 2000, 2001, 2002a; Johard *et al.*, 2001).

Recent approaches in functional genomics have contributed to the deorphaning of a large number of insect GPCRs. Functional analyses have been designed for screening sets of GPCRs and ligands belonging to a phylogenetic clade. This approach assumes ligand–receptor coevolution (see Section 14.6) and utilizes an established ligand–receptor pair as a baseline for formulation of testable hypotheses.

Such an approach was taken to identify likely receptors for peptide ligands having C-terminal PRXamide motifs (Park *et al.*, 2002). The PRXa peptides in *Drosophila* and other insects fall into three classes: pyrokinins including the pheromone biosynthesis activating neuropeptide (PBAN) (–FXPRXa) (Raina *et al.*, 1989; Matsumoto *et al.*, 1990), cardioactive CAP2b-like peptides (–FPRXa) (Morris *et al.*, 1982; Huesmann *et al.*, 1995), and ETHs (–PRXa) (Zitnan *et al.*, 1996; Adams and Zitnan, 1997; Park *et al.*, 1999, 2002). In the vertebrates, PRXa motifs are found in the peptides NMU, vasopressin (AVP), and pancreatic polypeptide (PP), for which receptors already have been identified (Thibonnier *et al.*, 1994; Fujii *et al.*, 2000; Howard *et al.*, 2000). Functional analyses demonstrated that *Drosophila* GPCRs related to the monophyletic NMU receptor group are activated by all three classes of *Drosophila* PRXa peptides (Figure 3). PRXa receptors are now being investigated in other species of insects, such as *Manduca*, where the physiological functions of the neuropeptides are better characterized (Kim and Adams, unpublished data), while genuine functions of the PRXamide receptors in *Drosophila* also are being investigated.

Drosophila GPCRs related to the vasopressin receptor failed to respond to PRXa peptides, but instead was activated by crustacean cardioactive peptide (CCAP), corazonin, and adipokinetic hormone (AKH), none of which falls into the PRXa peptide group (Park *et al.*, 2002). *Drosophila* sulfakinin receptors also were identified through the logic of ligand–receptor coevolution upon finding homology in ligands between vertebrate cholecystokinin/gastrin and *Drosophila* sulfakinin (Kubiak *et al.*, 2002). A *Drosophila* neuropeptide F receptor was identified, and found to belong to one of four *Drosophila* GPCRs in the neuropeptide Y receptor subgroup (Garczynski *et al.*, 2002; Mertens *et al.*, 2002).

Another productive approach for deorphaning GPCRs is reverse physiology (see Section 14.5). Receptors for allatostatin type A (DAR-1) (Birgul *et al.*, 1999) and allatostatin type C (Drostar1, Drostar2) (Kreienkamp *et al.*, 2002a) were identified through isolation from tissue extracts of native

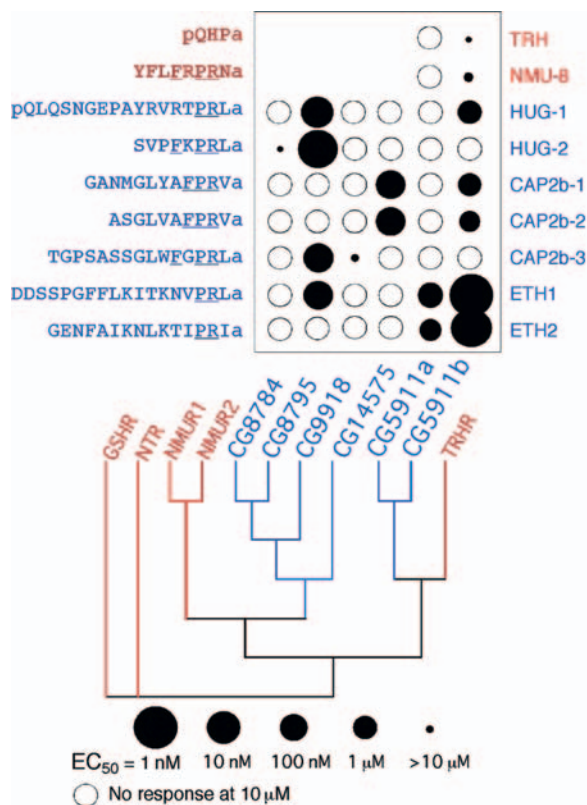


Figure 3 Ligand–receptor specificity for *Drosophila* PRXamide and related peptides. The amino acid sequence of each ligand is shown at the left, with the names of each peptide on the right. Circles indicate relative activity of ligands to each receptor. The larger the circle, the more potent is the peptide. Empty circles indicate “no response” at concentrations up to 10 μM, the highest concentration tested. The tree and names of neuropeptide receptors are: GSHR, GH secretagogue receptor; NTR, neurotensin receptor; NMUR1, neuromedin U receptor 1; NMUR2, neuromedin U receptor 2; TRHR, thyrotropin releasing hormone receptor; *Drosophila* PRXamide receptor GPCRs with CG numbers. Red letters are vertebrate peptides and GPCRs. Blue letters are *Drosophila* PRXamide peptides and GPCRs. (Data from Park, Y., Kim, Y.J., Adams, M.E., 2002. Identification of G protein-coupled receptors for *Drosophila* PRXamide peptides, CCAP, corazonin, and AKH supports a theory of ligand–receptor coevolution. *Proc. Natl Acad. Sci. USA* 99, 11423–11428; and Park, Y., Kim, Y.J., Dupriez, V., Adams, M.E., 2003. Two subtypes of ecdysis triggering hormone receptor in *Drosophila melanogaster*. *J. Biol. Chem.* 278, 17710–17715).

neuropeptide ligands that activate the receptor. Closely related genes in *Drosophila* and other insect species subsequently cloned and expressed, and showed their sensitivity to related allatostatin peptides (Lenz *et al.*, 2000, 2001; Auerswald *et al.*, 2001; Larsen *et al.*, 2001; Secher *et al.*, 2001). The reverse physiology approach also was successfully employed in identification of the *Drosophila* FMRFamide receptor (Meeusen *et al.*, 2002) and the leucokinin receptor in the snail *Lymnaea* (Cox

et al., 1997). Subsequent searches for leucokinin receptor orthologous genes in insects led to identification of novel leucokinin receptors in the cattle tick, *Boophilus microplus* (Holmes *et al.*, 2000, 2003) and in *Drosophila* (Radford *et al.*, 2002).

A gene homologous to the adenosine-like receptor has been found in both *Drosophila* and *Anopheles* genomes (Brody and Cravchik, 2000; Hill *et al.*, 2002a), but biological data are unavailable as yet. The findings in *Drosophila* will facilitate GPCR studies in other arthropod species where biological functions of those neuropeptides have been well characterized (see Section 14.5).

14.4.3. Family B: Secretin-Like Receptors

Insect GPCRs belonging to family B consist of secretin-like receptors (five genes in *Drosophila*) (Table 3), the *methuselah* group (~11 genes), human epididymis-specific protein 6 (HE6; two genes), and latrophilin (one gene) (Brody and Cravchik, 2000; Hewes and Taghert, 2001; Hill *et al.*, 2002a). Two insect neuropeptides, diuretic hormone and amnesiac (Feany and Quinn, 1995; Zhong, 1995; Zhong and Pena, 1995), are similar to corticotropin releasing factor (CRF) and the pituitary adenylate cyclase activating peptide (PACAP), respectively, of vertebrate neuropeptides that are grouped with receptors in the family B. All five secretin-like receptors in the *Drosophila* family B (Brody and Cravchik, 2000; Hewes and Taghert, 2001) appear to have orthologs in the *Anopheles* genome (Hill *et al.*, 2002a).

The first receptor for an insect diuretic hormone (DH-R) was identified by screening a *Manduca* expression library with a tritiated ligand (Reagan, 1994). The sequence of the *Manduca* DH-R revealed that it is an ortholog of the vertebrate CRF receptor (Hewes and Taghert, 2001). Activation of the *Manduca* DH-R receptor expressed in COS-7 and Sf9 insect cells triggered cAMP elevation (Reagan, 1995). An orthologous gene from the house cricket, *Acheta domesticus*, was cloned and characterized in a functional expression system (Reagan, 1996). The orthologous *Drosophila* DH-R sequence reveals two closely related genes as candidates (CG32843 and CG4395), but no experimental evidence has appeared as yet.

The *methuselah* (*mth*) group of receptors, found by screening for *Drosophila* mutants with extended lifespan (Lin *et al.*, 1998), comprises a large group of GPCRs unique to insects. Phylogenetic analysis of the closely related species *A. gambiae* and *D. melanogaster* revealed recent expansions in the number of copies of the *mth* genes (Hill *et al.*, 2002a). Examination of *Drosophila mth* mutants suggested that

this gene product controls synaptic efficacy at the glutamatergic neuromuscular junction by increasing the rate of neurotransmitter exocytosis from the presynaptic terminal (Song *et al.*, 2002). The crystal structure of the *mtb* ectodomain revealed potential ligand binding features in a shallow inter-domain groove, although the ligand(s) have yet to be identified (West *et al.*, 2001).

Two genes encoding the GPCRs grouped with the human epididymis-specific protein 6 (HE6) were identified in *Drosophila*, but not in *Anopheles* (Brody and Cravchik, 2000; Hill *et al.*, 2002a). This receptor in vertebrates has an unusually long extracellular region characteristic of cell adhesion proteins, indicating potential roles in cell adhesion and signaling (Stacey *et al.*, 2000; Kierszenbaum, 2003). The genomes of *Drosophila* and *Anopheles* each have genes orthologous to vertebrate latrophilin. Vertebrate latrophilin is a putative receptor or binding protein for α -latrotoxin, a major component of black widow spider (genus *Lactrodectus*) venom. It appears to be involved in triggering calcium independent exocytosis, thus is also known as CIRC (calcium-independent receptor for α -latrotoxin) (Henkel and Sankaranarayanan, 1999). Latrophilin is thought to be a component of a large NMDA-receptor-associated signaling complex (Kreienkamp *et al.*, 2002b).

14.4.4. Family C: Metabotropic Glutamate Receptor and GABA_B Receptor

Along with its well-known role in excitatory neuromuscular transmission, the neurotransmitter glutamate mediates both excitation and inhibition within the insect CNS (Parmentier *et al.*, 1998; Washio, 2002). Likewise, GABAergic inhibitory transmission occurs both in the peripheral and central nervous systems. In the CNS, these transmitters mediate both fast and slow transmission via ionotropic and metabotropic receptors, respectively. While our understanding of glutamate and GABA transmission in invertebrates is derived mostly from work on ionotropic receptors, metabotropic receptors are receiving increased attention because of new information gathered from genome sequences.

Analysis of the *Drosophila* genome revealed two genes encoding putative metabotropic glutamate receptors (mGluR) and three genes for metabotropic GABA_B receptors, along with two other related genes not yet annotated (Brody and Cravchik, 2000). The *Anopheles* genome shows orthologous relationships with corresponding *Drosophila* genes in this group (Hill *et al.*, 2002a).

Drosophila mGluR receptors appear to function in the CNS by coupling to G_i, inhibiting activation of

adenylyl cyclase (Parmentier *et al.*, 1996, 1998), although linkage to K⁺ and Ca²⁺ channel modulation downstream could serve excitatory roles as well.

GABA is a major inhibitory neurotransmitter in the CNS of both vertebrates and invertebrates. Postsynaptic, ionotropic GABA_A and GABA_C receptors mediate fast inhibitory responses, while slow transmission occurs via metabotropic GABA_B receptors. In vertebrates, GABA_B receptors mediate reduction of transmitter release presynaptically (Slesinger *et al.*, 1997) and hyperpolarization postsynaptically (Isaacson and Vitten, 2003). Pharmacological studies of the GABA_B receptor in the cockroach *Periplaneta americana* demonstrated hyperpolarizing, ionotropic responses of the fast coxal depressor motor neuron (Bai and Sattelle, 1995).

Heterodimerization of GABA_BR1 and R2 subunits is necessary for constitution of a functional receptor in vertebrates (Jones *et al.*, 1998; Kaupmann *et al.*, 1998; White *et al.*, 1998). Furthermore, it is proposed that ligand binding involves only the GABA_BR1 subunit (Kniazeff *et al.*, 2002). Orthologous relationships of the subtypes GABA_BR1 and R2 are extended to *Drosophila* and the nematode *Caenorhabditis elegans* (Kniazeff *et al.*, 2002). As in the vertebrates, functional *Drosophila* GABA_B receptors require heterodimerization of GABA_BR1 and R2 subunits (Mezler *et al.*, 2001). A third *Drosophila* subunit, GABA_BR3, appears to be an insect-specific subtype expressed in the CNS.

14.4.5. Other GPCRs: Odorant Receptor, Gustatory Receptor, Atypical 7TM Proteins

Analysis of genomic sequences in *Drosophila* and *Anopheles* indicates that the largest groups of GPCRs in insects are odorant and gustatory receptors (~70 genes for each) (Clyne *et al.*, 1999, 2000; Scott *et al.*, 2001; Hill *et al.*, 2002a). These receptors appear to have evolved quickly and comprise largely divergent subgroups having little if any conserved amino acid sequences in most cases. Evidence for functionality as odorant receptors has been provided by heterologous expression and bioassay (Stortkuhl and Kettler, 2001; Wetzel *et al.*, 2001). A gustatory receptor sensing trehalose was identified from a *Drosophila* mutant (Ishimoto *et al.*, 2000; Dahanukar *et al.*, 2001). Homology based cDNA library screening led to cloning of a number of putative odorant receptors in *Heliothis virescens*, and to demonstration of tissue-specific expression in antennae. This work thus has expanded olfactory receptor studies from *Drosophila* to other insect species (Krieger *et al.*, 2002).

Another group of putative receptors having seven transmembrane topology is classified separately as

“atypical 7TM proteins.” This class includes frizzled (*fz*), smoothed, and bride of sevenless (*bos*), most of which were initially identified as *Drosophila* mutants. The function of *fz*, four homologous copies of which are found in *Drosophila* and *Anopheles*, appears to be in establishing morphogenic symmetry and cell polarity during development (Bhanot *et al.*, 1999; Strutt, 2001).

14.5. Intracellular Signaling Pathways Triggered by GPCRs

Activation of GPCRs by extracellular ligands at the cell surface leads to a rich diversity of intracellular signal transduction pathways, beginning with activation of heterotrimeric guanine nucleotide-binding proteins (G-proteins), comprised of α , β , and γ subunits (Figure 1). The α subunit binds guanosine nucleotides, GDP in the resting state and GTP following activation of the GPCR with which the G-protein is associated. Upon GPCR activation, GTP binding to the α subunit of the G-protein leads to dissociation of the α and $\beta\gamma$ subunits. The $\beta\gamma$ dimer does not dissociate further under physiological conditions. Both α and $\beta\gamma$ subunits can bind independently to target proteins to initiate cellular responses.

The type of intracellular signaling that ensues GPCR activation depends on the associated G protein, most importantly the $G\alpha$ subunit. Coupling with $G\alpha_s$ activates adenylyl cyclase (AC), whereas coupling with $G\alpha_i$ and $G\alpha_o$ suppresses AC activity. Coupling with $G\alpha_q$ activates phospholipase C, initiating phosphoinositide turnover and intracellular Ca^{2+} mobilization. Prediction of coupling specificity between GPCR and G protein subtypes has been attempted using precedent data from vertebrate GPCRs (Bourne, 1997; Wess, 1998; Horn *et al.*, 2000; Moller *et al.*, 2001). To what extent such methods are predictive for insect GPCR signaling remains an open question.

Numerous instances of $G\beta\gamma$ signaling also have been documented in the context of $G\alpha_{i/o}$ signaling. The target proteins for $G\beta\gamma$ include AC, PLC β , potassium channels, and phosphatidylinositol 3-kinase. Perhaps the best known of these is K channel modulation following parasympathetic activation of heart muscarinic acetylcholine receptors to slow heartbeat frequency (Kofuji *et al.*, 1995). This response results from dissociation of $G\alpha_i$ and $G\beta\gamma$, with the latter acting directly as a ligand to open G protein coupled inwardly rectifying potassium channels (GIRK) and hyperpolarize myocardial cells (Reuveny *et al.*, 1994; Wickman *et al.*, 1994). This signaling pathway provides a particularly clear example of how direct

G protein modulation of ion channels leads to an important physiological response.

Analysis of the *Drosophila* genome sequence reveals seven, six, and two genes that are homologous to vertebrate $G\alpha$, $G\beta$, and $G\gamma$ subunits, respectively (Table 4). The seven $G\alpha$ homologous genes are classified as $G\alpha_q$, $G\alpha_s$, and $G\alpha_{i/o}$, the latter including $G\alpha_t$, the well-known transducin involved in phototransduction (Simon *et al.*, 1991; Neves *et al.*, 2002) (Figure 4). Genes homologous to the $G\beta$ subunit appear to have larger diversity than their vertebrate counterparts (Figure 4). Only two copies of the $G\gamma$ subunit are found in *Drosophila* (Figure 4). Alternatively spliced forms of these genes appear to provide additional flexibility to signaling systems (de Sousa *et al.*, 1989; Ray and Ganguly, 1994; Talluri *et al.*, 1995).

Immunolocalization of G protein α subunits in *Drosophila* demonstrated that expression of $G\alpha_q$, $G\alpha_s$, and $G\alpha_{i/o}$ is highly restricted in the CNS (Wolfgang *et al.*, 1990). Expression in photoreceptor synaptic terminals and antennal lobes suggests functional roles in transmission of primary sensory information. Distinct expression patterns in embryonic stages implies involvement of G protein-mediated sensory transmission in embryonic development (Wolfgang *et al.*, 1991). Expression profiles of $G\alpha$ subtypes in *M. sexta* also were examined by immunohistochemistry, where they were shown to be expressed in subsets of CNS cells (Copenhaver *et al.*, 1995).

A salient characteristic of GPCR-induced signaling is the opportunity for amplification along multiple steps of the pathway. For example, a single GPCR binding event leads to dissociation of α and $\beta\gamma$ subunits, each of which can trigger downstream signaling pathways. In instances where the G protein subunit triggers second messengers through activation of enzymes such as AC or PLC, a second level of amplification occurs. Downstream activation of additional enzymes such as kinases or phosphatases provides a third level of amplification. Thus activation of a very small number of GPCRs can elicit highly significant biochemical responses in target cells. The diversity of downstream signal transduction pathways is summarized in several reviews, and generalized current concepts based vertebrate studies may well be applicable to insects (Simon *et al.*, 1991; Morris and Malbon, 1999; Neves *et al.*, 2002).

14.6. Assignment of GPCR Functions

GPCRs regulate a plethora of cellular functions by modulating the activities of diverse intracellular

Table 4 *Drosophila* trimeric G protein subunits G α , G β , and G γ

Group	Name	Synonym	Expression pattern	Reference
<i>Gα subunit</i>				
Gq	G α 49B	CG17759, dgq, DGq α , DG α q	Chemosensory cells, photoreceptor cells	Lee <i>et al.</i> (1990, 1994), Talluri <i>et al.</i> (1995), Bahner <i>et al.</i> (2000), Ratnaparkhi <i>et al.</i> (2002)
	CG17760			
Gi/o	G α o47A	CG2204, DG α o, dgo, Go α	Brain, ovary, cardioblast, CNS	de Sousa <i>et al.</i> (1989), Schmidt <i>et al.</i> (1989), Wolfgang <i>et al.</i> (1990, 1991), Fremion <i>et al.</i> (1999)
	G α i65A	CG10060, G α i, Gi α , DG α 1,	CNS	Wolfgang <i>et al.</i> (1990, 1991)
Gs	G α 73B	CG12232, Gf α	Embryonic stage, midgut	Quan <i>et al.</i> (1993)
	G α s60A	CG2835, G α s, Gs α , Dgs α , dgs, Gs	Neuropil in CNS	Wolfgang <i>et al.</i> (1990, 1991, 2001)
	Concertina	CG17678, cta	Embryonic mesoderm	Parks and Wieschaus (1991)
<i>Gβ subunit</i>				
	CG2812			
	CG3004			
	EG:86E4.3	CG17766		
	G β 13F	CG10545, dg β , G β , G-protein β 13F	Eye	Yarfitz <i>et al.</i> (1988, 1991)
	G β 5			
	G β 76C	CG8770		
<i>Gγ subunit</i>				
	G γ 1	CG8261, D-G γ 1, dg1 α	CNS	Ray and Ganguly (1994)
	G γ 30A	CG18511, CG3694, G γ e	Eye	Schulz <i>et al.</i> (1999)

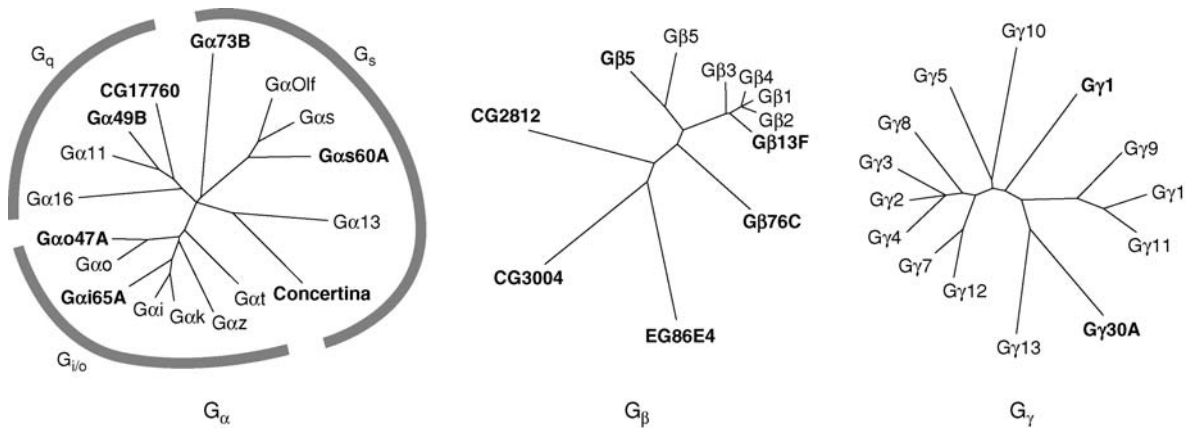


Figure 4 Phylogram for *Drosophila* G proteins with vertebrate G proteins. *Drosophila* G proteins are in bold letters.

target proteins, including ion channels, metabolic enzymes, and transporters. These actions lead to cellular responses important for embryonic, postembryonic, and reproductive development, homeostatic processes, responses to environmental stimuli, and induction of various context-driven behavioral states.

Strategies for functional identification of GPCRs have gone through several phases since the initial breakthroughs with β -adrenergic and muscarinic acetylcholine receptors (Dixon *et al.*, 1986; Kubo

et al., 1986; Yarden *et al.*, 1986). These first discoveries began with direct protein purification and partial amino acid sequencing of receptors with known ligands. This allowed for construction of oligonucleotide probes, cDNA library screening, and eventual cloning and sequencing of entire receptors. The next wave of GPCR identification occurred through expression cloning of, among others, neurokinin2 and serotonin receptors using early stage oocytes of the African clawed toad, *Xenopus laevis* (Masu *et al.*, 1987; Julius *et al.*,

1988). Taking advantage of sequence information from earlier work, homology cloning based on nucleotide sequence identity provided relatively easy and productive approaches to further receptor identification. Such homology searches led to the identification of the first insect GPCR, the muscarinic acetylcholine receptor (Shapiro *et al.*, 1989). Finally, availability of entire genome sequences from an increasing number of organisms has led to an explosive phase of *in silico* discoveries of putative GPCRs (Kim and Carlson, 2002), while homology search using cDNA library screening and degenerate polymerase chain reaction (PCR) is still highly effective for GPCR identification in species for which whole genome sequences are not yet available.

Whereas early efforts focused on receptors with known function, the recent era of genome discovery has generated numerous “orphan receptors” with unknown ligands and uncertain functions. For example, large numbers of putative GPCR in insects have been identified and analyzed from the genome sequences of *Drosophila* and *Anopheles* (Hewes and Taghert, 2001; Hill *et al.*, 2002a). Homology analysis of putative *Drosophila* GPCRs and particularly for neuropeptide receptors (Hewes and Taghert, 2001) has provided a foundation for GPCR deorphaning based on comparisons with mammalian genomes. This analysis, combined with improvements in heterologous expression systems and reporter constructs, has increased the rate of GPCR deorphaning during the past several years.

Deorphaning putative GPCRs through identification of endogenous ligands depends primarily on heterologous expression within a suitable bioassay system, and characterization of interactions between candidate ligands through biochemical or functional methods. The choice of expression and assay systems for a given GPCR gene entail practical considerations based on knowledge of previous studies. Since the assay primarily focuses on ligand–receptor interaction rather than endogenous downstream pathways, it often uses an exogenously introduced reporter system. The most frequently used expression systems have been the *Xenopus* oocyte expression system and various cell lines. The expression system needs to be chosen according to certain criteria: paucity of receptor endogenous to the expression system, which might cross-react with ligands under investigation, robust expression of the heterologous receptor, localization of the receptor on the cell membrane, appropriate posttranslational processing, and efficient coupling to downstream signaling pathways for activation of reporters. Tables 1–3 summarize assay systems currently used for studies of insect GPCRs.

14.6.1. Functional GPCR Assays: Coupling with Reporters

Given the diversity of functions regulated by GPCRs, it is not surprising that they are targeted by a high percentage of pharmaceutical drugs currently on the market. For this reason, considerable efforts have been devoted to development of methods for high-throughput screening of novel GPCR agonists and antagonists. It seems likely that similar approaches may lead to future insecticidal agents.

The most straightforward functional assays for GPCRs in oocytes or cell lines register Ca^{2+} mobilization as an increased fluorescence or luminescence. This cellular response is most commonly a result of $G\alpha_q$ activation and phosphoinositol metabolism, leading to IP₃ mediated Ca^{2+} release from intracellular stores (Neves *et al.*, 2002). Commonly used Ca^{2+} sensors generate fluorescence or luminescence signals (see Section 14.5.3). Many orphan GPCRs are coupled to other $G\alpha$ subunits that do not mobilize calcium. Consequently, efforts have been made to utilize more promiscuous G proteins coupled to the PLC β pathway. One very successful method has been the use of a $G\alpha_q$ variant known as $G\alpha_{16}$, which couples widely divergent GPCRs to the phosphoinositide turnover pathway via PLC, leading to Ca^{2+} mobilization (review: Stables *et al.*, 1997; Kostenis, 2001) (Figures 5 and 6). For example, expression of a *Drosophila* odorant receptor with exogenous $G\alpha_{16}$ in *Xenopus* oocytes was the system used to identify its cognate odorant ligand (Wetzel *et al.*, 2001). Other approaches have involved engineering of chimeric G proteins (e.g., $G\alpha_{16/2}$) which couple a diversity of GPCRs to PLC β (Liu *et al.*, 2003).

For fluorescence assays, cells expressing GPCRs are pretreated with membrane permeant calcium indicators such as Fura2-AM, fast green AM, or fluo-3 AM (Meeusen *et al.*, 2002; Choi *et al.*, 2003; Johnson *et al.*, 2003a). For luminescence responses in cells expressing aequorin, pretreatment with the cofactor coelenterazine is necessary. Fluorescence or luminescence microplate readers having injection ports can be used for introduction of the cells into microplate wells that contain various concentrations of ligand. For high-throughput assay, simultaneous injection and reading of a plate for 96 or 384 wells also has been developed (Le Poul *et al.*, 2002).

Coupling to G_s normally leads to increases in AC activity. Experiments with the *Xenopus* oocyte expression system have demonstrated that $G\alpha_s$ activation can gate exogenous inwardly rectifying potassium channels GIRK1 and GIRK2 through the direct action of $G\beta\gamma$ (Kofuji *et al.*, 1995; Lim *et al.*,

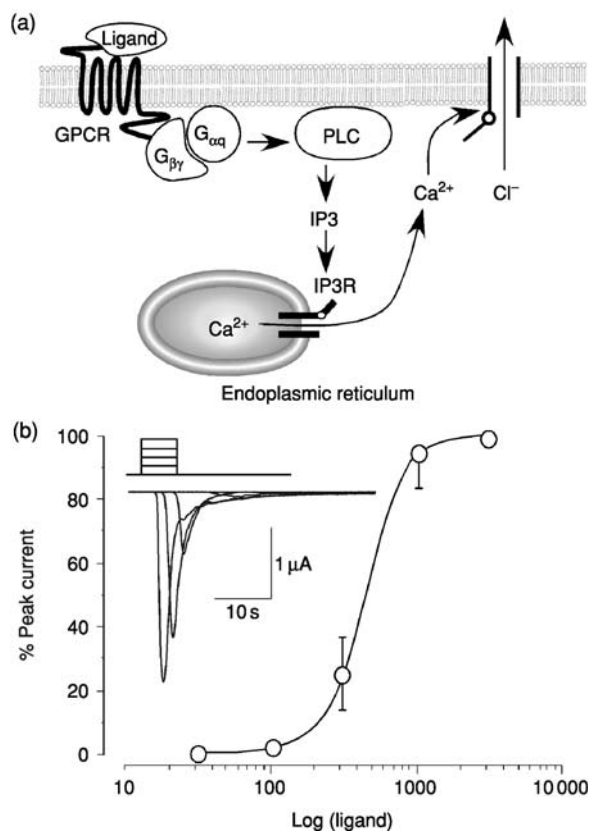


Figure 5 (a) A presumed signaling pathway in the *Xenopus* oocyte assay system for orphan GPCRs. GPCR expressed from injected cRNA transduce signal through phospholipase C (PLC), inositol triphosphate (IP3), and IP3 receptor triggering Ca^{2+} mobilization from intracellular Ca^{2+} storage. Ca^{2+} activates chloride channels, resulting in inward currents. (b) Progressively higher concentrations of ligands generate larger currents. Mean values and corresponding standard deviations from multiple experiments are plotted as a function of ligand concentration.

1995; Peleg *et al.*, 2002). Expression of these exogenous GIRK subtypes in the proper stoichiometry can lead to robust inward K^{+} currents, and hence provide a convenient assay for activation of $\text{G}\alpha_s$.

Other approaches for detecting $\text{G}\alpha_s$ activation have included measurements of increased cAMP levels (i.e., serotonin receptor 5HT₇) (Witz *et al.*, 1990; Saudou *et al.*, 1992; Obosi *et al.*, 1996). For $\text{G}\alpha_i$ activation, inhibition of AC activity can be detected through a reduction in forskolin-inducible cAMP increase (i.e., tyramine receptors) (Kutsukake *et al.*, 2000; Poels *et al.*, 2001).

14.6.2. Expression of GPCRs in *Xenopus* Oocytes

Advantages of using unfertilized *Xenopus* oocytes are many, not the least of which is the absence of endogenous GPCRs (Lee and Durieux, 1998). Injection of complementary RNA prepared with 5' and

3' untranslated regions of *Xenopus* β -globin gene results in sufficient expression in stage 5 or 6 oocytes for robust bioassays (Jespersen *et al.*, 2002). The oocyte contains sufficient machinery to link activation of exogenously introduced GPCRs to cellular responses.

The most routine use of oocytes is coupling of heterologously expressed GPCRs with endogenous G_q , leading to mobilization of intracellular Ca^{2+} through PLC β activation and phosphoinositide turnover. This results in opening of Ca^{2+} dependent Cl^{-} channels on the oocyte plasma membrane and large inward currents that can be quantified by the two-electrode voltage clamp technique (Figure 5). This assay can be made considerably more flexible through the introduction of $\text{G}\alpha_{16}$, chimeric G proteins, or coupling to GIRK K^{+} channels.

A precise perfusion system is required for generation of large sets of data using oocytes. Park *et al.* (2002) used a liquid chromatography pump and associated Rheodyne injection valve, supplying a continuous flow of buffer at a flow rate of 1.2 ml min^{-1} . Various concentrations of ligands were applied in standard $100 \mu\text{l}$ volumes of ligand through the injection valve, providing a 5 s exposure of ligand solution to the oocyte (Park *et al.*, 2002). This system allows precise application of a given concentration of ligand in a continuous flow of buffer, while caution must be exercised in the use of lipophilic ligands that may persist in the walls of the perfusion system even after extensive washes.

A challenge using *Xenopus* oocytes for GPCR assay is rapid desensitization following receptor activation. The degree of desensitization is highly variable and dependent on the specific GPCR expressed (Park *et al.*, 2002). In the case of *Drosophila* ETH receptors, the assay in oocytes was confounded by desensitization (Park, unpublished data). Even very low concentrations that did not elicit measurable currents were sufficient to cause desensitization. In such a case, alternative expression and assay systems using cell lines are preferable, where measurement of β -arrestin activity has proved to be a very effective method (see Section 14.6.4).

14.6.3. Assay of GPCR Activation in Cell Lines

Expression of GPCRs in cell lines currently is the method of choice both for deorphaning receptors and for large-scale, rapid-throughput drug screening for agonists and antagonists. Considerations in the choice of cell lines include high-level expression and proper processing of the exogenous GPCR gene, knowledge of the endogenous signaling systems operating, and the efficacy of the reporter system.

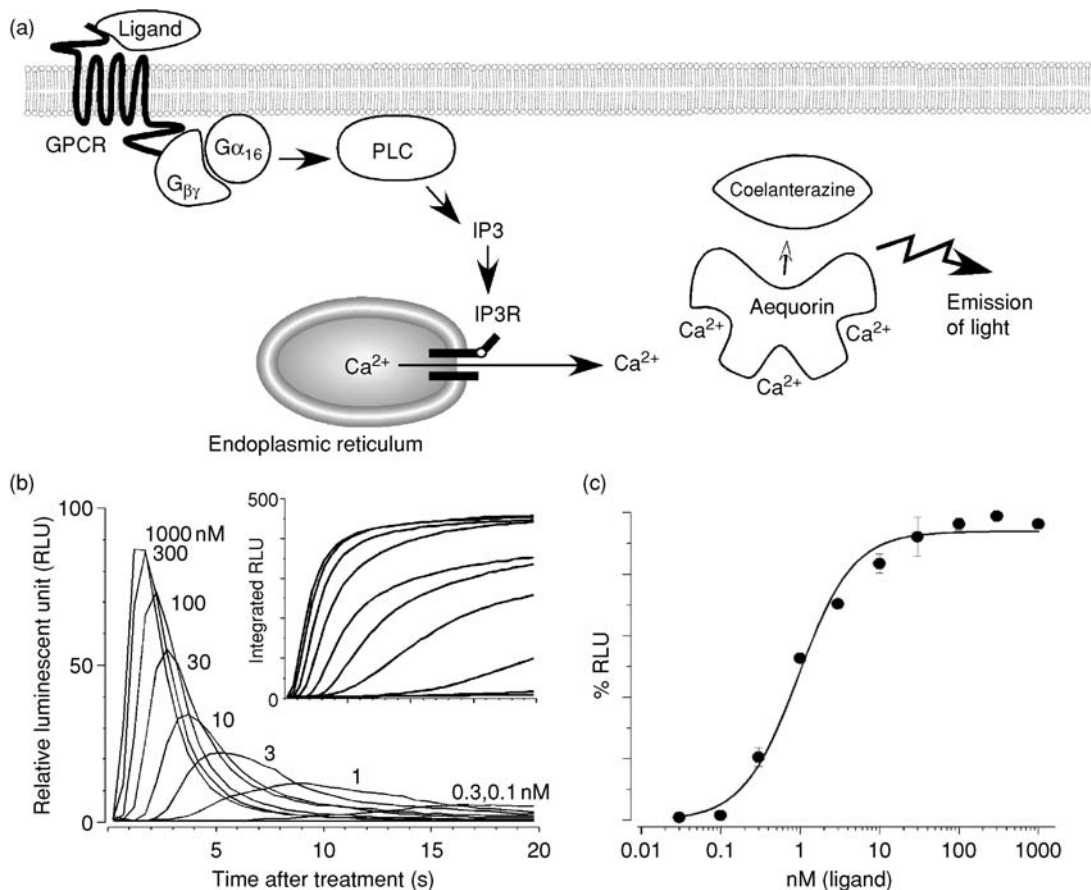


Figure 6 (a) A presumed signaling pathway operating in the aequorin assay for orphan GPCRs. The expressed heterologous GPCR transduces ligand binding through $G\alpha_{16}$, phospholipase C (PLC), inositol triphosphate (IP_3), and IP_3 receptor, resulting in Ca^{2+} mobilization from intracellular stores. Binding of Ca^{2+} to aequorin with the cofactor coelenterazine results in emission of light. (b) Progressively higher concentrations of ligand generate a more intense light response. (c) Integrated luminescence during a given time is used to generate the concentration–response curve.

Cell lines generally used for expression of insect GPCRs are Chinese hamster ovary (CHO-K1) cells, human embryonic kidney (HEK) cells, and Schneider's *Drosophila* (S2) cells (see **Tables 1** and **2**). All of these cells are known for their efficient coupling of GPCR activation to endogenous downstream signaling pathways. For instance, GPCRs expressed in CHO-K1 cells can be coupled to the PLC–calcium mobilization pathway, presumably via activation of G_q . A summary of downstream coupling mechanisms and signaling pathways for GPCRs in each cell line can be found at <http://www.tumor-gene.org>. Characteristics of *Drosophila* S2 cells are found in two recent publications (Van Poyer *et al.*, 2001; Torfs *et al.*, 2002b).

Both ligand binding assays and functional assays can be performed using cell line expression systems, depending on the need and situation. Affinity constants, receptor density, and kinetics of binding of heterologously expressed receptors can be investigated by using radioligands and compared with data

obtained from native receptors (e.g., Johnson *et al.*, 2003b; Meeusen *et al.*, 2002). An advantage of the binding assay is that the biochemical properties of the expressed GPCR can be determined independent of coupling efficiency to downstream signaling and reporter activation.

14.6.4. Other GPCR Reporter Assays

While functional assays for GPCRs have been improved and refined through introduction of various reporter molecules into the expression system, rapid desensitization can severely attenuate responses. An effective strategy for dealing with this problem is to detect the interactions of β -arrestin with the GPCR. In this instance, desensitization of the GPCR can be monitored using bioluminescence resonance energy transfer (BRET). GPCR fused to *Renilla* luciferase is used for the cell line stably expressing arrestin fused to green fluorescent protein (GFP) (Barak *et al.*, 1997; Bertrand *et al.*, 2002). Activation of the GPCR leads to interaction between the

arrestin::GFP and GPCR::luciferase resulting in the BRET from the donor luciferase to the acceptor GFP. GPCR activation can be also measured by downstream gene regulation. Activation of GPCR coupled to AC and elevation of cAMP is monitored by expression of reporter gene controlled by cAMP responsive elements (CREs) (Chen *et al.*, 1995; Durocher *et al.*, 2000). It is thought that the CRE is activated through both $G\alpha_s$ and $G\alpha_q$ pathways (Durocher *et al.*, 2000). A simpler approach is to simply follow migration of a β -arrestin-GFP fusion protein to the cell membrane following GPCR activation (Barak *et al.*, 1997; Johnson *et al.*, 2003b).

The use of heterologous expression systems to obtain pharmacological profiles of GPCRs should be interpreted with appropriate caution, since it is often likely that critical transduction components unique to the natural physiological system will be absent. Thus the ligand affinities of certain GPCRs may be under the strong influence of native G protein subtypes with which they are associated (Kostenis, 2001). Even more complexity can be introduced in instances where relative stoichiometries of receptors to other cellular signaling components affect the assay system (Kenakin, 1997, 2003). Finally, the availability of specific G proteins appears to affect ligand binding affinity, as in the case of dopamine receptors (Reale *et al.*, 1997; Cordeaux and Hill, 2002; Gazi *et al.*, 2003).

14.6.5. Identifying Biological Functions of GPCRs

Understanding the biological function of a particular signaling pathway has been advanced through examination of ligand actions in large insects amenable to physiological experimentation. Important tools for endocrinological studies have been classical physiological experiments involving ligation, surgical extirpation and implantation of organs and tissues, parabiosis, and transplantations followed by bioassay-guided chemical isolation of the ligand on a particular system. Study of receptors mediating these processes provides further in-depth understanding of the mode of action of chemical signals at the molecular and cellular levels.

Biochemical and pharmacological data obtained through heterologous expression of a GPCR provides the first step in understanding molecular mechanisms of a signaling pathway and association of a putative authentic receptor with its ligand and physiological actions. Experimental evidence supporting an endogenous ligand-receptor interaction could include: highest affinity functional or biochemical interaction between the ligand and the receptor (usually at nanomolar to picomolar

concentrations of ligand), spatial and temporal expression patterns of the GPCR that concurs with biological data, and mutant phenotypes that are in accordance with biological function of the signaling pathway.

Chemical isolation of unknown ligands for heterologously expressed orphan GPCRs is an increasingly useful method for functional analysis, especially with recently developed tools in proteomics. This approach is referred as reverse physiology (Birgul *et al.*, 1999) or reverse pharmacology (Civelli *et al.*, 2001) where the natural ligand for a receptor is sought. This approach contrasts with general approaches in which receptors are sought for a given ligand. Reverse physiology was successful in associating the peptide allatostatin A with its physiological receptor by isolating the peptide activating a *Drosophila* GPCR that is homologous to the somatostatin receptor of vertebrates (Birgul *et al.*, 1999). Three steps of peptide purification by cation-exchange column and reverse phase chromatography succeeded in isolation of a fraction active on the GPCR of interest. The final active fraction purified was analyzed by a matrix-assisted laser desorption ionization post source decay (MALDI-PSD) time-of-flight spectrum for obtaining the sequence. A similar approach was successful for deorphaning *Drosophila* CG2114 as an FMRFamide receptor (Meeusen *et al.*, 2002).

Multidisciplinary approaches have been successful in a number of cases. Dow and his group studied signaling systems *Drosophila* Malpighian tubules mediating diuretic functions (review: Dow and Davies, 2003). Three neuroendocrine peptides and their signaling pathways implicated in the activation of fluid secretion were examined: cardioacceleratory peptide, diuretic hormone, and leucokinin. A combination of physiology, pharmacology, and *Drosophila* genetics elucidated downstream signaling pathways at the molecular and cellular levels. Specific GPCRs activated by leucokinin (CG10626) (Radford *et al.*, 2002) and cardioacceleratory peptide (CG14575) (Park *et al.*, 2002) were identified in functional assays. In the case of the leucokinin receptor, immunohistochemistry has been used to study its expression in stellate cells, a subtype of Malpighian tubule epithelial cell previously shown to respond to leucokinin by Ca^{2+} mobilization activating chloride current and fluid secretion (Rosay *et al.*, 1997; Radford *et al.*, 2002).

14.7. Conclusions

We find ourselves in an exciting era of exponential growth of knowledge regarding GPCR structure

and function. Knowledge of these receptors obtained in basic science could well be applicable to the development of innovative pest control measures. Disruption of neuropeptide signaling systems through use of peptide hormone mimetics targeting the GPCRs also shows increasing promise (Nachman *et al.*, 2001, 2002a, 2002b). Chemical properties of pyrokinin analogs have been improved for increased cuticle penetrability and for resistance to peptidases (Nachman *et al.*, 2002a, 2000b). A novel method for delivering compounds to target GPCRs was attempted through use of a fusion protein combining lectin with allatostatin (Fitches *et al.*, 2002). By this means, allatostatin is delivered to insect hemolymph following oral ingestion and inhibits feeding and growth of the insect tomato moth (*Lacanobia oleracea*). With developing new biotechnology, GPCRs, particularly those mediating peptide signaling, may be excellent targets for pest control strategies providing an optimal range of specificity.

References

- Adams, M.E., Zitnan, D., 1997. Identification of ecdysis-triggering hormone in the silkworm *Bombyx mori*. *Biochem. Biophys. Res. Commun.* 230, 188–191.
- Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., *et al.*, 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucl. Acids Res.* 25, 3389–3402.
- Alvarez, C.E., Robison, K., Gilbert, W., 1996. Novel Gq alpha isoform is a candidate transducer of rhodopsin signaling in a *Drosophila* testes-autonomous pacemaker. *Proc. Natl Acad. Sci. USA* 93, 12278–12282.
- Andretic, R., Hirsh, J., 2000. Circadian modulation of dopamine receptor responsiveness in *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA* 97, 1873–1878.
- Angers, S., Salahpour, A., Bouvier, M., 2002. Dimerization: an emerging concept for G protein-coupled receptor ontogeny and function. *Annu. Rev. Pharmacol. Toxicol.* 42, 409–435.
- Arakawa, S., Gocayne, J.D., McCombie, W.R., Urquhart, D.A., Hall, L.M., *et al.*, 1990. Cloning, localization, and permanent expression of a *Drosophila* octopamine receptor. *Neuron* 4, 343–354.
- Auerswald, L., Birgul, N., Gäde, G., Kreienkamp, H.J., Richter, D., 2001. Structural, functional, and evolutionary characterization of novel members of the allatostatin receptor family from insects. *Biochem. Biophys. Res. Commun.* 282, 904–909.
- Baggerman, G., Cerstiaens, A., De Loof, A., Schoofs, L., 2002. Peptidomics of the larval *Drosophila melanogaster* central nervous system. *J. Biol. Chem.* 277, 40368–40374.
- Bahner, M., Sander, P., Paulsen, R., Huber, A., 2000. The visual G protein of fly photoreceptors interacts with the PDZ domain assembled INAD signaling complex via direct binding of activated Galpha(q) to phospholipase cbeta. *J. Biol. Chem.* 275, 2901–2904.
- Bai, D., Sattelle, D., 1995. A GABA_B receptor on an identified insect motor neurone. *J. Exp. Biol.* 198, 889–894.
- Baker, J.D., Truman, J.W., 2002. Mutations in the *Drosophila* glycoprotein hormone receptor, rickets, eliminate neuropeptide-induced tanning and selectively block a stereotyped behavioral program. *J. Exp. Biol.* 205, 2555–2565.
- Barak, L.S., Ferguson, S.S., Zhang, J., Caron, M.G., 1997. A beta-arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation. *J. Biol. Chem.* 272, 27497–27500.
- Benton, M.J., Ayala, F.J., 2003. Dating the tree of life. *Science* 300, 1698–1700.
- Bertrand, L., Parent, S., Caron, M., Legault, M., Joly, E., *et al.*, 2002. The BRET2/arrestin assay in stable recombinant cells: a platform to screen for compounds that interact with G protein-coupled receptors (GPCRs). *J. Recept. Signal Transduct. Res.* 22, 533–541.
- Bhanot, P., Fish, M., Jemison, J.A., Nusse, R., Nathans, J., *et al.*, 1999. Frizzled and Dfrizzled-2 function as redundant receptors for Wingless during *Drosophila* embryonic development. *Development* 126, 4175–4186.
- Birgul, N., Weise, C., Kreienkamp, H.J., Richter, D., 1999. Reverse physiology in *Drosophila*: identification of a novel allatostatin-like neuropeptide and its cognate receptor structurally related to the mammalian somatostatin/galanin/opioid receptor family. *EMBO J.* 18, 5892–5900.
- Blake, A.D., Anthony, N.M., Chen, H.H., Harrison, J.B., Nathanson, N.M., *et al.*, 1993. *Drosophila* nervous system muscarinic acetylcholine receptor: transient functional expression and localization by immunocytochemistry. *Mol. Pharmacol.* 44, 716–724.
- Blenau, W., Balfanz, S., Baumann, A., 2000. Amtyr1: characterization of a gene from honeybee (*Apis mellifera*) brain encoding a functional tyramine receptor. *J. Neurochem.* 74, 900–908.
- Blenau, W., Baumann, A., 2001. Molecular and pharmacological properties of insect biogenic amine receptors: lessons from *Drosophila melanogaster* and *Apis mellifera*. *Arch. Insect Biochem. Physiol.* 48, 13–38.
- Blenau, W., Erber, J., Baumann, A., 1998. Characterization of a dopamine D1 receptor from *Apis mellifera*: cloning, functional expression, pharmacology, and mRNA localization in the brain. *J. Neurochem.* 70, 15–23.
- Bockaert, J., Pin, J.P., 1999. Molecular tinkering of G protein-coupled receptors: an evolutionary success. *EMBO J.* 18, 1723–1729.
- Borowsky, B., Adham, N., Jones, K.A., Raddatz, R., Artymyshyn, R., *et al.*, 2001. Trace amines: identification of a family of mammalian G protein-coupled receptors. *Proc. Natl Acad. Sci. USA* 98, 8966–8971.
- Bourne, H.R., 1997. How receptors talk to trimeric G proteins. *Curr. Opin. Cell Biol.* 9, 134–142.
- Brady, A.E., Limbird, L.E., 2002. G protein-coupled receptor interacting proteins: emerging roles in localization and signal transduction. *Cell. Signal.* 14, 297–309.

- Briscoe, A.D., 2001. Functional diversification of lepidopteran opsins following gene duplication. *Mol. Biol. Evol.* 18, 2270–2279.
- Briscoe, A.D., 2002. Homology modeling suggests a functional role for parallel amino acid substitutions between bee and butterfly red- and green-sensitive opsins. *Mol. Biol. Evol.* 19, 983–986.
- Briscoe, A.D., Chittka, L., 2001. The evolution of color vision in insects. *Annu. Rev. Entomol.* 46, 471–510.
- Brody, T., Cravchik, A., 2000. *Drosophila melanogaster* G protein-coupled receptors. *J. Cell Biol.* 150, F83–F88.
- Cazzamali, G., Grimmelikhuijzen, C.J., 2002. Molecular cloning and functional expression of the first insect FMRFamide receptor. *Proc. Natl Acad. Sci. USA* 99, 12073–12078.
- Cazzamali, G., Saxild, N., Grimmelikhuijzen, C., 2002. Molecular cloning and functional expression of a *Drosophila* corazonin receptor. *Biochem. Biophys. Res. Commun.* 298, 31–36.
- Chen, W., Shields, T.S., Stork, P.J., Cone, R.D., 1995. A colorimetric assay for measuring activation of Gs- and Gq-coupled signaling pathways. *Analyt. Biochem.* 226, 349–354.
- Choi, M.Y., Fuerst, E.J., Rafaeli, A., Jurenka, R., 2003. Identification of a G protein-coupled receptor for pheromone biosynthesis activating neuropeptide from pheromone glands of the moth *Helicoverpa zea*. *Proc. Natl Acad. Sci. USA* 100, 9721–9726.
- Chothia, C., Gough, J., Vogel, C., Teichmann, S.A., 2003. Evolution of the protein repertoire. *Science* 300, 1701–1703.
- Chou, W.H., Hall, K.J., Wilson, D.B., Wideman, C.L., Townson, S.M., et al., 1996. Identification of a novel *Drosophila* opsin reveals specific patterning of the R7 and R8 photoreceptor cells. *Neuron* 17, 1101–1115.
- Chou, W.H., Huber, A., Bantrop, J., Schulz, S., Schwab, K., et al., 1999. Patterning of the R7 and R8 photoreceptor cells of *Drosophila*: evidence for induced and default cell-fate specification. *Development* 126, 607–616.
- Civelli, O., Nothacker, H.P., Saito, Y., Wang, Z., Lin, S.H., et al., 2001. Novel neurotransmitters as natural ligands of orphan G-protein-coupled receptors. *Trends Neurosci.* 24, 230–237.
- Clyne, P.J., Warr, C.G., Carlson, J.R., 2000. Candidate taste receptors in *Drosophila*. *Science* 287, 1830–1834.
- Clyne, P.J., Warr, C.G., Freeman, M.R., Lessing, D., Kim, J., et al., 1999. A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in *Drosophila*. *Neuron* 22, 327–338.
- Colas, J.F., Launay, J.M., Kellermann, O., Rosay, P., Maroteaux, L., 1995. *Drosophila* 5-HT₂ serotonin receptor: coexpression with fushi-tarazu during segmentation. *Proc. Natl Acad. Sci. USA* 92, 5441–5445.
- Colas, J.F., Launay, J.M., Vonesch, J. L., Hickel, P., Maroteaux, L., 1999. Serotonin synchronises convergent extension of ectoderm with morphogenetic gastrulation movements in *Drosophila*. *Mech. Devel.* 87, 77–91.
- Copenhaver, P.F., Horgan, A.M., Nichols, D.C., Rasmussen, M.A., 1995. Developmental expression of heterotrimeric G proteins in the nervous system of *Manduca sexta*. *J. Neurobiol.* 26, 461–484.
- Cordeaux, Y., Hill, S.J., 2002. Mechanisms of cross-talk between G-protein-coupled receptors. *Neurosignals* 11, 45–57.
- Cordeaux, Y., Nickolls, S.A., Flood, L.A., Graber, S.G., Strange, P.G., 2001. Agonist regulation of D(2) dopamine receptor/G protein interaction: evidence for agonist selection of G protein subtype. *J. Biol. Chem.* 276, 28667–28675.
- Cowman, A.F., Zuker, C.S., Rubin, G.M., 1986. An opsin gene expressed in only one photoreceptor cell type of the *Drosophila* eye. *Cell* 44, 705–710.
- Cox, K.J., Tensen, C.P., Van der Schors, R.C., Li, K.W., van Heerikhuizen, H., et al., 1997. Cloning, characterization, and expression of a G-protein-coupled receptor from *Lymnaea stagnalis* and identification of a leucokinin-like peptide, PSFHSWSamide, as its endogenous ligand. *J. Neurosci.* 17, 1197–1205.
- Dahanukar, A., Foster, K., van der Goes van Naters, W.M., Carlson, J.R., 2001. A Gr receptor is required for response to the sugar trehalose in taste neurons of *Drosophila*. *Nat. Neurosci.* 4, 1182–1186.
- Darlison, M.G., Richter, D., 1999. Multiple genes for neuropeptides and their receptors: co-evolution and physiology. *Trends Neurosci.* 22, 81–88.
- de Sousa, S.M., Hoveland, L.L., Yarfitz, S., Hurley, J.B., 1989. The *Drosophila* Go alpha-like G protein gene produces multiple transcripts and is expressed in the nervous system and in ovaries. *J. Biol. Chem.* 264, 18544–18551.
- Dixon, R.A., Kobilka, B.K., Strader, D.J., Benovic, J.L., Dohlman, H.G., et al., 1986. Cloning of the gene and cDNA for mammalian beta-adrenergic receptor and homology with rhodopsin. *Nature* 321, 75–79.
- Dolzer, J., Krannich, S., Fischer, K., Stengl, M., 2001. Oscillations of the transepithelial potential of moth olfactory sensilla are influenced by octopamine and serotonin. *J. Exp. Biol.* 204, 2781–2794.
- Dow, J.A.T., Davies, S.A., 2003. Integrative physiology and functional genomics of epithelial function in a genetic model organism. *Physiol. Rev.* 83, 687–729.
- Durocher, Y., Perret, S., Thibaudeau, E., Gaumond, M.H., Kamen, A., et al., 2000. A reporter gene assay for high-throughput screening of G-protein-coupled receptors stably or transiently expressed in HEK293 EBNA cells grown in suspension culture. *Analyt. Biochem.* 284, 316–326.
- Ebert, P.R., Rowland, J.E., Toma, D.P., 1998. Isolation of seven unique biogenic amine receptor clones from the honey bee by library scanning. *Insect Mol. Biol.* 7, 151–162.
- Egerod, K., Reynisson, E., Hauser, F., Cazzamali, G., Williamson, M., et al., 2003a. Molecular cloning and functional expression of the first two specific insect myosuppressin receptors. *Proc. Natl Acad. Sci. USA* 100, 9808–9813.

- Egerod, K., Reynisson, E., Hauser, F., Williamson, M., Cazzamali, G., *et al.*, 2003b. Molecular identification of the first insect proctolin receptor. *Biochem. Biophys. Res. Commun.* 306, 437–442.
- Eriksen, K.K., Hauser, F., Schiott, M., Pedersen, K.M., Sondergaard, L., *et al.*, 2000. Molecular cloning, genomic organization, developmental regulation, and a knock-out mutant of a novel leu-rich repeats-containing G protein-coupled receptor (DLGR-2) from *Drosophila melanogaster*. *Genome Res.* 10, 924–938.
- Evans, P.D., 1980. Biogenic amines in the insect nervous system. *Adv. Insect Physiol.* 15, 317–473.
- Evans, P.D., 1993. Molecular studies on insect octopamine receptors. In: Pichon, Y. (Ed.), *Comparative Molecular Neurobiology*. Birkhauser, Basel, pp. 286–296.
- Feany, M.B., Quinn, W.G., 1995. A neuropeptide gene defined by the *Drosophila* memory mutant amnesiac. *Science* 268, 869–873.
- Feiler, R., Harris, W.A., Kirschfeld, K., Wehrhahn, C., Zuker, C.S., 1988. Targeted misexpression of a *Drosophila* opsin gene leads to altered visual function. *Nature* 333, 737–741.
- Feng, G., Hannan, F., Reale, V., Hon, Y.Y., Kousky, C.T., *et al.*, 1996. Cloning and functional characterization of a novel dopamine receptor from *Drosophila melanogaster*. *J. Neurosci.* 16, 3925–3933.
- Feng, G., Reale, V., Chatwin, H., Kennedy, K., Venard, R., *et al.*, 2003. Functional characterization of a neuropeptide F-like receptor from *Drosophila melanogaster*. *Eur. J. Neurosci.* 18, 227–238.
- Filipek, S., Teller, D.C., Palczewski, K., Stenkamp, R., 2003. The crystallographic model of rhodopsin and its use in studies of other G protein-coupled receptors. *Annu. Rev. Biophys. Biomol. Struct.* 32, 375–397.
- Fitches, E., Audsley, N., Gatehouse, J.A., Edwards, J.P., 2002. Fusion proteins containing neuropeptides as novel insect control agents: snowdrop lectin delivers fused allatostatin to insect haemolymph following oral ingestion. *Insect Biochem. Mol. Biol.* 32, 1653–1661.
- Fremion, F., Astier, M., Zaffran, S., Guillen, A., Homburger, V., *et al.*, 1999. The heterotrimeric protein Go is required for the formation of heart epithelium in *Drosophila*. *J. Cell Biol.* 145, 1063–1076.
- Fryxell, K.J., Meyerowitz, E.M., 1987. An opsin gene that is expressed only in the R7 photoreceptor cell of *Drosophila*. *EMBO J.* 6, 443–451.
- Fujii, R., Hosoya, M., Fukusumi, S., Kawamata, Y., Habata, Y., *et al.*, 2000. Identification of neuromedin U as the cognate ligand of the orphan G protein-coupled receptor FM-3. *J. Biol. Chem.* 275, 21068–21074.
- Gäde, G., Hoffmann, K.H., Spring, J.H., 1997. Hormonal regulation in insects: facts, gaps, and future directions. *Physiol. Rev.* 77, 963–1032.
- Garczynski, S.F., Brown, M.R., Shen, P., Murray, T.F., Crim, J.W., 2002. Characterization of a functional neuropeptide F receptor from *Drosophila melanogaster*. *Peptides* 23, 773–780.
- Gazi, L., Nickolls, S.A., Strange, P.G., 2003. Functional coupling of the human dopamine D(2) receptor with Galpha(i1), Galpha(i2), Galpha(i3) and Galpha(o) G proteins: evidence for agonist regulation of G protein selectivity. *Br. J. Pharmacol.* 138, 775–786.
- Goh, C.S., Cohen, F.E., 2002. Co-evolutionary analysis reveals insights into protein–protein interactions. *J. Mol. Biol.* 324, 177–192.
- Gomes, I., Jordan, B.A., Gupta, A., Rios, C., Trapaidze, N., *et al.*, 2001. G protein coupled receptor dimerization: implications in modulating receptor function. *J. Mol. Med.* 79, 226–242.
- Gotzes, F., Balfanz, S., Baumann, A., 1994. Primary structure and functional characterization of a *Drosophila* dopamine receptor with high homology to human D1/5 receptors. *Recept. Channels* 2, 131–141.
- Gotzes, F., Baumann, A., 1996. Functional properties of *Drosophila* dopamine D1-receptors are not altered by the size of the N-terminus. *Biochem. Biophys. Res. Commun.* 222, 121–126.
- Guerrero, F.D., 1997. Transcriptional expression of a putative tachykinin-like peptide receptor gene from stable fly. *Peptides* 18, 1–5.
- Ha, S.D., Kataoka, H., Suzuki, A., Kim, B.J., Kim, H.J., *et al.*, 2000. Cloning and sequence analysis of cDNA for diuretic hormone receptor from *Bombyx mori*. *Mol. Cells* 10, 13–17.
- Han, K.A., Millar, N.S., Davis, R.L., 1998. A novel octopamine receptor with preferential expression in *Drosophila* mushroom bodies. *J. Neurosci.* 18, 3650–3658.
- Han, K.A., Millar, N.S., Grotewiel, M.S., Davis, R.L., 1996. DAMB, a novel dopamine receptor expressed specifically in *Drosophila* mushroom bodies. *Neuron* 16, 1127–1135.
- Hannan, F., Hall, L.M., 1996. Temporal and spatial expression patterns of two G-protein coupled receptors in *Drosophila melanogaster*. *Invertebr. Neurosci.* 2, 71–83.
- Hardie, R.C., Raghu, P., 2001. Visual transduction in *Drosophila*. *Nature* 413, 186–193.
- Hauser, F., Nothacker, H.P., Grimmelikhuijzen, C.J., 1997. Molecular cloning, genomic organization, and developmental regulation of a novel receptor from *Drosophila melanogaster* structurally related to members of the thyroid-stimulating hormone, follicle-stimulating hormone, luteinizing hormone/choriogonadotropin receptor family from mammals. *J. Biol. Chem.* 272, 1002–1010.
- Hearn, M.G., Ren, Y., McBride, E.W., Reveillaud, I., Beinborn, M., *et al.*, 2002. A *Drosophila* dopamine 2-like receptor: molecular characterization and identification of multiple alternatively spliced variants. *Proc. Natl Acad. Sci. USA* 99, 14554–14559.
- Henkel, A.W., Sankaranarayanan, S., 1999. Mechanisms of alpha-latrotoxin action. *Cell Tissue Res.* 296, 229–233.
- Hermans, E., Challiss, R.A., 2001. Structural, signalling and regulatory properties of the group I metabotropic glutamate receptors: prototypic family C G-protein-coupled receptors. *Biochem. J.* 359, 465–484.
- Hewes, R.S., Taghert, P.H., 2001. Neuropeptides and neuropeptide receptors in the *Drosophila melanogaster* genome. *Genome Res.* 11, 1126–1142.

- Hill, C.A., Fox, A.N., Pitts, R.J., Kent, L.B., Tan, P.L., *et al.*, 2002a. G protein-coupled receptors in *Anopheles gambiae*. *Science* 298, 176–178.
- Hill, E.S., Iwano, M., Gatellier, L., Kanzaki, R., 2002b. Morphology and physiology of the serotonin-immunoreactive putative antennal lobe feedback neuron in the male silkworm *Bombyx mori*. *Chem. Senses* 27, 475–483.
- Holmes, S.P., Barhoumi, R., Nachman, R.J., Pietrantonio, P.V., 2003. Functional analysis of a G protein-coupled receptor from the Southern cattle tick *Boophilus microplus* (Acari: Ixodidae) identifies it as the first arthropod myokinin receptor. *Insect Mol. Biol.* 12, 27–38.
- Holmes, S.P., He, H., Chen, A.C., Ivie, G.W., Pietrantonio, P.V., 2000. Cloning and transcriptional expression of a leucokinin-like peptide receptor from the southern cattle tick, *Boophilus microplus* (Acari: Ixodidae). *Insect Mol. Biol.* 9, 457–465.
- Horn, F., van der Wenden, E.M., Oliveira, L., IJzerman, A.P., Vriend, G., 2000. Receptors coupling to G proteins: is there a signal behind the sequence? *Proteins* 41, 448–459.
- Howard, A.D., Wang, R., Pong, S.-S., Mellin, T.N., Strack, A., *et al.*, 2000. Identification of receptors for neuromedin U and its role in feeding. *Nature* 406, 70–74.
- Huesmann, G.R., Cheung, C.C., Loi, P.K., Lee, T.D., Swiderek, K.M., *et al.*, 1995. Amino acid sequence of CAP-2b, an insect cardioacceleratory peptide from the tobacco hawkmoth *Manduca sexta*. *FEBS Lett.* 371, 311–314.
- Isaacson, J.S., Vitten, H., 2003. GABA_B receptors inhibit dendrodendritic transmission in the rat olfactory bulb. *J. Neurosci.* 23, 2032–2039.
- Ishimoto, H., Matsumoto, A., Tanimura, T., 2000. Molecular identification of a taste receptor gene for trehalose in *Drosophila*. *Science* 289, 116–119.
- Iversen, A., Cazzamali, G., Williamson, M., Hauser, F., Grimelikhuijzen, C.J., 2002. Molecular identification of the first insect ecdysis triggering hormone receptors. *Biochem. Biophys. Res. Commun.* 299, 924–931.
- Jespersen, T., Grunnet, M., Angelo, K., Klaerke, D.A., Olesen, S.P., 2002. Dual-function vector for protein expression in both mammalian cells and *Xenopus laevis* oocytes. *Biotechniques* 32, 536–538, 540.
- Johard, H.A., Muren, J.E., Nichols, R., Larhammar, D.S., Nassel, D.R., 2001. A putative tachykinin receptor in the cockroach brain: molecular cloning and analysis of expression by means of antisera to portions of the receptor protein. *Brain Res.* 919, 94–105.
- Johnson, E.C., Bohn, L.M., Barak, L.S., Birse, R.T., Nassel, D.R., *et al.*, 2003a. Identification of *Drosophila* neuropeptide receptors by G protein-coupled receptors-beta-arrestin2 interactions. *J. Biol. Chem.* 278, 52172–52178.
- Johnson, E.C., Garczynski, S.F., Park, D., Crim, J.W., Nassel, D.R., *et al.*, 2003b. Identification and characterization of a G protein-coupled receptor for the neuropeptide proctolin in *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA* 100, 6198–6203.
- Jones, K.A., Borowsky, B., Tamm, J.A., Craig, D.A., Durkin, M.M., *et al.*, 1998. GABA(B) receptors function as a heteromeric assembly of the subunits GABA(B)R1 and GABA(B)R2. *Nature* 396, 674–679.
- Josefsson, L.G., 1999. Evidence for kinship between diverse G-protein coupled receptors. *Gene* 239, 333–340.
- Julius, D., MacDermott, A.B., Axel, R., Jessell, T.M., 1988. Molecular characterization of a functional cDNA encoding the serotonin 1c receptor. *Science* 241, 558–564.
- Kaupmann, K., Malitschek, B., Schuler, V., Heid, J., Froestl, W., *et al.*, 1998. GABA(B)-receptor subtypes assemble into functional heteromeric complexes. *Nature* 396, 683–687.
- Kenakin, T., 1997. Differences between natural and recombinant G protein-coupled receptor systems with varying receptor/G protein stoichiometry. *Trends Pharmacol. Sci.* 18, 456–464.
- Kenakin, T., 2003. A guide to drug discovery: predicting therapeutic value in the lead optimization phase of drug discovery. *Nature Rev. Drug Discov.* 2, 429–438.
- Kierszenbaum, A.L., 2003. Epididymal G protein-coupled receptor (GPCR): two hats and a two-piece suit tailored at the GPS motif. *Mol. Reprod. Devel.* 64, 1–3.
- Kim, J., Carlson, J.R., 2002. Gene discovery by e-genetics: *Drosophila* odor and taste receptors. *J. Cell Sci.* 115, 1107–1112.
- Kniazeff, J., Galvez, T., Labesse, G., Pin, J.P., 2002. No ligand binding in the GB2 subunit of the GABA(B) receptor is required for activation and allosteric interaction between the subunits. *J. Neurosci.* 22, 7352–7361.
- Kofuji, P., Davidson, N., Lester, H.A., 1995. Evidence that neuronal G-protein-gated inwardly rectifying K⁺ channels are activated by G beta gamma subunits and function as heteromultimers. *Proc. Natl Acad. Sci. USA* 92, 6542–6546.
- Kohout, T.A., Lefkowitz, R.J., 2003. Regulation of G protein-coupled receptor kinases and arrestins during receptor desensitization. *Mol. Pharmacol.* 63, 9–18.
- Kolakowski, L.F., Jr., 1994. GCRDb: a G-protein-coupled receptor database. *Recept. Channels* 2, 1–7.
- Kostenis, E., 2001. Is Galpha16 the optimal tool for fishing ligands of orphan G-protein-coupled receptors? *Trends Pharmacol. Sci.* 22, 560–564.
- Kreienkamp, H.J., Larusson, H.J., Witte, I., Roeder, T., Birgul, N., *et al.*, 2002a. Functional annotation of two orphan G-protein-coupled receptors, Drostar1 and -2, from *Drosophila melanogaster* and their ligands by reverse pharmacology. *J. Biol. Chem.* 277, 39937–39943.
- Kreienkamp, H.J., Soltau, M., Richter, D., Bockers, T., 2002b. Interaction of G-protein-coupled receptors with synaptic scaffolding proteins. *Biochem. Soc. Trans.* 30, 464–468.
- Krieger, J., Raming, K., Dewer, Y.M., Bette, S., Conzelmann, S., *et al.*, 2002. A divergent gene family encoding candidate olfactory receptors of the moth *Heliothis virescens*. *Eur. J. Neurosci.* 16, 619–628.

- Kubiak, T.M., Larsen, M.J., Burton, K. J., Bannow, C.A., Martin, R.A., *et al.*, 2002. Cloning and functional expression of the first *Drosophila melanogaster* sulfakinin receptor DSK-R1. *Biochem. Biophys. Res. Commun.* 291, 313–320.
- Kubo, T., Fukuda, K., Mikami, A., Maeda, A., Takahashi, H., *et al.*, 1986. Cloning, sequencing and expression of complementary DNA encoding the muscarinic acetylcholine receptor. *Nature* 323, 411–416.
- Kutsukake, M., Komatsu, A., Yamamoto, D., Ishiwa-Chigusa, S., 2000. A tyramine receptor gene mutation causes a defective olfactory behavior in *Drosophila melanogaster*. *Gene* 245, 31–42.
- Larsen, M.J., Burton, K.J., Zantello, M.R., Smith, V.G., Lowery, D.L., *et al.*, 2001. Type A allatostatins from *Drosophila melanogaster* and *Diptera punctata* activate two *Drosophila* allatostatin receptors, DAR-1 and DAR-2, expressed in CHO cells. *Biochem. Biophys. Res. Commun.* 286, 895–901.
- Le Poul, E., Hisada, S., Mizuguchi, Y., Dupriez, V.J., Burgeon, E., *et al.*, 2002. Adaptation of aequorin functional assay to high throughput screening. *J. Biomol. Screen.* 7, 57–65.
- Lee, A., Durieux, M.E., 1998. The use of *Xenopus laevis* oocytes for the study of G protein-coupled receptors. In: Lynch, K.R. (Ed.), Identification and Expression of G Protein-Coupled Receptors. Wiley-Liss, New York, pp. 73–96.
- Lee, Y.J., Dobbs, M.B., Verardi, M.L., Hyde, D.R., 1990. *dgq*: a *Drosophila* gene encoding a visual system-specific G alpha molecule. *Neuron* 5, 889–898.
- Lee, Y.J., Shah, S., Suzuki, E., Zars, T., O'Day, P.M., *et al.*, 1994. The *Drosophila dgq* gene encodes a G alpha protein that mediates phototransduction. *Neuron* 13, 1143–1157.
- Lenz, C., Williamson, M., Grimmelhuijzen, C.J., 2000. Molecular cloning and genomic organization of a second probable allatostatin receptor from *Drosophila melanogaster*. *Biochem. Biophys. Res. Commun.* 273, 571–577.
- Lenz, C., Williamson, M., Hansen, G.N., Grimmelhuijzen, C.J., 2001. Identification of four *Drosophila* allatostatins as the cognate ligands for the *Drosophila* orphan receptor DAR-2. *Biochem. Biophys. Res. Commun.* 286, 1117–1122.
- Li, X.J., Wolfgang, W., Wu, Y.N., North, R.A., Forte, M., 1991. Cloning, heterologous expression and developmental regulation of a *Drosophila* receptor for tachykinin-like peptides. *EMBO J.* 10, 3221–3229.
- Li, X.J., Wu, Y.N., North, R.A., Forte, M., 1992. Cloning, functional expression, and developmental regulation of a neuropeptide Y receptor from *Drosophila melanogaster*. *J. Biol. Chem.* 267, 9–12.
- Lim, N.F., Dascal, N., Labarca, C., Davidson, N., Lester, H.A., 1995. A G protein-gated K channel is activated via beta 2-adrenergic receptors and G beta gamma subunits in *Xenopus* oocytes. *J. Gen. Physiol.* 105, 421–439.
- Lin, Y.J., Seroude, L., Benzer, S., 1998. Extended lifespan and stress resistance in the *Drosophila* mutant *methuselah*. *Science* 282, 943–946.
- Liu, A.M., Ho, M.K., Wong, C.S., Chan, J.H., Pau, A.H., *et al.*, 2003. Galpha(16/z) chimeras efficiently link a wide range of G protein-coupled receptors to calcium mobilization. *J. Biomol. Screen.* 8, 39–49.
- Masu, Y., Nakayama, K., Tamaki, H., Harada, Y., Kuno, M., *et al.*, 1987. cDNA cloning of bovine substance-K receptor through oocyte expression system. *Nature* 329, 836–838.
- Matsumoto, S., Kitamura, A., Nagasawa, H., Kataoka, H., Orikasa, C., *et al.*, 1990. Functional diversity of a neurohormone produced by the subesophageal ganglion: molecular identity of melanization and reddish coloration hormone and pheromone biosynthesis activating neuropeptide. *J. Insect Physiol.* 36, 427–432.
- Maxwell, G.D., Tait, J.F., Hildebrand, J.G., 1978. Regional synthesis of neurotransmitter candidates in the CNS of the moth *Manduca sexta*. *Comp. Biochem. Physiol.* C 61, 109–119.
- McBride, E.W., Reveillaud, I., Ren, Y., Kopin, A.S., 2001. Molecular cloning, genomic organization and expression of a *Drosophila* cholecystokinin-like receptor. *Annu. Drosophila Res. Conf.* 42, 432C.
- Meeusen, T., Mertens, I., Clynen, E., Baggerman, G., Nichols, R., *et al.*, 2002. Identification in *Drosophila melanogaster* of the invertebrate G protein-coupled FMRFamide receptor. *Proc. Natl Acad. Sci. USA* 99, 15363–15368.
- Mertens, I., Meeusen, T., Huybrechts, R., De Loof, A., Schoofs, L., 2002. Characterization of the short neuropeptide F receptor from *Drosophila melanogaster*. *Biochem. Biophys. Res. Commun.* 297, 1140–1148.
- Mezler, M., Muller, T., Raming, K., 2001. Cloning and functional expression of GABA(B) receptors from *Drosophila*. *Eur. J. Neurosci.* 13, 477–486.
- Moller, S., Vilo, J., Croning, M.D., 2001. Prediction of the coupling specificity of G protein coupled receptors to their G proteins. *Bioinformatics* 17, S174–S181.
- Monnier, D., Colas, J.F., Rosay, P., Hen, R., Borrelli, E., *et al.*, 1992. NKD, a developmentally regulated tachykinin receptor in *Drosophila*. *J. Biol. Chem.* 267, 1298–1302.
- Montell, C., 1999. Visual transduction in *Drosophila*. *Annu. Rev. Cell Devel. Biol.* 15, 231–268.
- Morris, A.J., Malbon, C.C., 1999. Physiological regulation of G protein-linked signaling. *Physiol. Rev.* 79, 1373–1430.
- Morris, H.R., Panico, M., Karplus, A., Lloyd, P.E., Riniker, B., 1982. Elucidation by FAB-MS of the structure of a new cardioactive peptide in *Aplysia*. *Nature* 300, 643–645.
- Nachman, R.J., Strey, A., Isaac, E., Pryor, N., Lopez, J.D., *et al.*, 2002a. Enhanced *in vivo* activity of peptidase-resistant analogs of the insect kinin neuropeptide family. *Peptides* 23, 735–745.
- Nachman, R.J., Teal, P.E., Strey, A., 2002b. Enhanced oral availability/pheromonotropic activity of

- peptidase-resistant topical amphiphilic analogs of pyrokinin/PBAN insect neuropeptides. *Peptides* 23, 2035–2043.
- Nachman, R.J., Teal, P.E., Ujvary, I., 2001. Comparative topical pheromotropic activity of insect pyrokinin/PBAN amphiphilic analogs incorporating different fatty and/or cholic acid components. *Peptides* 22, 279–285.
- Nassel, D.R., 2002. Neuropeptides in the nervous system of *Drosophila* and other insects: multiple roles as neuromodulators and neurohormones. *Prog. Neurobiol.* 68, 1–84.
- Neves, S.R., Ram, P.T., Iyengar, R., 2002. G protein pathways. *Science* 296, 1636–1639.
- Nishi, S., Hsu, S.Y., Zell, K., Hsueh, A.J., 2000. Characterization of two fly LGR (leucine-rich repeat-containing, G protein-coupled receptor) proteins homologous to vertebrate glycoprotein hormone receptors: constitutive activation of wild-type fly LGR1 but not LGR2 in transfected mammalian cells. *Endocrinology* 141, 4081–4090.
- Noguchi, H., Hayakawa, Y., 2001. Dopamine is a key factor for the induction of egg diapause of the silkworm, *Bombyx mori*. *Eur. J. Biochem.* 268, 774–780.
- O'Tousa, J.E., Baehr, W., Martin, R.L., Hirsh, J., Pak, W.L., *et al.*, 1985. The *Drosophila* *ninaE* gene encodes an opsin. *Cell* 40, 839–850.
- Obosi, L.A., Schuette, D.G., Europe-Finner, G.N., Beadle, D.J., Hen, R., *et al.*, 1996. Functional characterisation of the *Drosophila* 5-HTdro1 and 5-HTdro2B serotonin receptors in insect cells: activation of a G(alpha)s-like protein by 5-HTdro1 but lack of coupling to inhibitory G-proteins by 5-HTdro2B. *FEBS Lett.* 381, 233–236.
- Orchard, I., Jan-Marino, R., ALange, A.B., 1993. A multifunctional role for octopamine in locust flight. *Annu. Rev. Entomol.* 38, 227–249.
- Palczewski, K., Kumasaka, T., Hori, T., Behnke, C.A., Motoshima, H., *et al.*, 2000. Crystal structure of rhodopsin: a G protein-coupled receptor. *Science* 289, 739–745.
- Park, Y., Kim, Y.J., Adams, M.E., 2002. Identification of G protein-coupled receptors for *Drosophila* PRXamide peptides, CCAP, corazonin, and AKH supports a theory of ligand–receptor coevolution. *Proc. Natl Acad. Sci. USA* 99, 11423–11428.
- Park, Y., Kim, Y.J., Dupriez, V., Adams, M.E., 2003. Two subtypes of ecdysis triggering hormone receptor in *Drosophila melanogaster*. *J. Biol. Chem.* 278, 17710–17715.
- Park, Y., Zitnan, D., Gill, S.S., Adams, M.E., 1999. Molecular cloning and biological activity of ecdysis-triggering hormones in *Drosophila melanogaster*. *FEBS Lett.* 463, 133–138.
- Parks, S., Wieschaus, E., 1991. The *Drosophila* gastrulation gene *concertina* encodes a G alpha-like protein. *Cell* 64, 447–458.
- Parmentier, M.L., Joly, C., Restituito, S., Bockaert, J., Grau, Y., *et al.*, 1998. The G protein-coupling profile of metabotropic glutamate receptors, as determined with exogenous G proteins, is independent of their ligand recognition domain. *Mol. Pharmacol.* 53, 778–786.
- Parmentier, M.L., Pin, J.P., Bockaert, J., Grau, Y., 1996. Cloning and functional expression of a *Drosophila* metabotropic glutamate receptor expressed in the embryonic CNS. *J. Neurosci.* 16, 6687–6694.
- Peleg, S., Varon, D., Ivanina, T., Dessauer, C.W., Dascal, N., 2002. G(alpha)(i) controls the gating of the G protein-activated K(+) channel, GIRK. *Neuron* 33, 87–99.
- Pierce, K.L., Lefkowitz, R.J., 2001. Classical and new roles of beta-arrestins in the regulation of G-protein-coupled receptors. *Nat. Rev. Neurosci.* 2, 727–733.
- Pietrantonio, P.V., Jagge, C., McDowell, C., 2001. Cloning and expression analysis of a 5HT7-like serotonin receptor cDNA from mosquito *Aedes aegypti* female excretory and respiratory systems. *Insect Mol. Biol.* 10, 357–369.
- Poels, J., Suner, M.M., Needham, M., Torfs, H., De Rijck, J., *et al.*, 2001. Functional expression of a locust tyramine receptor in murine erythroleukaemia cells. *Insect Mol. Biol.* 10, 541–548.
- Pophof, B., 2000. Octopamine modulates the sensitivity of silkworm pheromone receptor neurons. *J. Comp. Physiol. A* 186, 307–313.
- Quan, F., Wolfgang, W.J., Forte, M., 1993. A *Drosophila* G-protein alpha subunit, Gf alpha, expressed in a spatially and temporally restricted pattern during *Drosophila* development. *Proc. Natl Acad. Sci. USA* 90, 4236–4240.
- Radford, J.C., Davies, S.A., Dow, J.A., 2002. Systematic G-protein-coupled receptor analysis in *Drosophila melanogaster* identifies a leucokinin receptor with novel roles. *J. Biol. Chem.* 277, 38810–38817.
- Raina, A.K., Jaffe, H., Kempe, T.G., Keim, P., Blacher, R.W., *et al.*, 1989. Identification of a neuropeptide hormone that regulates sex pheromone production in female moths. *Science* 244, 796–798.
- Ratnaparkhi, A., Banerjee, S., Hasan, G., 2002. Altered levels of Gq activity modulate axonal pathfinding in *Drosophila*. *J. Neurosci.* 22, 4499–4508.
- Ray, K., Ganguly, R., 1994. Organization and expression of the *Drosophila melanogaster* D-G gamma 1 gene encoding the G-protein gamma subunit. *Gene* 148, 315–319.
- Raymond, V., Hamon, A., Grau, Y., Lapied, B., 1999. DmGluRA, a *Drosophila* metabotropic glutamate receptor, activates G-protein inwardly rectifying potassium channels in *Xenopus* oocytes. *Neurosci. Lett.* 269, 1–4.
- Reagan, J.D., 1994. Expression cloning of an insect diuretic hormone receptor, a member of the calcitonin/secretin receptor family. *J. Biol. Chem.* 269, 9–12.
- Reagan, J.D., 1995. Functional expression of a diuretic hormone receptor in baculovirus-infected insect cells: evidence suggesting that the N-terminal region of diuretic hormone is associated with receptor activation. *Insect Biochem. Mol. Biol.* 25, 535–539.

- Reagan, J.D., 1996. Molecular cloning and function expression of a diuretic hormone receptor from the house cricket, *Acheta domesticus*. *Insect Biochem. Mol. Biol.* 26, 1–6.
- Reale, V., Hannan, F., Hall, L.M., Evans, P.D., 1997. Agonist-specific coupling of a cloned *Drosophila melanogaster* D1-like dopamine receptor to multiple second messenger pathways by synthetic agonists. *J. Neurosci.* 17, 6545–6553.
- Reuveny, E., Slesinger, P.A., Inglese, J., Morales, J.M., Iniguez-Lluhi, J.A., et al., 1994. Activation of the cloned muscarinic potassium channel by G protein beta gamma subunits. *Nature* 370, 143–146.
- Ridley, M., 1993. Evolution. Blackwell Scientific Publications, Oxford.
- Riehle, M.A., Garczynski, S.F., Crim, J.W., Hill, C.A., Brown, M.R., 2002. Neuropeptides and peptide hormones in *Anopheles gambiae*. *Science* 298, 172–175.
- Robb, S., Cheek, T.R., Hannan, F.L., Hall, L.M., Midgley, J.M., et al., 1994. Agonist-specific coupling of a cloned *Drosophila* octopamine/tyramine receptor to multiple second messenger systems. *EMBO J.* 13, 1325–1330.
- Roeder, T., 2002. Biochemistry and molecular biology of receptors for biogenic amines in locusts. *Microsc. Res. Tech.* 56, 237–247.
- Roeder, T., 2003. Metabotropic histamine receptors: nothing for invertebrates? *Eur. J. Pharmacol.* 466, 85–90.
- Rosay, P., Davies, S.A., Yu, Y., Sozen, A., Kaiser, K., et al., 1997. Cell-type specific calcium signalling in a *Drosophila* epithelium. *J. Cell Sci.* 110, 1683–1692.
- Rosenkilde, C., Cazzamali, G., Williamson, M., Hauser, F., Sondergaard, L., et al., 2003. Molecular cloning, functional expression, and gene silencing of two *Drosophila* receptors for the *Drosophila* neuropeptide pyrokinin-2. *Biochem. Biophys. Res. Commun.* 309, 485–494.
- Saifullah, A.S., Tomioka, K., 2002. Serotonin sets the day state in the neurons that control coupling between the optic lobe circadian pacemakers in the cricket *Gryllus bimaculatus*. *J. Exp. Biol.* 205, 1305–1314.
- Salcedo, E., Huber, A., Henrich, S., Chadwell, L.V., Chou, W.H., et al., 1999. Blue- and green-absorbing visual pigments of *Drosophila*: ectopic expression and physiological characterization of the R8 photoreceptor cell-specific Rh5 and Rh6 rhodopsins. *J. Neurosci.* 19, 10716–10726.
- Saudou, F., Amlaiky, N., Plassat, J.L., Borrelli, E., Hen, R., 1990. Cloning and characterization of a *Drosophila* tyramine receptor. *EMBO J.* 9, 3611–3617.
- Saudou, F., Boschert, U., Amlaiky, N., Plassat, J.L., Hen, R., 1992. A family of *Drosophila* serotonin receptors with distinct intracellular signalling properties and expression patterns. *EMBO J.* 11, 7–17.
- Schmidt, C.J., Garen-Fazio, S., Chow, Y.K., Neer, E.J., 1989. Neuronal expression of a newly identified *Drosophila melanogaster* G protein alpha 0 subunit. *Cell Reg.* 1, 125–134.
- Schneider, L.E., Taghert, P.H., 1988. Isolation and characterization of a *Drosophila* gene that encodes multiple neuropeptides related to Phe-Met-Arg-Phe-NH₂ (FMRFamide). *Proc. Natl Acad. Sci. USA* 85, 1993–1997.
- Schneider, L.E., Taghert, P.H., 1990. Organization and expression of the *Drosophila* Phe-Met-Arg-Phe-NH₂ neuropeptide gene. *J. Biol. Chem.* 265, 6890–6895.
- Schulz, S., Huber, A., Schwab, K., Paulsen, R., 1999. A novel Ggamma isolated from *Drosophila* constitutes a visual G protein gamma subunit of the fly compound eye. *J. Biol. Chem.* 274, 37605–37610.
- Scott, K., Brady, R., Jr., Cravchik, A., Morozov, P., Rzhetsky, A., et al., 2001. A chemosensory gene family encoding candidate gustatory and olfactory receptors in *Drosophila*. *Cell* 104, 661–673.
- Secher, T., Lenz, C., Cazzamali, G., Sorensen, G., Williamson, M., et al., 2001. Molecular cloning of a functional allatostatin gut/brain receptor and an allatostatin preprohormone from the silkworm *Bombyx mori*. *J. Biol. Chem.* 276, 47052–47060.
- Shapiro, R.A., Wakimoto, B.T., Subers, E.M., Nathanson, N.M., 1989. Characterization and functional expression in mammalian cells of genomic and cDNA clones encoding a *Drosophila* muscarinic acetylcholine receptor. *Proc. Natl Acad. Sci. USA* 86, 9039–9043.
- Shichida, Y., Imai, H., 1998. Visual pigment: G-protein-coupled receptor for light signals. *Cell. Mol. Life Sci.* 54, 1299–1315.
- Simon, M.I., Strathmann, M.P., Gautam, N., 1991. Diversity of G proteins in signal transduction. *Science* 252, 802–808.
- Slesinger, P.A., Stoffel, M., Jan, Y.N., Jan, L.Y., 1997. Defective gamma-aminobutyric acid type B receptor-activated inwardly rectifying K⁺ currents in cerebellar granule cells isolated from weaver and Girk2 null mutant mice. *Proc. Natl Acad. Sci. USA* 94, 12210–12217.
- Song, W., Ranjan, R., Dawson-Scully, K., Bronk, P., Marin, L., et al., 2002. Presynaptic regulation of neurotransmission in *Drosophila* by the g protein-coupled receptor methuselah. *Neuron* 36, 105–119.
- Stables, J., Green, A., Marshall, F., Fraser, N., Knight, E., et al., 1997. A bioluminescent assay for agonist activity at potentially any G-protein-coupled receptor. *Analyt. Biochem.* 252, 115–126.
- Stacey, M., Lin, H.H., Gordon, S., McKnight, A.J., 2000. LNB-TM7, a group of seven-transmembrane proteins related to family-B G-protein-coupled receptors. *Trends Biochem. Sci.* 25, 284–289.
- Staubli, F., Jorgensen, T.J., Cazzamali, G., Williamson, M., Lenz, C., et al., 2002. Molecular identification of the insect adipokinetic hormone receptors. *Proc. Natl Acad. Sci. USA* 99, 3446–3451.
- Stortkuhl, K.F., Kettler, R., 2001. Functional analysis of an olfactory receptor in *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA* 98, 9381–9385.

- Strutt, D.I., 2001. Asymmetric localization of frizzled and the establishment of cell polarity in the *Drosophila* wing. *Mol. Cell* 7, 367–375.
- Talluri, S., Bhatt, A., Smith, D.P., 1995. Identification of a *Drosophila* G protein alpha subunit (dGq alpha-3) expressed in chemosensory cells and central neurons. *Proc. Natl Acad. Sci. USA* 92, 11475–11479.
- Teller, D.C., Okada, T., Behnke, C.A., Palczewski, K., Stenkamp, R.E., 2001. Advances in determination of a high-resolution three-dimensional structure of rhodopsin, a model of G-protein-coupled receptors (GPCRs). *Biochemistry* 40, 7761–7772.
- Thibonnier, M., Auzan, C., Madhun, Z., Wilkins, P., Berti-Mattera, L., et al., 1994. Molecular cloning, sequencing, and functional expression of a cDNA encoding the human V-1a vasopressin receptor. *J. Biol. Chem.* 269, 3304–3310.
- Torfs, H., Detheux, M., Oonk, H.B., Akerman, K.E., Poels, J., et al., 2002a. Analysis of C-terminally substituted tachykinin-like peptide agonists by means of aequorin-based luminescent assays for human and insect neurokinin receptors. *Biochem. Pharmacol.* 63, 1675–1682.
- Torfs, H., Oonk, H.B., Broeck, J.V., Poels, J., Van Poyer, W., et al., 2001. Pharmacological characterization of STKR, an insect G protein-coupled receptor for tachykinin-like peptides. *Arch. Insect Biochem. Physiol.* 48, 39–49.
- Torfs, H., Poels, J., Detheux, M., Dupriez, V., Van Loy, T., et al., 2002b. Recombinant aequorin as a reporter for receptor-mediated changes of intracellular Ca²⁺-levels in *Drosophila* S2 cells. *Invertebr. Neurosci.* 4, 119–124.
- Torfs, H., Shariatmadari, R., Guerrero, F., Parmentier, M., Poels, J., et al., 2000. Characterization of a receptor for insect tachykinin-like peptide agonists by functional expression in a stable *Drosophila* Schneider 2 cell line. *J. Neurochem.* 74, 2182–2189.
- Townson, S.M., Chang, B.S., Salcedo, E., Chadwell, L.V., Pierce, N.E., et al., 1998. Honeybee blue- and ultraviolet-sensitive opsins: cloning, heterologous expression in *Drosophila*, and physiological characterization. *J. Neurosci.* 18, 2412–2422.
- Vaidehi, N., Floriano, W.B., Trabanino, R., Hall, S.E., Freddolino, P., et al., 2002. Prediction of structure and function of G protein-coupled receptors. *Proc. Natl Acad. Sci. USA* 99, 12622–12627.
- van Kesteren, R.E., Tensen, C.P., Smit, A.B., van Minnen, J., Kolakowski, L.F., et al., 1996. Co-evolution of ligand-receptor pairs in the vasopressin/oxytocin superfamily of bioactive peptides. *J. Biol. Chem.* 271, 3619–3626.
- Van Poyer, W., Torfs, H., Poels, J., Swinnen, E., De Loof, A., et al., 2001. Phenolamine-dependent adenylyl cyclase activation in *Drosophila* Schneider 2 cells. *Insect Biochem. Mol. Biol.* 31, 333–338.
- Vanden Broeck, J., Vulsteke, V., Huybrechts, R., De Loof, A., 1995. Characterization of a cloned locust tyramine receptor cDNA by functional expression in permanently transformed *Drosophila* S2 cells. *J. Neurochem.* 64, 2387–2395.
- von Nickisch-Roseneck, E., Krieger, J., Kubick, S., Laage, R., Strobel, J., et al., 1996. Cloning of biogenic amine receptors from moths (*Bombyx mori* and *Heliothis virescens*). *Insect Biochem. Mol. Biol.* 26, 817–827.
- Washio, H., 2002. Glutamate receptors on the somata of dorsal unpaired median neurons in cockroach, *Periplaneta americana*, thoracic ganglia. *Zool. Sci.* 19, 153–162.
- Wess, J., 1998. Molecular basis of receptor/G-protein-coupling selectivity. *Pharmacol. Therapeut.* 80, 231–264.
- West, A.P., Jr., Llamas, L.L., Snow, P.M., Benzer, S., Bjorkman, P.J., 2001. Crystal structure of the ectodomain of Methuselah, a *Drosophila* G protein-coupled receptor associated with extended lifespan. *Proc. Natl Acad. Sci. USA* 98, 3744–3749.
- Wetzel, C.H., Behrendt, H.J., Gisselmann, G., Stortkuhl, K.F., Hovemann, B., et al., 2001. Functional expression and characterization of a *Drosophila* odorant receptor in a heterologous cell system. *Proc. Natl Acad. Sci. USA* 98, 9377–9380.
- White, J.H., Wise, A., Main, M.J., Green, A., Fraser, N.J., et al., 1998. Heterodimerization is required for the formation of a functional GABA(B) receptor. *Nature* 396, 679–682.
- Wickman, K.D., Iniguez-Lluhl, J.A., Davenport, P.A., Taussig, R., Krapivinsky, G.B., et al., 1994. Recombinant G-protein beta gamma-subunits activate the muscarinic-gated atrial potassium channel. *Nature* 368, 255–257.
- Witz, P., Amlaiky, N., Plassat, J.L., Maroteaux, L., Borrelli, E., et al., 1990. Cloning and characterization of a *Drosophila* serotonin receptor that activates adenylate cyclase. *Proc. Natl Acad. Sci. USA* 87, 8940–8944.
- Wolfgang, W.J., Hoskote, A., Roberts, I.J., Jackson, S., Forte, M., 2001. Genetic analysis of the *Drosophila* Gs(alpha) gene. *Genetics* 158, 1189–1201.
- Wolfgang, W.J., Quan, F., Goldsmith, P., Unson, C., Spiegel, A., et al., 1990. Immunolocalization of G protein alpha-subunits in the *Drosophila* CNS. *J. Neurosci.* 10, 1014–1024.
- Wolfgang, W.J., Quan, F., Thambi, N., Forte, M., 1991. Restricted spatial and temporal expression of G-protein alpha subunits during *Drosophila* embryogenesis. *Development* 113, 527–538.
- Yarden, Y., Rodriguez, H., Wong, S.K., Brandt, D.R., May, D.C., et al., 1986. The avian beta-adrenergic receptor: primary structure and membrane topology. *Proc. Natl Acad. Sci. USA* 83, 6795–6799.
- Yarfitz, S., Niemi, G.A., McConnell, J.L., Fitch, C.L., Hurley, J.B., 1991. A G beta protein in the *Drosophila* compound eye is different from that in the brain. *Neuron* 7, 429–438.
- Yarfitz, S., Provost, N.M., Hurley, J.B., 1988. Cloning of a *Drosophila melanogaster* guanine nucleotide

- regulatory protein beta-subunit gene and characterization of its expression during development. *Proc. Natl Acad. Sci. USA* 85, 7134–7138.
- Zhong, Y., 1995. Mediation of PACAP-like neuropeptide transmission by coactivation of Ras/Raf and cAMP signal transduction pathways in *Drosophila*. *Nature* 375, 588–592.
- Zhong, Y., Pena, L.A., 1995. A novel synaptic transmission mediated by a PACAP-like neuropeptide in *Drosophila*. *Neuron* 14, 527–536.
- Zitnan, D., Kingan, T.G., Hermesman, J., Adams, M. E., 1996. Identification of ecdysis-triggering hormone from an epitracheal endocrine system. *Science* 271, 88–91.

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15 Insect Transformation for Use in Control

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15.1. Introduction

In the previous series of *Comprehensive Insect Physiology, Biochemistry, and Pharmacology*, the corresponding article by Whitten (1985) dealing, briefly, with insect transformation technology raised two points about the problems that the extension of this technology into the field would most likely face. The prescience of these forecasts is even more remarkable given that it was written shortly after the development of a genetic transformation technology for *Drosophila melanogaster* and so was authored some 10 years before the repeatable transposable element-mediated transformation of nondrosophilid pest insect species.

In writing this review we have chosen not to regurgitate information already presented in recent reviews on the subject (Robinson and Franz, 2000; Atkinson, 2002; Handler, 2002; Robinson, 2002; Wimmer, 2003; Robinson *et al.*, 2004), and we will not discuss the issue of the development of a regulatory framework for the use of this technology. Two recent reports on the regulation of the release of transgenic arthropods have recently been published and the reader is referred to these for illumination as to the state of this issue (National Research Council, 2002; Pew Initiative on Food and Biotechnology, 2004). It would also be difficult to improve on Whitten's (1985) review, which comprehensively examined the history, conceptual basis, and application of genetic control in insects. Rather, under examination are two points raised by Whitten as a means to assess critically the current status of the application of genetically engineered insects to help solve problems in medicine and agriculture.

It must first be said that, some 20 years after the publication of Whitten's review, there is not one

single example of the use of genetically engineered pest insects in the field. This is despite the first report of the transformation of an anopheline mosquito by Miller *et al.* (1987), and then, 8 years later, the first of several published reports ultimately describing five new transposable elements used to transform a range of nondrosophilid insect species, e.g., the Mediterranean fruit fly, *Ceratitis capitata*, and the yellow fever mosquito, *Aedes aegypti* (review: Robinson *et al.*, 2004).

In his 1985 review, Whitten stated:

Clearly it is unlikely that our technical ability to transform a species will prove lacking: rather, it is our uncertainty concerning what genetic modifications are desirable that will usually prove to be the major impediment.

The current state of the field supports this prediction. Repeatable genetic transformation technologies for nondrosophilid insects have now existed for 9 years. While the development of these did prove to be more problematic than expected and awaited the discovery of new transposable elements such as *Mimos* (Franz *et al.*, 1994), *Mos1* (Medhora *et al.*, 1991), *piggyBac* (Cary *et al.*, 1989), and *Hermes* (Warren *et al.*, 1994), the application of these technologies to practical problems in medical, veterinary, and economic entomology has not occurred. Several reasons exist for this, the most important one being the difficulty in discovering genetic and biochemical systems that might prove to be effective genetic control strategies in the field. Even as recently as 2001, the most elegant examples of using genetic-based strategies to selectively eliminate females (e.g., for the augmentation of a sterile insect technology) occurred in *D. melanogaster* and with experimental population sizes several orders

of magnitude smaller than those required for the mass-rearing of insects in sterile insect technique (SIT) programs (Heinrich and Scott, 2000; Thomas *et al.*, 2000). Both experiments utilized conventional *P* element-mediated genetic transformation of *Drosophila*. As discussed, research into the manipulation of genetic and biochemical systems has produced interesting advances to prevent the transmission of pathogens through mosquitoes and, using model systems, proof of principle that transmission of pathogens can be diminished in genetically modified mosquitoes has been demonstrated (Ito *et al.*, 2002; Moreira *et al.*, 2002). While significant for the control of mosquitoes and the human diseases they vector, these experiments have little bearing on the development of corresponding technology in agriculturally important insect species, such as *C. capitata* and the many lepidopteran species that feed on crops. Strategies for introducing and testing genetic markers, such as the green fluorescent protein (GFP), into an SIT strain for the purpose of allowing easy identification of this strain from the field strain, have been discussed and such strains have been generated in the Caribbean fruit fly, *Anastrepha suspensa*, in which the green fluorescence has been found to be detectable up to 4 weeks postmortem (Handler, 2002). Strains having similar properties have also been developed in *C. capitata* (Franz, personal communication). The stability of this protein, although very important for its use as a marker, has raised concerns about the transfer of the protein to nontarget organisms through predation. Significant effort has been expended towards unraveling the genetic basis of sexual development in *C. capitata* with the goal being to use these genes and their promoters as the basis of new genetic sexing strategies. Important genes such as *double-sex* and *transformer* have been isolated and characterized, allowing comparisons to be made of their function with their structural homologs in *D. melanogaster* (Saccone *et al.*, 2002). Sex-specific promoters from *C. capitata* have also been isolated (Christophides *et al.*, 2000). To date, however, no genetically engineered strains of *C. capitata* designed for a genetic sexing strategy have been generated or tested. In lepidopterans, the only progress in pest species has been the genetic transformation of *Pectinophora gossypiella* using the *piggyBac* element and the consequent application for permits to examine the robustness of this genetically engineered strain in outdoor cage experiments (Peloquin *et al.*, 2000).

Another factor contributing to the slow development of the technology is that genetic

transformation of nondrosophilid insects is still not a routine robust technology. In this regard Ashburner *et al.* (1998) defined robustness:

by the property that anyone skilled in the art can carry out the procedure, as is the case with transformation of *Drosophila*, a technique that has been performed in hundreds of laboratories world-wide.

Indeed, one of the reasons that *C. capitata* and *A. aegypti* are most frequently the subject of transformation experiments is the relative ease with which their embryos can be handled and injected. Combined with this is the very small number of laboratories in which nondrosophilid transformation is practiced. This is compounded by, in the case of tephritid fruit flies, quarantine laws that prevent many pest species from being reared in regions where they are classified as being exotic, thereby ruling out many research groups from developing genetically engineered strains of tephritids. Some 9 years after the successful demonstration of *Mimos* transposable element-mediated transformation of *C. capitata* by Loukeris *et al.* (1995), the comparison with the rapid spread of *D. melanogaster* transformation protocols through the *Drosophila* community following the publication of this technique in 1982 by Rubin and Spradling (1982) could not be more stark. By 1991, *Drosophila* transformation was a routine technique that permeated *Drosophila* genetics. This has not been achieved as yet with mosquitoes, let alone many tephritids, lepidopterans, or coleopterans.

Another reason concerns the nature of the transposable elements used to transform nondrosophilid species. As mentioned above, the identification of new transposable elements such as *Mos1*, *Mimos*, *Hermes*, *piggyBac*, and *Tn5* (Rowan *et al.*, 2004), was a major step in achieving genetic transformation of these species. With the exception of *Tn5*, which has been developed as a transformation agent of bacteria, we know little about the genetic and biochemical behavior of these elements, particularly with respect to their behavior in nonhost insect species. Possible problems about deploying transposable elements, and the transgenes contained within them, was forecast by Whitten (1985):

Thus the selfishness of DNA is clearly subject to rather strict rules of etiquette, presumably imposed by evolutionary constraints on its host. These constraints may force those workers who see practical benefits flowing from the availability of transposable element vectors for genomic modification of particular plants or animals, to tailor a system suited to the special genetic constraints of their particular candidate species.

Recently, the behavior of *Hermes*, *piggyBac*, and *Mos1* elements in *A. aegypti* was described by O'Brochta *et al.* (2003) while the immobility of *Mos1* in transgenic lines of *A. aegypti* was reported by Wilson *et al.* (2003). These studies reveal that, while these transposable elements can be used to genetically transform this mosquito species, they are then rendered relatively immobile in following generations. Furthermore, the mechanism of *Hermes* element transposition appears to differ between germline nuclei and somatic nuclei of *A. aegypti*. Both mechanisms are transposase dependent, yet cut-and-paste transposition occurs in somatic nuclei while a mechanism that results in the transposition of both the transposable element and flanking sequences occur in the germline. This difference in mechanisms of *Hermes* element transposition has not been seen in any of the higher dipteran species – *D. melanogaster* (O'Brochta *et al.*, 1996; Guimond *et al.*, 2003), *C. capitata* (Michel *et al.*, 2001), and *Stomoxys calcitrans* (Lehane *et al.*, 2000) – in which *Hermes* has been used as a gene vector. It is clear we have a very limited knowledge of how any of these transposable elements function either in their original host species, or in species into which they are introduced. This lack of knowledge, combined with the problem of identifying genetic systems they would carry into a population so as to be used in genetic control programs, has contributed to the inability to bring these transgenic-based control programs to fruition. In particular, those who propose transposable element-based strategies for spreading beneficial transgenes through field populations of insects need to take into account that the ability of a given element to transform a species does not necessarily mean that it can also subsequently spread through this same species. That the *P* element of *D. melanogaster* can do this in this species is no guarantee that other elements can also do the same. Kidwell and Ribeiro (1992) specify three critical parameters needed for a transposable element to spread through a population. These are:

- the basic reproductive rate of the insects possessing the transposable element;
- the infectivity of the element (which is a function of its transposition frequency, its ability to undergo replicative transposition, and its propensity to transpose to loci unlinked to the original donor site); and
- the size of the target insect population.

In a subsequent article (Ribeiro and Kidwell, 1994), they proposed seven questions that needed to be resolved if one is to believe that a transposable

element can spread genes through an insect population. These are: (1) the instability of chimeric transposable elements containing transgenes, (2) stabilization of these transgenes following their fixation in the target population (if, indeed, this is possible), (3) the biochemical properties of the transposase, (4) the possibility of repression systems becoming active as the copy number of the transposable element increases in the genome, (5) position effects, (6) the promoters chosen to control expression of the transgene, and (7) the length of the chimeric transposable element. It must be emphasized that none of these determines how well a given transposable element will function as a gene vector in achieving transformation in the first place. It is also poignant that we know next to nothing about the answers to any of these questions for the four “new” transposable elements used to transform pest insect species.

15.2. The Status of Transgenic-Based Insect Control Programs

What then, is the current status of programs in which transgenic insects have been proposed to utilize transgenic insects? They can be categorized into three broad categories:

1. Those that are designed to impose a genetic load on the target population in order to reduce population size.
2. Those that are designed to only eliminate the pest phenotype of the insects in a receiving population without altering population size.
3. Those that are designed to increase the fitness of biological control agents or increase the production of products excreted by insects, such as honey or silk.

15.2.1. Load Imposition on the Target Population

The sterile insect technique is the classic example of controlling insects through the imposition of a genetic load. The method was developed during the 1940s and 1950s and is now a mature and widely accepted method for insect population suppression and, in some cases, eradication. The pest species is mass-reared and irradiated to induce dominant lethal mutations and rearrangements at high frequencies in the germlines of these insects. The mass-reared insects, preferably only males, are released into the receiving, field population where they mate with wild fertile insects. Fertile females mating with released sterile males are nonproductive with a net reproductive rate (R_0) of zero. In many species a small

proportion of the females mate more than once so the net effect on R_0 is a function of sperm competitiveness in multiple-mated females. Successive releases in multiple generations continues to drive R_0 down until either the population size is below its economic threshold (at which point it does not impact upon the economy of the relevant commodity) or until it is eradicated. The advantages of using this method of control have been discussed elsewhere (Hendrichs, 2000) but clearly rest on the species specificity of the method and its low environmental impact. Nonetheless implementing this “clean” control method faces some constraints including fitness costs associated with the development of specific strains and the effects of mass rearing, radiation, handling, and release on many aspects of field behavior (Robinson and Franz, 2000). Transgenic technology has the potential to positively impact existing SIT programs by improving aspects of insect production and monitoring and also has the potential to increase the use of SIT to include species that have otherwise been intractable to this technology.

Perhaps the biggest impact transgenic insect technology could have on any SIT program is to create strains of insects that permit the removal or elimination of females. The most significant effect that this would have on an SIT program is the reduction of the costs of mass-rearing and release because fewer insects need to be reared and released. In other programs, for example those that might involve vectors of human disease, removal of females prior to release may be essential since females would still be capable of biting and transmitting disease. Transgenic technology offers numerous options for creating strains that permit the selective removal of females prior to release. Conceptually the problem is one of expressing genes in one sex or the other that can be easily selected for or against. Using transgenic technology specificity of expression can be achieved by a variety of ways. First, expression can be controlled through the use of sex-specific promoters in combination with genes resulting in lethality. Sex-specific promoters from the vitellogenin and yolk protein genes have been characterized, and are examples of female-specific promoters that have been tested in transgenic insects with respect to possible applications in insect control programs (Heinrich and Scott, 2000; Kokoza *et al.*, 2000; Thomas *et al.*, 2000). These promoters are active in adult stages and would have limited application in SIT programs. They would not be useful for eliminating females at the larval stage and so any cost savings resulting from rearing both sexes would still be accrued. For eliminating females prior to mass-rearing, the challenge

remains to identify promoters or other regulatory sequences that selectively eliminate females during, or immediately following, embryogenesis.

15.2.2. Challenges of Long-Term Gene Introduction into Natural Populations

Transgenic strategies relying on load imposition to reduce or eliminate a population involve the short-term introduction of “effector” genes (usually dominant or conditionally dominant lethals) into the gene pool of native populations. As described above for the SIT, approaches relying on inundative releases of nongenetically engineered mass-reared insects carrying dominant lethal mutations have been successful. More subtle approaches have been proposed whereby deleterious genes can be transmitted over the course of a few generations before the load effects are encountered (Schliekelman and Gould, 2000a, 2000b). The most ambitious plans involve the permanent and stable alteration of the genotypes of wild insects. These ideas present enormous and novel challenges to insect geneticists. Introducing new laboratory-produced genotypes into populations is relatively simple (e.g., inundative releases of radiation-sterilized flies), but maintaining those genotypes in the population and, in fact, having them increase in frequency is an unprecedented undertaking in applied insect genetics.

An allele can increase in frequency in populations for a variety of reasons. For example, if there is a fitness advantage associated with a genotype then over time this allele is expected to become more abundant. If selection pressures are sufficiently high then the forces of natural selection can result in relatively rapid changes in allele frequencies. The global spread of insecticide resistance in *Culex* mosquitoes is one of many such examples of selection driven increase in gene frequencies (Raymond, 1991). The fitness costs associated with transgenic mosquitoes are largely unknown and is an area of research in need of attention. A few reports on the ability of transgenics to compete with wild conspecifics or on fitness estimates based on life table analyses, consistently indicate that the process of transgenesis decreases the fitness of the host insect (Catteruccia *et al.*, 2003; Irvin *et al.*, 2004). The sources of these fitness costs have not been precisely determined but are expected to be partitioned between costs associated with inbreeding during the process of creating a transgenic line of insects, transgene expression, and mutagenesis associated with transgene integration. It should be remembered, however, that genetic sexing strains generated through standard Mendelian genetics are less fit than

wild-type strains but are still successfully used in SIT programs. In *Drosophila* it has been estimated that transposing *P* elements results in a 1% reduction in viability per integration event, on average (Eanes *et al.*, 1988). Thus, transgene integration is likely to result in fitness costs to the host organism. Expression of transgenes might also have effects on the fitness of the host organism. General costs associated with the metabolic load imposed by transgene expression are expected but have not been specifically measured (Billingsley, 2003). Individual transgenes, including marker genes, which have been generally considered to be “neutral,” are likely to have costs associated with their expression. It is reasonable to assume, like others (Tiedje *et al.*, 1989) that transgenic insects will be less fit relative to the nontransgenic host insect. Understanding the magnitude of fitness costs associated with transgenesis will permit insect geneticists to allow for any fitness costs in a given experiment. However, given that natural selection is unlikely to favor the increase in frequency of transgenes in populations, insect geneticists are still faced with the problem of driving a deleterious gene into populations quickly and effectively enough to alleviate the problem, economic or medical, caused by the target pest species.

Deleterious genes can be spread through populations by a number of mechanisms. For example, hitchhiking is a genetic phenomenon whereby genes closely linked to a particular gene under positive selection are also selected despite potentially negative fitness costs associated with them. Hitchhiking is a well-established phenomenon and insect geneticists could conceivably exploit it if a gene under strong positive selection could be identified and isolated. An alternative to hitchhiking is to link the deleterious transgene to a genetic element with transmission-skewing properties. Genetic segregation distorter systems and transposable elements have enhanced transmission potential under certain conditions. That is, organisms heterozygous for such elements give rise to a disproportionate number of gametes with the element or gene as compared to what would be expected based on simple Mendelian inheritance. A notable example of a transposable element with the ability to rapidly infiltrate populations of naive insects is the *P* element of *D. melanogaster*. Indeed, the natural history of *P* has been promoted to the status of a paradigm, and optimistic views by some vector biologists about the prospects of driving anti-*Plasmodium* genes into native populations of *Anopheles gambiae*, thereby eliminating malaria transmission, are based on exploiting the anticipated mobility

properties of a *P*-like transposable element. It has been implicitly assumed that solution of the gene transformation problem in nondrosophilid insects through the development of transposable element based gene vectors would simultaneously solve the problem of gene drivers. This is an erroneous assumption.

Transposable elements differ in their abilities to spread through populations as illustrated by the differences observed between actively transposing *hobo* and *P* elements in *D. melanogaster*. The dynamics of movement and the final state achieved was element specific. A given element, such as the *P* element, may have drastically different drive potential in one species as compared to another. For example, while *P* elements effectively invade and spread in small laboratory cages of *D. melanogaster* they are relatively ineffective in *D. simulans* (Kimura and Kidwell, 1994).

There are a number of properties of a transposable element that are likely to influence its ability to spread through wild populations and these have already been listed based on previous studies (Kidwell and Ribeiro, 1992; Ribeiro and Kidwell, 1994). If we focus on the mechanism of transposition, then, clearly, activity of an element alone is not sufficient for an element to spread. Spread will require that transposition is associated with replication of the element to insure a net gain in allele frequency. Elements that transpose by a cut-and-paste mechanism are not expected to spread unless they are replicated in association with transposition. Class II transposable elements employ a number of mechanisms by which they can replicate. Element excision leaves a double-stranded break in the host DNA that is highly reactive and can initiate strand invasion and recombination using the sister chromatid as a template. Template-directed gap repair results in a copy of the moving element replacing the copy that has excised as a result of transposition. The frequency with which this type of repair takes place varies from element to element. The timing of transposition during the cell cycle is also a factor. Class II elements can also be replicated during the transposition process by timing their jumps to correspond to S phase of the cell cycle. If an element transposes after it has been replicated and targets an unreplicated region of a chromosome there will be a net gain of one element in the genome of one of the resulting mitotic products. The *Mos1* element of *D. mauritiana* appears to transpose during S phase in transgenic lines of *A. aegypti*; however, the stability of this element in these insects limits its use as a genetic drive mechanism in at least this species (O'Brochta *et al.*, 2003). The proximity of the new

insertion sites relative to the donor site is also an important factor in determining the ability of the transposable element to spread. A highly active element that moves only, or even predominately, to tightly linked sites would not be a suitable agent to spread genes through an insect population, while an element with a reduced transposition rate that moved to unlinked loci either on the same or different chromosomes would be a viable spreading agent.

As mentioned above (see Section 15.1), at present little or no information exists as to the mobility properties of the four transposable elements used to genetically transform nondrosophilid insect species. Indeed, even for the relatively well-characterized *P* element of *D. melanogaster*, little is known about its mode of movement within and between chromosomes. This element does show a tendency to insert in or near the 5' ends of genes, perhaps due to a relaxation of the DNA double helix during gene transcription. It has also been suggested that this element recognizes a structural feature at the insertion site rather than a strict canonical motif (Liao *et al.*, 2000). This may well be true of other transposable elements and may well be an important factor in determining transposable element spread, but this remains an underexplored area of research.

15.2.3. Engineering of Beneficial Insects

Progress in this area has been limited to the stable introduction of genes, using the *piggyBac* transposable element, into the silkworm, *Bombyx mori* (Tamurua *et al.*, 2000). Initially these experiments have been proof of principle experiments in which enhanced green fluorescent protein (EGFP) was used as a genetic marker to demonstrate that transformation could be achieved. Recently, Imamura *et al.* (2003) demonstrated that the GAL4/UAS system functions sufficiently in transgenic *B. mori* to enable tissue-specific expression of a reporter gene to occur. Transformation frequencies using the *piggyBac* transposable element as the gene vector were in the order of several percent. These experiments pave the way for gene identification using enhancer trapping in *Bombyx* which will further elevate the use of this species as a model system for other lepidopteran species. The extension of these techniques into practical benefits of silk production remains a challenge. While there have been reports of sperm-mediated transformation of the honeybee, *Apis mellifera* (Robinson *et al.*, 2000), this technology has not yet been exploited by the honey industry and, since honey is a food, genetic engineering of its source may encounter public

resistance. Similarly, the initial report of genetic transformation of the predatory mite *Metaseiulus occidentalis*, which is used as a biological control agent, has not been pursued in field applications (Presnail and Hoy, 1992).

15.3. Conclusion

Genetic transformation technologies have been successfully extended into selected nondrosophilid species using transposable elements. This significant and exciting success has, however, been confined to the laboratory where it has enabled novel genotypes to be constructed and tested. These technologies have yet to be extended to the field, despite many years elapsing since genetic transformation protocols were first established for key pest species such as *C. capitata* and *A. aegypti*. If the full potential and benefits of genetic modification of medically and agriculturally significant insect species is to be realized then there must be a stronger linkage between the formulation of ideas and the subsequent timely and safe testing of these in transgenic insect strains in the laboratory, and in the field. Key to this is improving the robustness of transgenic technology in these insect species. Alternatively, the wisdom of establishing a handful of insect transformation centers that would provide this service to the community needs to be explored. This may be particularly attractive for species, such as *A. gambiae*, that remain difficult to transform. Providing a central transformation center may encourage researchers to develop and test new concepts, confident that at least the transgenic insects containing the desired transgenes will be routinely produced in a timely manner. It is critical to demonstrate in the laboratory and then in field cage experiments clear and concrete examples of how transgenic insect technology is beneficial to the general public so that arguments about the benefits of these new approaches can be clearly made to this interested and undoubtedly concerned audience.

References

- Ashburner, M., Hoy, M.A., Peloquin, J.J., 1998. Prospects for the genetic transformation of arthropods. *Insect Mol. Biol.* 7, 201–213.
- Atkinson, P.W., 2002. Genetic engineering in insects of agricultural importance. *Insect Biochem. Mol. Biol.* 32, 1237–1242.
- Billingsley, P.F., 2003. Environmental constraints on the physiology of transgenic mosquitoes. In: Takken, W., Scott, T.W. (Eds.), *Ecological Aspects for Application of*

- Genetically Modified Mosquitoes. Kluwer Academic Publishers, Dordrecht, pp. 149–161.
- Cary, L.C., Goebel, M., Corsaro, B.G., Wang, H.G., Rosen, E., *et al.*, 1989. Transposon mutagenesis of baculoviruses: analysis of *Trichoplusia ni* transposon IFP2 insertions within the FP-locus of nuclear polyhedrosis viruses. *Virology* 172, 156–169.
- Catteruccia, F., Godfray, H.C., Crisanti, A., 2003. Impact of genetic manipulation on the fitness of *Anopheles stephensi* mosquitoes. *Science* 299, 1225–1227.
- Christophides, G.K., Livadaras, I., Savakis, C., Komitopoulou, K., 2000. Two medfly promoters that have originated by recent gene duplication drive distinct sex, tissue and temporal expression patterns. *Genetics* 156, 173–182.
- Eanes, W.F., Wesley, C., Hey, J., Houle, D., Ajioka, J.W., 1988. The fitness consequences of *P* element insertion in *Drosophila melanogaster*. *Genet. Res.* 52, 17–26.
- Franz, G., Loukeris, T.G., Dialektaki, G., Thompson, C.R., Savakis, C., 1994. Mobile *Mimos* elements from *Drosophila hydei* encode a two-exon transposase with similarity to the paired DNA-binding domain. *Proc. Natl Acad. Sci. USA* 91, 4746–4750.
- Guimond, N.D., Bideshi, D.K., Pinkerton, A.C., Atkinson, P.W., O'Brochta, D.A., 2003. Patterns of *Hermes* element transposition in *Drosophila melanogaster*. *Mol. Gen. Genom.* 268, 779–790.
- Handler, A.M., 2002. Prospects for using genetic transformation for improved SIT and new biocontrol methods. *Genetica* 116, 137–149.
- Heinrich, J.C., Scott, M.J., 2000. A repressible female-specific lethal genetic system for making transgenic strains suitable for a sterile-release program. *Proc. Natl Acad. Sci. USA* 97, 8229–8232.
- Hendrichs, J., 2000. Use of the Sterile Insect Technique against key insect pests. *Sust. Devel. Int.* 2, 75–79.
- Imamura, M., Nakai, J., Inoue, S., Quan, G.X., Kanda, T., *et al.*, 2003. Targeted gene expression using the GAL4/UAS system in the silkworm *Bombyx mori*. *Genetics* 165, 1329–1340.
- Irvin, N., Hoddle, M.S., O'Brochta, D.A., Carey, B., Atkinson, P.W., 2004. Assessing fitness costs for transgenic *Aedes aegypti* expressing the green fluorescent protein marker and transposase genes. *Proc. Natl Acad. Sci. USA* 101, 891–896.
- Ito, J., Ghosh, A., Moreira, L.A., Wimmer, E.A., Jacobs-Lorena, M., 2002. Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite. *Nature* 417, 387–388.
- Kidwell, M.G., Ribeiro, J.M.C., 1992. Can transposable elements be used to drive refractoriness genes into vector populations? *Parasitol. Today* 8, 325–329.
- Kimura, K., Kidwell, M.G., 1994. Differences in *P* element population dynamics between the sibling species *Drosophila melanogaster* and *Drosophila simulans*. *Genet. Res.* 63, 27–38.
- Kokoza, V., Ahmed, A., Cho, W.L., Jasinskiene, N., James, A.A., *et al.*, 2000. Engineering blood-meal activated systemic immunity in the yellow fever mosquito, *Aedes aegypti*. *Proc. Natl Acad. Sci. USA* 97, 9144–9149.
- Lehane, M.J., Atkinson, P.W., O'Brochta, D.A., 2000. *Hermes*-mediated genetic transformation of the stable fly, *Stomoxys calcitrans*. *Insect Mol. Biol.* 9, 531–538.
- Liao, G.C., Rehm, E.J., Rubin, G.M., 2000. Insertion site preferences of the *P* transposable element in *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA* 97, 3347–3451.
- Loukeris, T.G., Livadaras, I., Arca, B., Zabalou, S., Savakis, C., 1995. Gene transfer into the Medfly, *Ceratitis capitata*, using a *Drosophila hydei* transposable element. *Science* 270, 2002–2005.
- Medhora, M., Maruyama, K., Hartl, D.L., 1991. Molecular and functional analysis of the mariner mutator element *Mos1* in *Drosophila*. *Genetics* 128, 311–318.
- Michel, K., Stamenova, A., Pinkerton, A.C., Franz, G., Robinson, A.S., *et al.*, 2001. *Hermes*-mediated germline transformation of the Mediterranean fruit fly, *Ceratitis capitata*. *Insect Mol. Biol.* 10, 155–162.
- Miller, L.H., Sakai, R.K., Romans, P., Gwadz, R.W., Kantoff, P., *et al.*, 1987. Stable integration and expression of a bacterial gene in the mosquito, *Anopheles gambiae*. *Science* 237, 779–781.
- Moreira, L.A., Ito, J., Ghosh, A., Devenport, M., Zieler, H., *et al.*, 2002. Bee venom phospholipase inhibits malaria parasite development in transgenic mosquitoes. *J. Biol. Chem.* 277, 40839–40843.
- National Research Council, 2002. Animal Biotechnology: Science-Based Concerns. The National Academies Press, Washington, DC.
- O'Brochta, D.A., Sethuraman, N., Wilson, R., Hice, R.H., Pinkerton, A.C., *et al.*, 2003. Gene vector and transposable element behavior in mosquitoes. *J. Exp. Biol.* 206, 3823–3834.
- O'Brochta, D.A., Warren, W.D., Saville, K.J., Atkinson, P.W., 1996. *Hermes*, a functional non-drosophilid gene vector from *Musca domestica*. *Genetics* 142, 907–914.
- Peloquin, J.J., Thibault, S.T., Miller, T.A., 2000. Genetic transformation of the pink bollworm *Pectinophora gossypiella* with the *piggyBac* element. *Insect Mol. Biol.* 9, 323–333.
- Pew Initiative on Food and Biotechnology, 2004. "Bugs in the System? Issues in the Science and Regulation of Genetically Modified Insects." Washington, DC, pp. 109.
- Presnail, J.K., Hoy, M.A., 1992. Stable genetic transformation of a beneficial arthropod, *Metaseiulus occidentalis* (Acari: Phytoseiidae), by a microinjection technique. *Proc. Natl Acad. Sci. USA* 89, 7732–7736.
- Raymond, M., 1991. Worldwide migration of amplified insecticide resistance genes in mosquitoes. *Nature* 350, 151–153.
- Ribeiro, J.M., Kidwell, M.G., 1994. Transposable elements as population drive mechanisms: specification of critical parameter values. *J. Med. Entomol.* 31, 10–16.
- Robinson, A.S., 2002. Mutations and their use in insect control. *Mutat. Res.* 511, 113–132.
- Robinson, A.S., Franz, G., Atkinson, P.W., 2004. Insect transgenesis and its potential role in agriculture and human health. *Insect Biochem. Mol. Biol.* 34, 113–120.

- Robinson, A.S., Franz, G., 2000. The application of transgenic insect technology in the sterile insect technique. In: Handler, A.M., James, A.A. (Eds.), *Insect Transgenesis: Methods and Applications*. CRC Press, Boca Raton, FL, pp. 307–318.
- Robinson, K.O., Ferguson, H.J., Cobey, S., Vassein, H., Smith, B.H., 2000. Sperm-mediated transformation of the honey bee, *Apis mellifera*. *Insect Mol. Biol.* 9, 625–634.
- Rowan, K.H., Orsetti, J., Atkinson, P.W., O'Brochta, D.A., 2004. *Tn5* as an insect gene vector. *Insect Biochem. Mol. Biol.* 34, 695–705.
- Rubin, G.M., Spradling, A.C., 1982. Genetic transformation of *Drosophila* with transposable element vectors. *Science* 218, 348–353.
- Saccone, G., Pane, A., Polito, L.C., 2002. Sex determination in flies, fruitflies and butterflies. *Genetica* 116, 15–23.
- Schliekelman, P., Gould, F., 2000a. Pest control by the introduction of a conditional lethal trait on multiple loci: potential, limitations, and optimal strategies. *J. Econ. Entomol.* 93, 1543–1565.
- Schliekelman, P., Gould, F., 2000b. Pest control by the release of insects carrying a female-killing allele on multiple loci. *J. Econ. Entomol.* 93, 1566–1579.
- Tamura, T., Thibert, C., Royer, C., Kanda, T., Abraham, E., *et al.*, 2000. Germline transformation of the silkworm *Bombyx mori* L. using a *piggyBac* transposon-derived vector. *Nature Biotechnol.* 18, 81–84.
- Thomas, D.D., Donnelly, C.A., Wood, R.J., Alphey, L.S., 2000. Insect population control using a dominant, repressible, lethal genetic system. *Science* 287, 2474–2476.
- Tiedje, J.M., Colwell, R.K., Grossman, Y.L., Hodson, R.E., Lenski, R.E., *et al.*, 1989. The planned introduction of genetically engineered organisms: ecological considerations and recommendations. *Ecology* 70, 298–315.
- Warren, W.D., Atkinson, P.W., O'Brochta, D.A., 1994. The *Hermes* transposable element from the housefly, *Musca domestica*, is a short inverted repeat-type element of the *hobo*, *Ac*, and *Tam3* (*hAT*) element family. *Genet. Res.* 64, 87–97.
- Whitten, M.J., 1985. The conceptual basis for genetic control. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 12. Pergamon, Oxford, pp. 465–528.
- Wilson, R., Orsetti, J., Klocko, A.K., Aluvihare, C., Peckham, E., *et al.*, 2003. Post-integration behavior of a *Mos1* mariner gene vector in *Aedes aegypti*. *Insect Biochem. Mol. Biol.* 33, 853–863.
- Wimmer, E.A., 2003. Innovations: applications of insect transgenesis. *Nature Rev. Genet.* 4, 225–232.

16 Insect Growth- and Development-Disrupting Insecticides

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16.1. Introduction

In 1967 Carrol Williams proposed that the term “third generation pesticide” be applied to the potential use of the insect juvenile hormone (JH) as an insecticide, and suggested that it would not only be environmentally benign but that the pest insects would also be unable to develop resistance. However, it took several years before the first commercial juvenile hormone analog (JHA) made its debut (reviews: Retnakaran *et al.*, 1985; Staal, 1975). Since then, several compounds that adversely interfere with the growth and development of insects have been synthesized, and have been collectively

referred to as “insect growth regulators (IGRs)” (review: Staal, 1982). Concerns over eco-toxicology and mammalian safety have resulted in a paradigm shift from the development of neurotoxic, broad-spectrum insecticides towards softer, more environmentally friendly pest control agents such as IGRs. This search has led to the discovery of chemicals that: (1) interfere with physiological and biochemical systems that are unique to either insects in particular or arthropods in general; (2) have insect-specific toxicity based on either molecular target site or vulnerability to a developmental stage; and (3) are safe to the environment and nontarget species. At

the physiological level, opportunities to develop insecticides with such characteristics existed, *inter alia*, in the endocrine regulation of growth, development, reproduction, and metamorphosis. The rationale was that if the pest insect is treated with a chemical analog, which mimics the action of hormones like JHs and ecdysteroids, at an inappropriate stage the hormonal imbalance would force the insect to go through abnormal development leading to mortality. It was also thought that because such chemicals would in many instances work via the receptor(s) of these hormones, it was less likely for the affected population to develop target site resistance. Yet other target sites are the biosynthetic steps of cuticle formation, which insects share with other arthropods. Adversely interfering with this process would result in the inability of the intoxicated insect to molt and undergo further development. Unlike neurotoxic insecticides that are fast acting, IGRs are in general slow acting, which might result in more damage to the crop. However, some IGRs, such as ecdysone agonists, induce feeding inhibition, significantly reducing the damage to below acceptable levels. The slow mode of action of some IGRs, such as chitin synthesis inhibitors, can be an advantage in controlling social insects, such as termites, where the material has to be carried to the brood and spread to other members.

It was in the early seventies that the first chitin synthesis inhibitor, a benzoylphenylurea, was discovered by scientists at Philips-Duphar BV, (now Crompton Corp., Weesp, The Netherlands), and marketed as Dimilin[®] by the Uniroyal Chemical Company (Crompton Corp. Middlebury, CT) in the USA. Since then several new analogs that interfere with one or more steps of cuticle synthesis have been synthesized and are being marketed for controlling various pests. These products are reviewed in Section 16.4, with emphasis on recent developments.

After the initial success with the synthesis of methoprene by Zoecon (Palo Alto, CA, USA) very few new JHAs with good control potential other than pyriproxyfen and fenoxycarb have been developed. These compounds have been particularly useful in targeting the eggs and embryonic development as well as larvae. The synthesis of pyriproxifen and fenoxycarb was a departure from the terpenoid structure of JHs and earlier JHAs, and is reviewed in Section 16.3. For extensive reviews on earlier JHAs the reader is referred to Retnakaran *et al.* (1985) and Staal *et al.* (1975).

Early attempts in the 1970s to synthesize insecticides with molting hormone (20-hydroxyecdysone)

activity failed because they were based on a cholesterol backbone, which resulted in chemical and metabolic instability of the steroid nucleus (Watkinson and Clarke, 1973). It took nearly two more decades before the first nonsteroidal ecdysone agonist, based on the bisacylhydrazine class of compounds, was synthesized (Hsu, 1991). Structure activity optimization of the first such compound over the subsequent few years led to the synthesis of four highly effective compounds that have since been commercialized (see Section 16.2.1). The reader is also referred to earlier reviews on the ecdysone agonist insecticides (Oberlander *et al.*, 1995; Dhadialla *et al.*, 1998).

For more basic information, the reader is referred to the chapters in this series on insect hormones (ecdysteroids, JHs) and cuticle synthesis (see **Chapter 12**) for further details on hormone physiology and chitin synthesis to augment the brief overviews presented in this chapter.

16.1.1. Physiological Role and Mode of Action of the Insect Molting Hormone

Amongst the animal kingdom, arthropods display a remarkable adaptability and diversity in inhabiting very different ecological niches. Between larval and adult stages of a given insect, an insect undergoes distinct developmental and morphological changes that help it to survive in different environments. For example, mosquito larvae are perfectly suited to surviving in an aqueous environment, whereas the adult mosquitos inhabit terrestrial and aerial environments. Larval or nymphal stages of most of the agricultural pests develop on host plant species, and the adult assumes an aerial space to look for food and a mate, returning to the host plant only to oviposit and start another life cycle.

The growth and development from one stage to another is regulated by two main hormones, the steroidal insect molting hormone, 20-hydroxyecdysone (20E; **Figure 1, 1**) and the sesquiterpenoid JH, of which there are five types (**Figure 15**). Even as an insect embryo grows, it undergoes embryonic molts, where each molt is regulated by 20E. The molting process continues through the larval and pupal stages culminating in the adult stage. While molting to accommodate growth is regulated by 20E, the development from an egg to a larva to a pupa to an adult is regulated by the timing and titers of JH (**Figure 17**; also reviewed extensively by Riddiford, 1996). In the adult stage, both these hormones, being pleiotropic, change their roles to regulating reproductive processes (Wyatt and Davey, 1996).

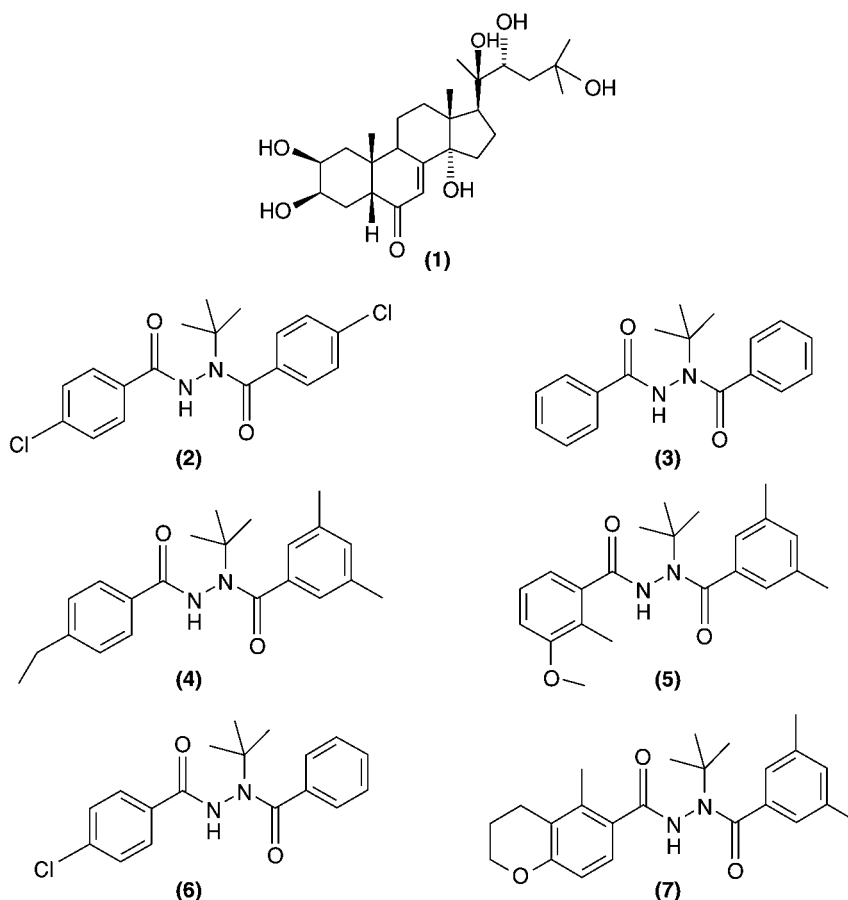


Figure 1 Chemical structures of 20-hydroxyecdysone (1), the first discovered symmetrically substituted dichloro-dibenzoylhydrazine (2), RH-5849 (3), tebufenozide (4), methoxyfenozide (5), halofenozide (6), and chromofenozide (7).

The molting process is initiated by an increase in the titer of 20E, and is completed following its decline and the release of eclosion hormone. In preparation for a molt and as the 20E titers increase, the larva stops feeding and apolysis of the epidermis from the old cuticle takes place leaving an ecdysial space that is filled with molting fluid containing inactive chitinolytic enzymes. During this time, the epidermal cells also reorganize in order that large quantities of protein can be synthesized for deposition of a new cuticle. This happens with up- and downregulation of a number of genes encoding a number of epidermal proteins and enzymes (Riddiford, 1994). The fall in the 20E titer triggers the activation of enzymes in the molting fluid for digestion of the procuticle underlying the old cuticle. This event is followed by the resorption of the molting fluid and preparation for ecdysis (Retnakaran *et al.*, 1997; Locke, 1998). Finally, when the 20E titer is cleared from the system, the eclosion hormone is released, which in turn results in the release of the ecdysis-triggering hormone, and all

these events lead to the ecdysis of the larva leaving behind the remnants of the old cuticle (Truman *et al.*, 1983; Zitnanova *et al.*, 2001). With the completion of ecdysis, feeding resumes and endocuticular deposition continues during the intermolt period.

16.1.1.1. Molecular basis of 20E action Of the two hormones 20E and JH, the molecular basis of 20E action is much better understood. The pioneering work of Clever and Karlson (1960) and Ashburner (1973) on the action of 20E to induce/repress “puffs” in the polytene chromosomes of *Chironomus* and *Drosophila* led Ashburner and co-workers to propose a model for ecdysteroid action (Ashburner *et al.*, 1974). According to this model, 20E binds to an ecdysone receptor to differentially regulate several classes of “early” and “late” genes. While the “early” genes are activated by the 20E–receptor complex, the “late” genes are repressed. The protein products of the “early” genes such as E75 and CHR3 derepress the expression of the “late” genes such as DOPA decarboxylase

(DDC) and at the same time repress their own expression.

16.1.1.2. Ecdysone receptors The ecdysone receptor complex is a heterodimer of two proteins, ecdysone receptor (EcR) and ultraspiracle (USP), which is a homolog of the mammalian retinoic acid receptor (RXR) (Yao *et al.*, 1992, 1995; Thomas *et al.*, 1993). In several insects, both EcR and USP exist in several transcriptional and splice variants, presumably for use in a stage- and tissue-specific way (review: Riddiford *et al.*, 2001). Both EcR and USP are members of the steroid receptor superfamily that have characteristic DNA and ligand binding domains. Ecdysteroids have been shown to bind to EcR only when EcR and USP exist as heterodimers (Yao *et al.*, 1993), although additional transcriptional factors are required for ecdysteroid dependent gene regulation (Arbeitman and Hogness, 2000; Tran *et al.*, 2000). Moreover, EcR can heterodimerize with RXR to form a functional ecdysteroid receptor complex in transfected cells (Yao *et al.*, 1992; Tran *et al.*, 2000). cDNAs encoding both EcR and USPs from a number of dipteran (Koelle *et al.*, 1991; Imhof *et al.*, 1993; Cho *et al.*, 1995; Kapitskaya *et al.*, 1996; Hannan and Hill, 1997, 2001; Veras *et al.*, 1999), lepidopteran (Kothapalli *et al.*, 1995; Swevers *et al.*, 1995), coleopteran (Mouillet *et al.*, 1997; Dhadialla and Tzertzinis, 1997), homopteran (Zhang *et al.*, 2003; Dhadialla *et al.*, unpublished data; Ronald Hill, personal communication), and orthopteran (Saleh *et al.*, 1998; Hayward *et al.*, 1999, 2003) insects, tick (Guo *et al.*, 1997) and crab (Chung *et al.*, 1998) have been cloned. Some of the EcRs and USPs have been characterized in ligand binding (Kothapalli *et al.*, 1995; Kapitskaya *et al.*, 1996; Dhadialla *et al.*, 1998) and cell transfection assays (Kumar *et al.*, 2002; Toya *et al.*, 2002). In all cases, the DNA binding domains (DBDs) of EcRs show a very high degree of homology and identity. However, homology between the ligand binding domains (LBDs) of EcRs varies from 70% to 90%, although all EcRs studied so far bind 20E and other active ecdysteroids. The DBDs of USPs are also highly conserved. The USP LBDs, however, show very interesting evolutionary dichotomy: the LBDs from the locust, *Locusta migratoria*, the mealworm beetle, *Tenebrio molitor*, the hard tick, *Amblyoma americanum*, and the fiddler crab, *Uca pugilator*, show about 70% identity with their vertebrate homolog, but the same sequences from dipteran and lepidopteran USPs show only about 45% identity with those from other arthropods and vertebrates (Guo *et al.*, 1997; Hayward *et al.*, 1999; Riddiford

et al., 2001). The functional significance of RXR-like LBDs in USPs of primitive arthropods is not well understood, because EcRs from the same insects still bind ecdysteroids (Guo *et al.*, 1997; Chung *et al.*, 1998; Hayward *et al.*, 2003; Dhadialla, unpublished data for *Tenebrio molitor* EcR and USP (TmEcR/TmUSP)).

The crystal structures of USPs from both *Heliothis virescens* and *Drosophila melanogaster* have been elucidated by two groups (Billas *et al.*, 2001; Clayton *et al.*, 2001). The crystal structure of USP is similar to its mammalian homolog RXR, except that USP structures show a long helix-1 to helix-3 loop and an insert between helices 5 and 6. These variations seem to lock USP in an inactive conformation by displacing helix 12 from the agonist conformation. Both groups found that crystal structures of the two USPs had large hydrophobic cavities, which contained phospholipid ligands.

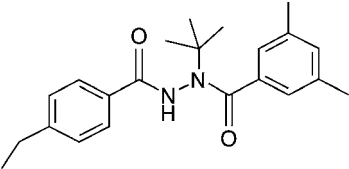
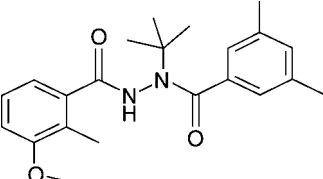
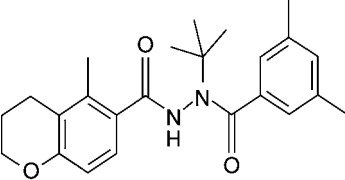
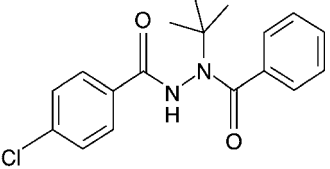
Finally, the crystal structures of *Heliothis virescens* EcR/USP (HvEcR/HvUSP) heterodimers liganded with an ecdysteroid or a nonsteroidal ecdysone agonist have been determined (Billas *et al.*, 2003; see Section 16.2.2.2 for more details). The crystal structure of liganded EcR/USP from the silverleaf whitefly, *Bemisia tabaci*, has also been determined and awaits publication (Ronald Hill, personal communication).

16.2. Ecdysteroid Agonist Insecticides

16.2.1. Discovery of Ecdysone Agonist Insecticides and Commercial Products

Although attempts to discover insecticides with an insect molting hormone activity were made in the early 1970s (Watkinson and Clarke, 1973), it was not until a decade later that the first bisacylhydrazine ecdysone agonist ((2) in **Figure 1**) was serendipitously discovered by Hsu (1991) at Rohm and Haas Company, Springs House, PA, USA. Several years later, after several chemical iterations of this early lead, a simpler, unsubstituted, but slightly more potent analog, RH-5849 ((3) in **Figure 1**), was discovered (Aller and Ramsay, 1988). Further work on the structure and activity of RH-5849, which had commercial-level broad spectrum activity against several lepidopteran, coleopteran, and dipteran species (Wing, 1988; Wing and Aller, 1990), resulted in more potent and cost-effective bisacylhydrazines with a high degree of selective pest toxicity (review: Dhadialla *et al.*, 1998). Of these, three bisacylhydrazine compounds, all substituted analogs of RH-5849, coded as RH-5992 (tebufenozide (4); **Figure 1**), RH-2485 (methoxyfenozide (5); **Figure 1**), and RH-0345 (halofenozide (6); **Figure 1**) have been

Table 1 Bisacylhydrazine insecticides with ecdysone mode of action

Structure	Common name	Coded as	Industry	Registered names	Pest spectrum
	Tebufenozide	RH-5992	Rohm and Haas Co., ^a Dow AgroSciences LLC ^b	MIMIC [®] , CONFIRM [®] , ROMDAN [®]	Lepidoptera
	Methoxyfenozide	RH-2485	Rohm and Haas Co., ^a Dow AgroSciences LLC ^b	INTREPID [®] , RUNNER [®] , PRODIGY [®] , FALCON [®]	Lepidoptera
	Chromofenozide	ANS-118, CM-001	Nippon Kayaku, Saitame, Japan and Sankyo, Ibaraki, Japan	MATRIC [®] , KILLAT [®]	Lepidoptera
	Halofenozide	RH-0345	Rohm and Haas Co., ^a Dow AgroSciences LLC ^b	MACH 2 [®]	Lepidoptera, Coleoptera

^aDiscovered and commercialized by Rohm and Haas Company, Spring House, PA, USA.

^bNow owned and commercialized by Dow AgroSciences, LLC., Indianapolis, IN, USA.

commercialized (Table 1). Both tebufenozide and methoxyfenozide are selectively toxic to lepidopteran larvae (Hsu, 1991). However, methoxyfenozide is more potent than tebufenozide, and is toxic to a wider range of lepidopteran pests of cotton, corn, and other agronomic pests (Ishaaya *et al.*, 1995; Le *et al.*, 1996; Trisyono and Chippendale, 1997). Halofenozide has a broader and an overall insect control spectrum somewhat similar to that of RH-5849, but with significantly higher soil-systemic efficacy against scarabid beetle larvae, cutworms, and webworms (RohMid LLC, 1996). Another bisacylhydrazine, chromafenozide (7) coded as ANS-118; Figure 1) was discovered and developed jointly by Nippon Kayaku Co., Ltd., Saitama, Japan and Sankyo Co., Ltd. Ibaraki, Japan (Yanagi *et al.*, 2000; Toya *et al.*, 2002; Table 1). Chromafenozide, registered under the trade names, MATRIC[®] and KILLAT[®], is commercialized for the control of lepidopteran larval pests of vegetables, fruits, vines, tea, rice, and ornamentals in Japan (Yanagi *et al.*, 2000; Reiji *et al.*, 2000).

16.2.1.1. Synthesis and structure–activity relationships (SAR) The synthesis of symmetrical and asymmetrical 1,2-dibenzoyl-1-*t*-butyl hydrazines can be achieved by the following two step reaction shown in Figure 2. In 1984, during the process of synthesizing compound number (1 in Figure 2), which was to be used for the synthesis of another class of compounds, Hsu (Rohm and Haas Company) obtained an additional undesired product (2 in Figure 2), 1,2-di (4-chlorobenzoyl)-1-*t*-butylhydrazine (A = 4-Cl). Testing of this undesired by-product revealed that it had ecdysteroid activity, and eventually led to the development of a bisacylhydrazine class of compounds. This serendipitous discovery can be attributed to the inquiring mind of Hsu, who decided to test this compound for biological activity even though it was a contaminant! By treating equivalent amounts of different benzoyl chlorides with 1 (benzoyl hydrazide), unsubstituted analogs (3) can be prepared as shown in Figure 2.

16.2.2. Bisacylhydrazines as Tools of Discovery

The discovery of bisacylhydrazine has been a catalyst in stimulating research on the biological activity of ecdysteroids and nonsteroidal ecdysone agonists in whole insects, tissues, and in cells (reviews: Dhadialla *et al.*, 1998; Oberlander *et al.*, 1995). Because bisacylhydrazines have a greater metabolic stability than 20E *in vivo*, it was possible to confirm and further understand the effects of rising and declining titers of 20E during growth and development (Retnakaran *et al.*, 1995).

With the cloning and sequencing of cDNAs encoding EcRs from different insects it became clear that there were homology differences in the LBDs of the cloned EcRs but the functional significance of these differences was not apparent. In almost all insects, the effect of 20E is manifested by an interaction with EcR in the ecdysone receptor complex. Therefore, it is paradoxical that tebufenozide and methoxyfenozide, which also manifest insect toxicity via interaction with EcR, are predominantly toxic to lepidopteran insects and to a lesser degree to other insects. Through the use of these ecdysone agonists it became clear that while 20E can bind to different LBDs of EcRs from different orders of insects, the same LBDs bound bisacylhydrazines with unequal affinities, which reflected their insect specificity (reviewed by Dhadialla *et al.*, 1998). It also became clear that while a relatively low but similar affinity ($K_d = 10\text{--}100\text{ nM}$) of 20E to different EcRs was functional, only high-affinity (nM range) interactions of tebufenozide and methoxyfenozide with EcR were functionally productive (Table 2). On the other hand, the low affinity of halofenozide was most likely compensated for by its high metabolic stability in susceptible insects.

16.2.2.1. Molecular modeling Molecular modeling studies on both EcR LBDs (Wurtz *et al.*, 2000; Kasuya *et al.*, 2003) and bisacylhydrazines (Chan

et al., 1990; Mohammed-Ali *et al.*, 1995; Hsu *et al.*, 1997; Cao *et al.*, 2001) were conducted, perhaps partly prompted by the molecular modeling and X-ray crystal structure data of LBDs of steroid receptors from vertebrates and the search for new classes of nonsteroidal ecdysone agonists/antagonists. The results suggested that the tertiary protein structure of EcR LBDs is similar to that of vertebrate steroid receptor LBDs, and that helix 12 forms a “mouse trap” for a ligand in the binding cavity (Figure 3; see also description of crystal structures of EcR and USP below and Chapter 7). Results of molecular modeling and *in silico* docking experiments with 20E and RH-5849 have provided conflicting information about the orientation of 20E in the binding pocket. However, the recent description of the X-ray crystal structure of ligand bound HvEcR/HvUSP LBDs has provided definite answers to this (see below).

16.2.2.2. Mutational analysis In a very elegant study, Kumar *et al.* (2002) extended the *in silico* molecular modeling approach to construct a homology model of *Choristoneura fumiferana* EcR (CfEcR) LBD with 20E docked in to predict amino acid residues involved in interaction with 20E. The authors identified 17 amino acid residues as possibly being critical for 20E binding. In order to confirm this prediction, all 17 of these amino acids were mutated to alanine, except for A110 (where the 110 residue is numbered starting from helix one, which otherwise is A393 in the full-length CfEcR), which was mutated to proline. When the single point mutated LBDs were tested in ligand binding assays and transactivation assays (in insect and mammalian cells and in mice *in vivo*) the A110P mutant was ineffective in the two assays for a response to ecdysteroids (ponasterone A and 20E). While there was a 30% decrease in transactivation assays for the nonsteroidal bisacylhydrazines, their

Table 2 Relative binding affinities of ponasterone A and bisacylhydrazine insecticides to EcR/USPs in cellular extracts of dipteran, lepidopteran, and coleopteran cells and (biological activities)

Ligand	K_d (nM)		
	<i>Drosophila Kc cells</i>	<i>Plodia interpunctella</i> ^a	<i>Leptinotarsa decemlineata</i> ^b
Ponasterone A	0.7	3	3
Tebufenozide	192	3 (predominantly lepidoptera active)	218
Methoxyfenozide	124	0.5 (predominantly lepidoptera active)	ND
Halofenozide	493	129 (lepidoptera active)	2162 (coleoptera active)

^aImaginal wing disc cell line.

^bEmbryonic cell line.

ND, not determined.

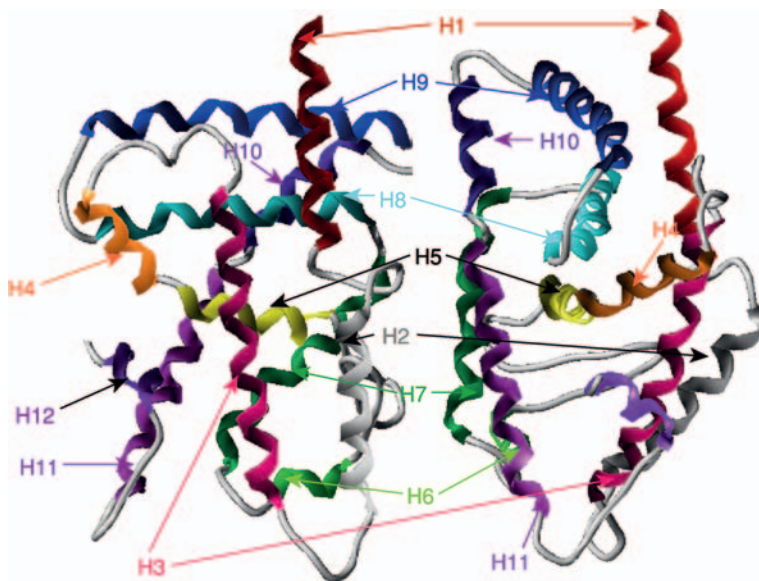


Figure 3 Different elevations of computer model(s) of the three-dimensional structure of the LBD of insect ecdysone receptors. The different helices are represented in different colors, and indicated by arrows and numbers. After a ligand has docked into the ligand binding pocket of EcR heterodimerized with USP, helix 12 closes on the binding cavity like a “mouse trap.”

ability to bind was unaffected. Changing A110 to leucine was as effective as the change to proline. Further work along these lines should make it possible not only to design new effective chemistries as ecdysone agonists/antagonists for pest control, but also to design EcR LBDs that result in productive binding with different chemistries, thus allowing their use in several gene switch applications.

The necessity of EcR and USP forming a heterodimer for ligand interaction coupled with the difficulty in producing large enough quantities of EcR and USP LBDs required for X-ray diffraction studies have been overcome by Billas *et al.* (2003). These authors reported the crystal structures of the LBDs of the moth *H. virescens* EcR-USP heterodimer in complex with the ecdysteroid ponasterone A and with a nonsteroidal, lepidopteran specific agonist, BY106830 (a bisacylhydrazine). Comparison of the crystal structures liganded with ponasterone A and BY106830 revealed that the two ligands occupy very different but slightly overlapping spaces in the ligand binding pockets. The overlap of the two ligands was observed on the side chain of ponasterone A with the *t*-butyl group and the benzoyl ring closest to it in BY106830. The presence of the *t*-butyl group and its occupation in a hydrophobic groove of the HvEcR LB pocket confers lepidopteran specificity on it. Further examination of the residues in this hydrophobic cavity revealed that V384 in helix 5, which was conserved in lepidopteran insect EcR LBDs and is replaced by methionine in other

insects, is essential for the lepidopteran specificity. The crystal structure observations supported the results of Kumar *et al.* (2002).

16.2.2.3. Ligand-dependent conformational changes Yao *et al.* (1995) used limited proteolysis to demonstrate that binding of muristerone A, a potent ecdysteroid, to *Drosophila melanogaster* EcR and USP (DmEcR/DmUSP) induces a conformation change in EcR that can be detected by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. In these experiments, incubation of muristerone A with DmEcR-³⁵S-methionine labeled/DmUSP proteins produced by transcription and translation of corresponding cDNAs in rabbit reticulocyte cell-free mixtures, afforded partial protection to EcR from trypsin as the protease. In an extension of this approach, Dhadialla *et al.* (unpublished data) used EcR-³⁵S-methionine labeled/USP proteins produced *in vitro* to demonstrate that muristerone A bound to DmEcR/DmUSP, *Aedes aegypti* EcR and USP (AeEcR/AeUSP), and CfEcR/CfUSP protected an EcR fragment of 36 kDa from limited proteolysis with trypsin, chymotrypsin, and proteinase K. On the other hand, the ability of tebufenozide (RH-5992) to induce a similar conformational change upon binding to EcR, which affords limited proteolytic protection as with muristerone A, correlated with its affinity to the various EcRs (Table 3). Interestingly, peptide fragments of a similar size (36 kDa)

Table 3 Size distribution of protease protected fragments of ³⁵S-labeled EcRs incubated in the presence of homologous USPs and an ecdysteroid, muristerone A, or a bisacylhydrazine, tebufenozide

Protease	Protected fragment size (kDa)					
	<i>DmEcR</i>		<i>AaEcR</i>		<i>CfEcR</i>	
	Muristerone A	Tebufenozide	Muristerone A	Tebufenozide	Muristerone A	Tebufenozide
Chymotrypsin	36		45	45	ND	ND
Trypsin	40		50	50	ND	ND
Proteinase K	36		36	36	36	36
<i>K_d</i> (nM)		336		28		0.5

ND, not determined.

Cloned EcR and USP cDNAs from the three insects, *Drosophila melanogaster* (Dm), *Aedes aegypti* (Aa), and *Choristoneura fumiferana* (Cf) were expressed using *in vitro* transcription and translations of the rabbit reticulocyte system. However, EcRs were *in vitro* labeled with ³⁵S-methionine. Both EcR and USPs from the same species were incubated in the presence of 1 μm muristerone A or 1 μm tebufenozide and binding allowed to reach equilibrium. At that point the binding reactions were aliquoted and subjected to increasing protease concentrations for 20 min at RT (limited proteolysis). The digests were analyzed by SDS-PAGE, and the labeled EcR bands visualized by fluorography. The *K_d* values are the same as those given in **Table 2**.

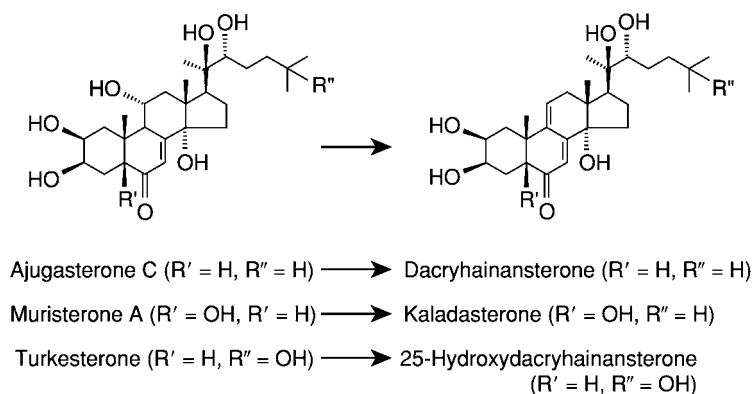


Figure 4 Schematic derivatization of ajugasterone C, muristerone A, and turkesterone to photo affinity ecdysteroid 7,9(11)-dien-6-ones, dacryhainansterone, kaladasterone, and 25-hydroxydacryhainansterone, respectively. (Adapted from Bourne, P.C., Whiting, P., Dhadialla, T.S., Hormann, R.E., Girault, J.-P., *et al.*, 2002. Ecdysteroid 7,9(11)-dien-6-ones as potential photoaffinity labels for ecdysteroid binding proteins. *J. Insect Sci.* 2, 1–11.

to the three EcRs with either muristerone A or tebufenozide (in the cases of AaEcR and CfEcR) bound were protected from proteolysis with proteinase K, suggesting similar ligand-induced conformational changes. Such a conformational change *in vivo* may be necessary for ligand-dependent transactivation of a gene.

With respect to the hypothesis of Billas *et al.* (2003) that in the EcR/USP heterodimer the EcR ligand binding pocket may conform to accommodate an active ligand, it is not clear if the changes seen in limited proteolysis experiments (above) are a result of just ligand-induced changes in the binding pocket or a result of ligand-induced changes in the whole of the EcR LBD.

16.2.2.4. Photoaffinity reagents for studying receptor–ligand interactions

Before the X-ray

crystal structures of the EcR/USP heterodimer were available, alternative approaches to understanding the interaction of ecdysteroid and nonsteroidal bisacylhydrazine ligands with residues in the EcR ligand binding pocket were explored. One such approach was to synthetically produce photoaffinity analogs of ecdysteroids (Bourne *et al.*, 2002) and bisacylhydrazines (Dhadialla *et al.*, unpublished data) as has been achieved for vertebrate steroids and their receptors (Katzenellenbogen and Katzenellenbogen, 1984). An ideal affinity labeling reagent should have: (1) a high affinity for the binding protein with low nonspecific binding to other proteins; (2) a photo-reactive functional group; and (3) a radiolabeled moiety for detection purposes.

Bourne *et al.* (2002) synthesized three ecdysteroid 7,9(11)-dien-7-ones (**Figure 4**; dacryhainansterone, 25-hydroxydacryhainansterone, and kaladasterone)

as photoaffinity ecdysteroids by dehydration of the corresponding 11α -hydroxy ecdysteroids (ajugasterone, turkesterone, and muristerone A, respectively). Of the three photoaffinity ecdysteroids, dacryhainansterone and kaladasterone showed the greatest potential for use in determining contact amino acid residues in EcR LBDs. All three dienone ecdysteroids retained their biological activity in the *D. melanogaster* B_{II} cell assay developed by Clement *et al.* (1993). However, upon irradiation with UV at 350 nm in the presence of bacterially expressed DmEcR/DmUSP and CfEcR/CfUSP, both dacryhainansterone and kaladasterone blocked subsequent specific binding of tritiated ponasterone A by >70%. These results clearly showed the potential of using radiolabeled dienones for further characterization of their interaction within EcR LBDs.

Photoaffinity analogs of bisacylhydrazines have also been produced and used to characterize their binding to EcRs from dipterans and lepidopterans (Dhadialla *et al.*, unpublished data). A tritiated benzophenone analog of methoxyfenozide (Figure 5), coded as RH-131039, displays the above-mentioned characteristic requirements of a photoaffinity reagent as well as the specificity to bind to lepidopteran EcR (CfEcR) with high affinity. Binding to dipteran EcR (DmEcR) could not be detected. Binding of either tritiated ponasterone A or tritiated RH-131039 to CfEcR/CfUSP could be competitively displaced using excess unlabeled RH-131039, tebufenozide, ponasterone A, or 20E. These results indicate that the ecdysteroids and the two nonsteroidal ecdysone agonists share a common binding site in the ligand binding pocket of CfEcR/CfUSP (Figure 6), an observation that has been confirmed by Billas *et al.* (2003) in their work on the crystal structure of liganded HvEcR/HvUSP. However, further experiments, which can now be done with the ecdysteroid and bisacylhydrazine photoaffinity compounds, need to be done to determine the ligand-receptor contact residues. Similar results were obtained for AaEcR/AaUSP, but not for DmEcR/DmUSP, indicating that even within an insect order, differences in the homology and folding of EcR LBDs is sufficient to discriminate between affinities of nonsteroidal ecdysone agonists. The bisacylhydrazine and ecdysteroid photoaffinity reagents described are additional useful tools that can be combined with data from chemical structure-activity relationships (SAR), analytical (liquid chromatography/mass spectrometry (LC/MS) and mass laser desorption ionization (MALDI)), and mutational analysis approaches to hypothesize and/or define the three-dimensional space of non-lepidopteran EcR LBDs. However, the availability of the liganded HvEcR/HvUSP LBD

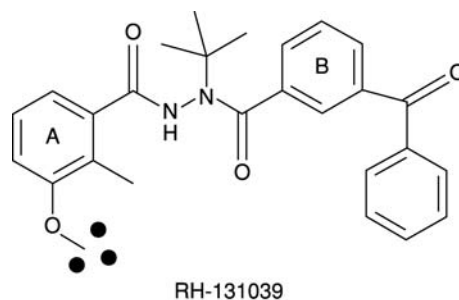


Figure 5 Structure of the tritiated photoaffinity benzophenone analog, RH-131039, of methoxyfenozide. The three dots on the methoxy substitution on the A-phenyl ring indicate tritiation of the methyl group. The benzophenone group on the B-phenyl ring can be activated with UV light for it to cross-link to the nearest amino acid residue.

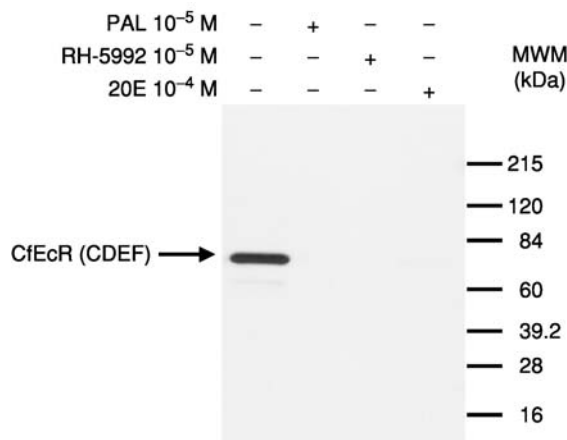


Figure 6 Fluorograph to show specific binding of tritiated RH-131039 (PAL; photo affinity ligand) to CDEF domain of *Choristoneura fumiferana* EcR (CfEcR) expressed by *in vitro* transcription and translation using rabbit reticulocyte system. CfEcR (CDEF domain) plus CfUSP was incubated with tritiated PAL in the presence of excess cold PAL, RH-5992, or 20E at the indicated concentrations under equilibrium binding conditions after which the reaction mixture was irradiated with UV_{350nm} for 20 min at 4°C. The reaction mixture was then mixed with SDS-PAGE sample buffer containing 1 mM DTT, heated for 5 min at 95°C and then subjected to SDS-PAGE. The gel was dried and fluorographed to get the above image. The numbers on the right indicate relative migration of molecular weight markers (MWM).

crystal structure provides the needed information for defining the three-dimensional ligand receptor interaction for homology modeling of such interactions with non-lepidopteran EcR LBDs, and for the design and discovery of new biologically potent ligands as insecticides for different insects orders.

16.2.3. Mode of Action of Bisacylhydrazines

In the earlier reviews on insecticides with ecdysteroid and JH activity (Oberlander *et al.*, 1995; Dhadialla *et al.*, 1998), much of the focus on mode

of action of ecdysone agonists was from data using RH-5849 and RH-5992. RH-5849, the unsubstituted bisacylhydrazine that was not commercialized, had a broader spectrum of insect toxicity than the four that have been commercialized. In this chapter, the focus will be on work published since 1996 on the commercialized bisacylhydrazine insecticides tebufenozide, methoxyfenozide, chromofenozide, and halofenozide. References will be made to earlier publications where relevant.

16.2.3.1. Bioassay A number of *in vitro* and *in vivo* assays have been used to study the effects and mode of action of the bisacylhydrazine insecticides (reviews: Oberlander *et al.*, 1995; Dhadialla *et al.*, 1998). Larvae of susceptible and nonsusceptible insects (described below), and a number of insect cell lines and dissected tissues have been used *in vitro* to understand the mode of action of bisacylhydrazines. Wing *et al.* (1988) were the first to use *D. melanogaster* embryonic Kc cells to demonstrate that like 20E, RH-5849 also induced aggregation and clumping of otherwise confluent cultures of Kc cells. Similar morphological effects of tebufenozide, methoxyfenozide, and halofenozide have also been demonstrated for cell lines derived from embryos or tissues of *Drosophila* (Clement *et al.*, 1993), the mosquito, *Aedes albopictus* (Smaghe *et al.*, 2003a), the midge, *Chironomus tentans* (Spindler-Barth *et al.*, 1991), the forest tent caterpillar, *Malacosoma disstria*, the spruce budworm, *Choristoneura fumiferana* (Sohi *et al.*, 1995), the Indian meal moth, *Plodia interpunctella* (Oberlander *et al.*, 1995), the cotton boll weevil, *Anthonomus grandis* (Dhadialla and Tzertzinis, 1997), the European corn borer, *Ostrinia nubilalis*, the Southwestern corn borer, *Diatraea grandiosella*, and the cotton bollworm, *Helicoverpa zea* (Trisyono *et al.*, 2000).

Some of these cell lines, imaginal wing discs, and larval claspers from susceptible insects have also been used to study the relative binding affinities, and biochemical and molecular effects of tebufenozide (Mikitani, 1996b), methoxyfenozide, or halofenozide (Table 4) and other ecdysone agonists

(Nakagawa *et al.*, 2002). Cytosolic and or nuclear extracts from 20E responsive cells and tissues containing functional ecdysone receptors, as well as bacterially expressed EcRs and USPs from different insects, have also been used to determine the relative binding affinities of bisacylhydrazines or to screen for new chemistries with similar mode of action in radiometric competitive receptor binding assays (Table 5).

Finally, in order to increase the throughput for screening a higher number of compounds, Tran *et al.* (2000) reconstituted ligand-dependent transactivation of *C. fumiferana* EcR in yeast. Reconstitution of the system required transforming yeast with four plasmids carrying cDNAs encoding CfEcR, CfUSP, or RXR γ , an activation factor, a glucocorticoid receptor interacting protein (GRIP), and a plasmid with four copies of an ecdysone response element (hsp27) in tandem fused to a reporter gene (Figure 7). The potency of a number of ecdysone agonists in transactivating the reporter gene in the reconstituted EcR ligand dependent assay correlated well with the known activities of the tested bisacylhydrazines in whole insect assays (Figure 8), indicating the utility of such assays for high throughput screening of new and novel chemistries with ecdysone mode of action. A similar yeast-based strain was also reconstituted using AaEcR/AaUSP (Tran *et al.*, 2001).

16.2.3.2. Whole organism effects The commercialized bisacylhydrazine insecticides are toxic to susceptible insects mainly as a result of ingestion. Toxicity via topical application is expressed only when very high doses are applied. The effects of bisacylhydrazines in susceptible insects have been studied in a number of insects (Slama, 1995; Smaghe *et al.*, 1996b, 1996c, 2002; Retnakaran *et al.*, 1997; Dhadialla *et al.*, 1998; Carton *et al.*, 2000). In general, because bisacylhydrazines are more metabolically stable *in vivo* than ecdysteroids and because they are true ecdysone agonists at the receptor level, ingestion of bisacylhydrazines creates “hyperecdysionism” in susceptible insects,

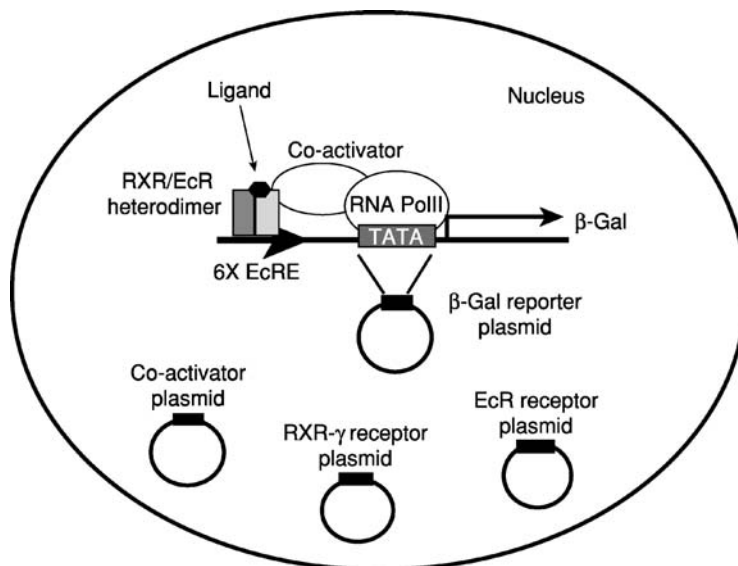
Table 4 Relative affinities of 20E and bisacylhydrazines in imaginal wing evagination assays

Species	20E	RH-5849	RH-5992	RH-2485	RH-0345	Reference
<i>Drosophilla melanogaster</i>	0.011 $\mu\text{mol l}^{-1}$	1 $\mu\text{mol l}^{-1}$	1.1 $\mu\text{mol l}^{-1}$	ND	ND	Farkas and Slama (1999)
<i>Chironomus tentans</i>	278 nM	27 nM	7.3 nM	ND	230 nM	Smaghe <i>et al.</i> (2002)
<i>Spodoptera littoralis</i>	291 nM	44500 nM	403 nM	10.9 nM	472 nM	Smaghe <i>et al.</i> (2000a)
<i>Galleria mellonella</i>	321 nM	865 nM	8.9 nM	ND	ND	Smaghe <i>et al.</i> (1996a)
<i>Leptinotarsa decemlineata</i>	60.7 nM	461 nM	757 nM	ND	ND	Smaghe <i>et al.</i> (1996a)

ND, not determined.

Table 5 Relative binding affinities of bisacylhydrazine compounds to cellular extracts or *in vitro* expressed EcR and USP proteins from insects of different orders

	<i>20E</i>	<i>Pon A</i>	<i>RH-5849</i>	<i>RH-5992</i>	<i>RH-2485</i>	<i>RH-0345</i>	Reference
<i>Drosophila melanogaster</i>	145 nM	0.9 nM		336 nM			Dhadialla, (unpublished data), Cherbas <i>et al.</i> (1988)
<i>Aedes aegypti</i>	28 nM	2.8 nM		30 nM			Dhadialla, unpublished data; Kapitskaya <i>et al.</i> (1996)
<i>Chironomus tentans</i>		0.35 ± 0.28 and 6.5 ± 2.4 nM					Grebe <i>et al.</i> (2000), Smagghe <i>et al.</i> (2001)
<i>Spodoptera littoralis</i>	158 nM			86.7 nM	24.3 nM		Smagghe <i>et al.</i> (2000a)
<i>Plodia interpunctella</i>	210 nM	3 nM		3 nM			Dhadialla, unpublished data
<i>Galleria mellonella</i>	106 nM		911 nM	22 nM			Smagghe <i>et al.</i> (1996a)
<i>Spodoptera frugiperda</i> (Sf9 cells)	166 nM	8.9 nM	363 nM	1.5 nM	3.5 nM		Nakagawa <i>et al.</i> (2000)
<i>Anthonomus grandis</i>	247 nM	6.1 nM		12 000 nM			Dhadialla and Tzertzinis (1997)
<i>Leptinotarsa decemlineata</i>	425 nM		740 nM	1316 nM			Smagghe <i>et al.</i> (1996a)
<i>Tenebrio molitor</i>		6 nM	>10 μM	>10 μM	>10 μM	>10 μM	Dhadialla, unpublished data
<i>Locusta migratoria</i>	1000 nM	1.18 nM	>10 μM	>10 μM	>10 μM	>10 μM	Hayward <i>et al.</i> (2003)
<i>Bamesia argentfolli</i>		8 nM	>10 μM	>10 μM	>10 μM	>10 μM	Dhadialla, unpublished data

**Figure 7** Schematic diagram showing a yeast cell transformed with plasmids carrying cDNAs encoding ecdysone receptor, RXR- γ , co-activator glucocorticoid receptor interaction protein (GRIP), and β -galactosidase (β -Gal) reporter gene fused to 6 heat shock protein 27 (hsp27) ecdysone response elements in a reconstituted and ecdysteroid (muristerone A) inducible high throughput screening system for discovery of new nonsteroidal ecdysone agonists.

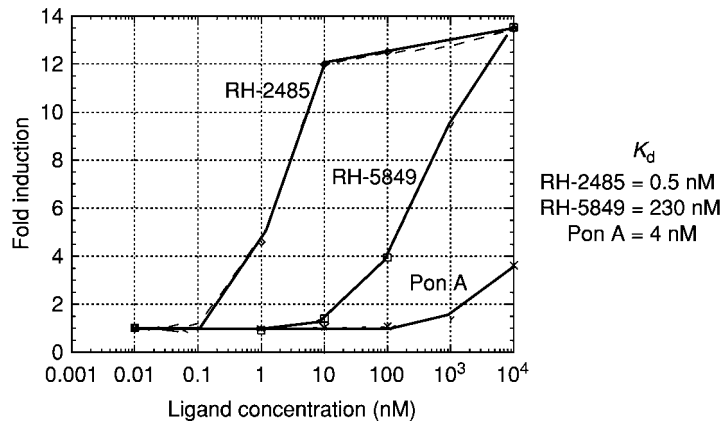


Figure 8 Dose-dependent induction of reporter (β -gal) gene in an ecdysone inducible, transformed yeast cell assay system, with RH-2485 (methoxyfenozide), RH-5849, and the phytoecdysteroid, ponasterone A. While the fold induction achieved with RH-2485 and RH-5849 correlate with their relative affinities (indicated on the right of the graph) for the ecdysone receptor (CfEcR) used in this study, the activation achieved by ponasterone does not correlate with its determined binding affinity to ecdysone receptor.

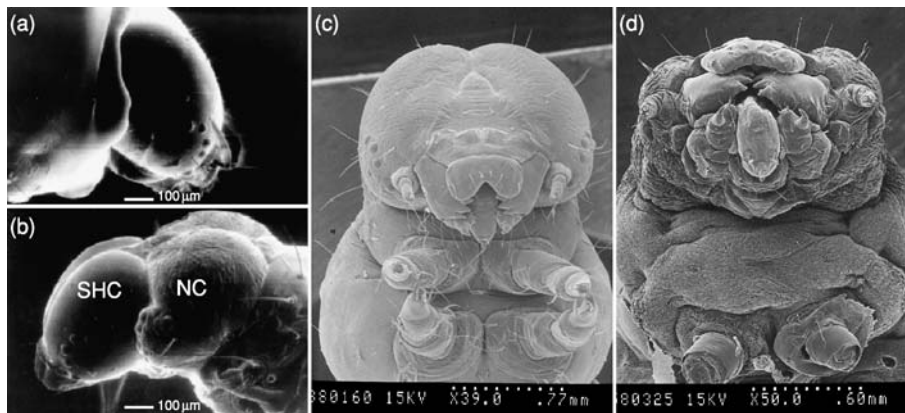


Figure 9 Scanning electron micrographs of control treated (a and c) and tebufenozide intoxicated (b and d) by ingestion (fava bean leaves dipped in a 10 ppm aqueous solution and 100 ng, respectively) second instar southern armyworm (a and b) and third instar spruce budworm (c and d) larvae 48 h after intoxication. In both control armyworm and budworm larvae (a and c), well formed sclerotized cuticle and mouthparts can be seen. In the intoxicated armyworm (b), the larvae is tricked into undergoing a precocious molt and the slipped head capsule (SHC) can be seen over the mouthparts. The new cuticle (NC) covering the head is soft, malformed, and unsclerotized. The soft unsclerotized mouthparts of the intoxicated spruce budworm (d) are shown after manually removing the slipped head capsule. In both cases, tebufenozide intoxicated larvae initiate a molt, slip head capsule, but are unable to complete a molt, and hence die of starvation, desiccation, and hemorrhage from the malformed unsclerotized new cuticle. (Adapted and composed from Dhadialla, T.S., Carlson, G.R., Le, D.P., 1998. New insecticides with ecdysteroidal and juvenile hormone activity. *Annu. Rev. Entomol.* 43, 545–569; as well as other unpublished electron micrographs.)

thus inducing effects and symptomology of a molt event. One of the first effects of bisacylhydrazine ingestion by susceptible larvae is feeding inhibition within 3–14 h (Slama, 1995; Smagghe *et al.*, 1996a; Retnakaran *et al.*, 1997), which prevents further plant damage. During this time, synthesis of a new cuticle begins and apolysis of the new cuticle from the old one takes place. Subsequently, the intoxicated larvae become moribund, slip their head capsule (Figures 9 and 10), and, in extreme cases, the hind gut may be extruded (Figure 11). The new cuticle is not tanned or sclerotized. One resulting consequence is that the mouth parts under the slipped

head capsule remain soft and mushy, preventing any crop damage even if the head capsule came off from mechanical or physical force. The larvae ultimately die as a result of their inability to complete a molt, starvation, and desiccation due to hemorrhage.

The reasons for the lethal precocious molt effects of bisacylhydrazines have been investigated at the ultrastructural level in *C. fumiferana* (Retnakaran *et al.*, 1997), in beet armyworm, *Spodoptera exigua* (Smagghe *et al.*, 1996c), in tomato looper, *Chrysodeixis chalcites* (Smagghe *et al.*, 1997), in the Colorado potato beetle, *Leptinotarsa*



Figure 10 Control (left) and tebufenozide intoxicated by ingestion (right) larvae of the white tussock moth. While the control larva continues normal growth and development, the tebufenozide intoxicated larva undergoes a precocious lethal molt. In this case the intoxicated larva is in the slipped head capsule stage.



Figure 11 Photomicrograph showing the severe growth inhibitory effects of ingested tebufenozide in spruce budworm larvae. While both larvae show slipped head capsules, one larva (bottom) shows an additional effect, extrusion of the gut.

decemlineata (Smagghe *et al.*, 1999c; Dhadialla and Antrim, unpublished observations), and in cultured abdominal sternites of the mealworm, *Tenebrio molitor* (Soltani *et al.*, 2002). Some general conclusions can be drawn from these studies. Examination of the cuticle following intoxication with any of the three bisacylhydrazines revealed

that the larvae synthesize a new cuticle that is malformed (Figure 12; Dhadialla and Antrim, unpublished data). Unlike during normal cuticle synthesis, the lamellate endocuticle deposition in bisacylhydrazine intoxicated larvae is disrupted and incomplete. The epidermal cells in intoxicated larvae have fewer microvilli, show hypertrophied Golgi complex and an increased number of vesicles compared to normal epidermal cells active in cuticle synthesis. The visual observations of precocious production of new cuticle have also been demonstrated by *in vivo* and *in vitro* experiments to demonstrate the inductive effects of tebufenozide and 20E on the amount of chitin in *S. exigua* larval cuticle and chitin synthesis in cultured claspers of *O. nubilalis*, respectively (Smagghe *et al.*, 1997).

At the physiological level the state of “hyperecdysionism,” coined by Williams (1967), manifested by bisacylhydrazines in intoxicated susceptible larvae is achieved by various mechanisms. Blackford and Dinan (1997) demonstrated that while larvae of the tomato moth, *Lacnobia oleracea*, detoxified ingested 20E as expected, it remained susceptible to ecdysteroid agonists RH-5849 and RH-5992. This suggested that the metabolic stability of the ecdysone agonists induced the “hyperecdysionism” state in the tomato moth larvae. In another study, RH-5849 was shown to repress steroidogenesis in the larvae of the blowfly, *Caliphora vicina*, as a result of its action on the ring gland (Jiang and Koolman, 1999). However, the production of ecdysteroids in abdominal sternites of *T. molitor*, cultured *in vitro* in the presence of RH-0345, increased compared to that in control cultured abdominal sternites (Soltani *et al.*, 2002). Ecdysteroid production, measured by an ecdysteroid enzyme immunoassay, by sternites cultured *in vitro* in the presence of 1–10 μM RH-0345 increased with increasing incubation times and concentrations of the bisacylhydrazine. Topical application of 10 μg RH-0345 to newly ecdysed pupae also caused a significant increase in hemolymph ecdysteroid amount as compared to control treated pupal hemolymph ecdysteroid levels. However, there was no effect in the timing of the normal ecdysteroid release in the hemolymph. Contrary to the *in vitro* and *in vivo* effects observed in the above *Tenebrio* study, Williams *et al.* (1997) observed that injection of RH-5849 into larvae of the tobacco hornworm, *Manduca sexta*, induced production of midgut cytosolic ecdysone oxidase and ecdysteroid phosphotransferase activities, which are involved in the inactivation of 20E. In addition, both 20E and RH-5849 caused induction of ecdysteroid 26-hydroxylase activity in the midgut mitochondria

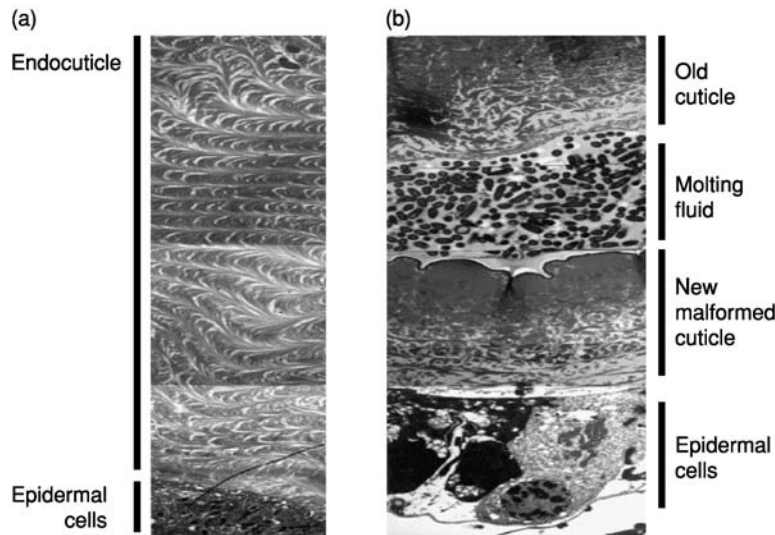


Figure 12 Transmission electron micrographs of the integument of control (a) and halofenozide (fava bean leaves dipped in a 100 ppm solution) ingested (b) second instar Colorado potato beetle larvae (same magnification). In the control integument, the epidermal cells appear normal (not clear in this figure) and the new cuticle is deposited in an ordered lamellate manner. In the integument of halofenozide intoxicated larva, the epidermal cells are highly vacuolated and the new cuticle is malformed, lacking ordered lamellate deposition. Although apolysis of the old cuticle has taken place, it is not shed, and electron dense granulated material (bacterial?) is seen in the ecdysial space. (Both micrographs taken at same magnification).

and microsomes. Subsequently, Williams *et al.* (2002) demonstrated that induction of ecdysteroid 26-hydroxylase by RH-5849, RH-5992 (tebufenozide), and RH-0345 (halofenozide) may be a universal action of bisacylhydrazines in susceptible lepidopteran larvae. These effects were not observed in nonsusceptible larvae of the waxmoth, *Galleria mellonella*, whose ecdysteroid receptor is capable of binding RH-5992 (Williams *et al.*, 2002). The binding in this case may not be sufficient for transactivation of genes that are involved in the molting process, and induced or repressed by 20E.

It is interesting to compare what happens at the molecular level in response to changing hormone levels in control insects compared to bisacylhydrazine intoxicated larvae as shown in Figure 13. Bisacylhydrazines, by virtue of their action via the ecdysone receptor, activate genes that are dependent upon increasing titers of 20E. However, those genes that are normally activated either by decreasing titers or absence of 20E are not expressed in the presence of ingested bisacylhydrazine in the hemolymph. During a normal molt cycle, the release of eclosion hormone to initiate the eclosion behavior in the molting larvae is dependent upon clearance of 20E titers in the hemolymph (Truman *et al.*, 1983). The presence of bisacylhydrazines in the hemolymph inhibits the release of eclosion hormone, thus resulting in an unsuccessful lethal molt.

The expression and repression of several genes during a molt cycle in lepidopteran (*M. sexta* and

C. fumiferana) larvae treated with RH-5992 have been investigated (Retnakaran *et al.*, 1995, 2001; Palli *et al.*, 1995, 1996, 1999). In control larvae, at the time of the rise in titer of 20E, as well as those treated with RH-5992, early genes such as MHR3 (in *Manduca*), CHR3 (in *Choristoneura*), and E75, (in *Drosophila*) are expressed. As 20E titers decline, DDC, which requires a transient exposure to 20E followed by its clearance, is expressed. In RH-5992, intoxicated larvae genes like DDC are not expressed, which prevents tanning and hardening of the already malformed new cuticle. During the intermolt period, when 20E is absent, genes like those for the 14 kDa larval cuticle protein (LCP14) that are normally repressed in the presence of 20E, are expressed. Once again, the prolonged presence of RH-5992 in the hemolymph prevents the expression of LCP14, and perhaps other genes that would normally be depressed in the absence of 20E. As a consequence of all the changes induced or repressed by RH-5992, and other bisacylhydrazine insecticides, the molting process is completely derailed at the ultrastructural, physiological and molecular level leading to precocious lethal molt in susceptible larvae.

Farkas and Slama (1999) studied the effects of RH-5849 and RH-5992 on chromosomal puffing, imaginal disc proliferation, and pupariation in larvae of *D. melanogaster*. In this study, they demonstrated that the two bisacylhydrazine compounds acted like 20E in the induction of early

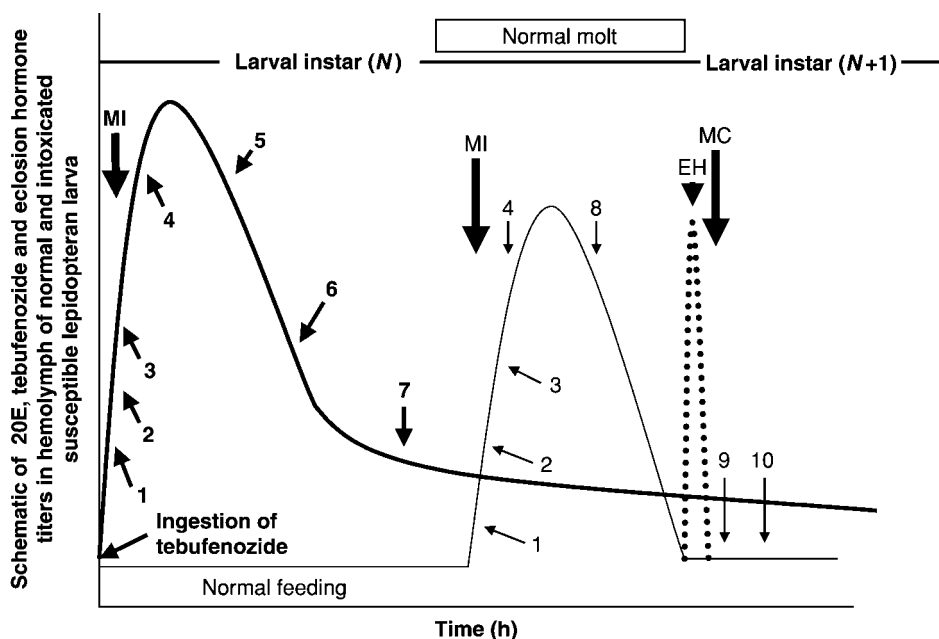


Figure 13 Schematic representation of titers 20E (thin line) and release of eclosion hormone (EH; dotted line), which triggers the ecdysis of the larva to complete a normal molt. The solid bold line represents relative titers of ingested tebufenozide in a susceptible lepidopteran larva (adapted from Dhadialla, unpublished data). Owing to the metabolic stability and amount of tebufenozide in the insect hemolymph, eclosion hormone, the release of which is normally dependent upon the complete decline of 20E, is not released and the intoxicated insect is not able to complete the molt, which leads to premature death. Both methoxyfenozide and halofenozide undergo similar metabolic fate, which is detrimental to intoxicated insect stage. The ecdysone agonists trigger a molt attempt anytime during the feeding stage of a susceptible larval instar. Events that take place during the molt and are dependent upon the increasing and decreasing titers of 20E are also shown. The numbers in bold and regular font represent different events triggered by tebufenozide and 20E, respectively. **1, 1**: Inhibition of feeding; **2, 2**: initiation of new cuticle synthesis; **3, 3**: apolysis of old cuticle from new cuticle resulting in an ecdysial space filled with molting fluid; **4, 4**: head capsule slippage; **MI**: molt initiated; **MC**: molt completed; **5**: derailment of the molting process; **6**: eclosion hormone is not released, and the larva stays trapped in its old cuticle and slipped head capsule covering the mouth parts (refer to Figure 9) causing it to starve; **7**: molt attempt is lethal and the ecdysone agonist intoxicated larvae dies of starvation, hemorrhage, and desiccation; **8**: cuticle formation continues and molting fluid starts to be resorbed; **9**: molt attempt is completed after release of EH and larva ecdyses into the next larval stage; **10**: new cuticle hardens and the mouth parts are sclerotized so that the larva may continue its growth and development into the next stage.

chromosomal puffs (74EF and 75B), and regression of pre-existing puffs (25AC, 68C) in larval salivary glands. In the chromosomal puff assay, the ED_{50} for bisacylhydrazine compounds were, however, an order of magnitude less than for 20E. Results of additional experiments in this study using different assays, i.e., glycoprotein glue secretion, imaginal disc evagination, and rescue of phenotypic expression in ecdysone deficient mutants showed the potencies of the bisacylhydrazines were two orders of magnitude lower than for 20E. In spite of the quantitative differences in the assays used, the results confirmed the ecdysone-mimetic action of the two bisacylhydrazines.

In a study to understand the role of ecdysteroids in the induction and maintenance of the pharate first instar diapause larvae of the gypsy moth, *Lymantria dispar*, Lee and Denlinger (1997) demonstrated that a diapause specific 55 kDa gut protein could be induced in ligature isolated larval abdomens

with injections of either 20E or RH-5992. They also demonstrated that the effect of KK-42, an imidazole derivative known to inhibit ecdysteroid biosynthesis, to prevent prediapausing pharate first instar larvae from entering diapause could be reversed by application of 20E or RH-5992. In this case, RH-5992 was two orders of magnitude more active than 20E.

Using 1,5-disubstituted imidazoles, Sonoda *et al.* (1995) demonstrated that the inductive effects of these compounds on precocious metamorphosis of *Bombyx mori* larvae could be reversed by tebufenozide.

16.2.4. Basis for Selective Toxicity of Bisacylhydrazine Insecticides

Unlike halofenozide, which is toxic to both coleopteran and lepidopteran larvae, both tebufenozide and methoxyfenozide are selectively toxic to lepidopteran larvae with a few exceptions of toxicity to

dipteran insects, like the midge, *C. tentans* (Smagghe *et al.*, 2002) and the mosquito species (Darvas *et al.*, 1998). This, at first, was a surprise, especially after the discovery that bisacylhydrazines are true ecdysone agonists, the activity of which, like that of 20E, is manifested via interaction with the ecdysone receptor complex. There are three basic reasons why an ingested insecticide may be differentially toxic to different insects: (1) metabolic differences between susceptible and nonsusceptible insects; (2) lack of transport to the target site in nonsusceptible insects; and (3) target site differences between susceptible and nonsusceptible insects.

Smagghe and Degheele (1994) found no differences in the pharmacokinetics and metabolism of ingested RH-5992 in two susceptible species, *Spodoptera exigua* and *S. exempta*, and a nonsusceptible insect, *Leptinotarsa decemlineata*. Similar results were obtained when the pharmacokinetics and metabolism of RH-5992 were investigated in larvae of susceptible *S. exempta* and the nonsusceptible Mexican bean beetle, *Epilachna verivesta* (Dhadialla and Thompson, unpublished results).

In competitive displacement EcR binding assays, it became apparent that one of the reasons for the differential selectivity of tebufenozide and methoxyfenozide might be due to differences in the LBDs of EcRs from different insects. While 20E binds to EcR complexes from insects from different orders with similar affinities, the affinity of tebufenozide and methoxyfenozide is very disparate for insects even within an insect order (Table 2). Hence, the very high affinity of tebufenozide and methoxyfenozide to lepidopteran EcR complexes correlates very well with their toxicity to larvae within this order, as well as those few amongst the Diptera. However, the same logic does not explain the toxicity of halofenozide and lack of toxicity of tebufenozide to coleopteran larvae, especially when the affinity of tebufenozide is higher than that of halofenozide for binding to EcRs from a coleopteran insect. Perhaps, in this case, the low binding affinity of halofenozide is compensated for by its greater metabolic stability (Smagghe and Degheele, 1994; Farinos *et al.*, 1999).

To determine if there are additional reasons at the cellular level for the selective toxicity of tebufenozide, Sundaram *et al.* (1998) made use of four lepidopteran and dipteran cell lines all of which are responsive to 20E indicating the presence of functional EcRs. A lepidopteran cell line from *C. fumiferana* midgut cells (CF-203) and a dipteran cell line (DM-2) derived from *D. melanogaster* embryos responded to 20E in a similar dose-dependent induction of the transcriptional factor, hormone receptor

3 (HR3) CHR3 and DHR3 respectively, which represents one of the early 20E inducible genes in the ecdysone signaling pathway. The lepidopteran CF-203 cells could produce CHR3 mRNA at 10^{-10} M RH-5992 concentration, as opposed to 10^{-6} M of this compound required for induction of very low amounts of DHR3 transcripts. In further experiments using ^{14}C -RH-5992, the authors were able to show that CF-203 cells accumulated and retained sixfold higher amounts of the radiolabeled compound than DM-2 cells over the same incubation period. Similar results were obtained with another lepidopteran cell line (MD-66) derived from *Malacosomma disstria*, and a dipteran cell line (Kc) from *D. melanogaster* embryos. This significantly higher retention of radiolabeled RH-5992 in lepidopteran cells compared to dipteran cells was in contrast to similar levels of retention of tritiated ponasterone A, a potent phytoecdysteroid. The extremely low amounts of RH-5992 in dipteran cells was due to an active efflux mechanism that was temperature-dependent and could be blocked with 10^{-5} M ouabain, an inhibitor of Na^+ , K^+ -ATPase.

In developing a yeast based ecdysteroid responsive reporter gene assay, Tran *et al.* (2001) had to use a yeast strain that was deficient in the *pdr5* and *snq2* genes, both of which are involved in multiple drug resistance, to increase the sensitivity of the transformed strain to both steroidal and nonsteroidal ecdysone agonists. Subsequently, Retnakaran *et al.* (2001) demonstrated that while the wild-type yeast *Saccharomyces cerevisiae* actively excluded tebufenozide, the *pdr5* deleted mutant strain retained significantly higher amounts of radiolabeled tebufenozide (Hu *et al.*, 2001; Retnakaran *et al.*, 2001). Retransformation of the mutant strain with the *pdr5* gene enabled the active exclusion of radiolabeled tebufenozide, thus once again suggesting a role for an ATP binding cassette transporter.

Some general conclusions can be drawn from the above studies regarding the selective insect toxicity of tebufenozide and methoxyfenozide. While the absence of significant differences in the pharmacokinetics and metabolism of these compounds in susceptible and nonsusceptible insects is not directly responsible for selective toxicity, it is indirectly, because the hormone levels of tebufenozide, after its ingestion by lepidopteran larvae, are about 2000-fold higher than the K_d equivalent for the lepidopteran EcR. The selective toxicity of tebufenozide and methoxyfenozide to lepidopteran larvae is probably due to the clearance of these agonists as shown in resistant cell lines where they are actively pumped out. It would be interesting to investigate if cells derived from *C. tentans* and mosquito tissues,

like lepidopteran cells, are devoid of an active mechanism to eliminate tebufenozide and methoxyfenozide, because ecdysone receptors from both these dipterans have high affinities for bisacylhydrazines (Kapitskaya *et al.*, 1996; Smaghe *et al.*, 2002) and are susceptible to tebufenozide (Smaghe *et al.*, 2002).

16.2.5. Spectrum of Activity of Commercial Products

16.2.6.1. Chromofenozide (MATRIC[®]; KILLAT[®]; ANS-118; CM-001) Jointly developed by Nippon Kayaku Co. Ltd (Saitama, Japan), and Sankyo, Co. Ltd. (Ibaraki, Japan), chromofenozide is the latest amongst the nonsteroidal ecdysone agonist insecticides registered for control of lepidopteran

pests on vegetables, fruits, vines, tea, rice, arboriculture, ornamentals, and other crops in Japan (Table 6). Chromofenozide has relatively safe mammalian, avian, and aquatic organism toxicology (Table 7). It also has no adverse effects on nontarget arthropods, including beneficials.

16.2.5.2. Halofenozide (MACH II; RH-0345) Unlike tebufenozide, methoxyfenozide, and chromofenozide, halofenozide is more soil systemic and has a broader pest spectrum of activity (Lepidoptera and Coleoptera; Table 6). It was developed for control of beetle grub and lepidopteran larval pests of turf in lawns and on golf courses.

Halofenozide gave excellent control of the Japanese beetle (*Popillia japonica*) and the oriental

Table 6 Spectrum of pest activity of the four commercial ecdysone agonist insecticides

<i>Tebufenozide and methoxyfenozide (Lepidoptera specific)</i>	<i>Methoxyfenozide (Lepidoptera specific)</i>	<i>Chromofenozide (Lepidoptera specific)</i>	<i>Halofenozide (Lepidoptera and Coleoptera specific)</i>
<i>Adoxophyes</i> spp.	<i>Clysia ambiguella</i>	<i>Spodoptera littura</i>	<i>Agrotis ipsilon</i>
<i>Anticarsia gemmatalis</i>	<i>Grapholitha molesta</i>	<i>Spodoptera exigua</i>	<i>Spodoptera frugiperda</i>
<i>Boarmia rhombodaria</i>	<i>Heliothis</i> spp.	<i>Spodoptera littoralis</i>	Sod webworm (<i>Crambinae</i>)
<i>Chilo suppressalis</i>	<i>Helicoverpa zea</i>	<i>Plutella xylostella</i>	<i>Anomala orientalis</i>
<i>Choristoneura</i> spp.	<i>Ostrinia nubilalis</i>	<i>Cnaphalocrosis medinalis</i>	<i>Maladera canstanea</i>
<i>Cnaphalocrosis medinalis</i>	<i>Phyllonorychter</i> spp.	<i>Ostrinia furnacalis</i>	<i>Ataenius spretulus</i>
<i>Cydia</i> spp.	<i>Tuta absoluta</i>	<i>Adoxophyes orana</i>	<i>Rhizotrogus majalis</i>
<i>Diatraea</i> spp.	<i>Keiferia lycopersicella</i>	<i>Heliothis virescens</i>	<i>Popillia japonica</i>
<i>Diaphania</i> spp.		<i>Lobesia botrana</i>	<i>Cotinis nitida</i>
<i>Hellula</i> spp., <i>Homona magnanima</i>		<i>Cydia pomonella</i>	<i>Cyclocephala</i> spp.
<i>Lobesia botrana</i>		<i>Chilo suppressalis</i>	<i>Phyllophaga</i> spp.
<i>Lymantria dispar</i>		<i>Anticarsia gemmatalis</i>	<i>Hyperodes near anthricinus</i>
<i>Planotortrix</i> spp.,			
<i>Platynota idaeusalis</i>			
<i>Plusia</i> spp., <i>Pseudoplusia includens</i>			
<i>Rhicoplusia nu</i> , <i>Sparganothis</i> spp.			
<i>Spodoptera</i> spp.			
<i>Trichoplusia ni</i>			
<i>Epiphyas postvittana</i> , <i>Grapholitha prunivora</i>			
<i>Memestra brassicae</i> , <i>Operophtera brumata</i>			

Table 7 Ecotoxicological profile of bisacylhydrazine insecticides

	<i>Tebufenozide</i>	<i>Methoxyfenozide</i>	<i>Chromofenozide</i>	<i>Halofenozide</i>
Avian: mallard duck, LC ₅₀ (8-day dietary)	>5000 mg kg ⁻¹	>5620 mg kg ⁻¹		>5000 mg kg ⁻¹
Bobwhite quail, LC ₅₀ (8-day dietary)	>5000 mg kg ⁻¹	>5620 mg kg ⁻¹	>5000 mg kg ⁻¹ (Japanese quail, 14-day)	4522 mg kg ⁻¹
Aquatic: bluegill sunfish, acute LC ₅₀ (96 h)	3.0 mg l ⁻¹	>4.3 mg l ⁻¹		>8.4 mg l ⁻¹
<i>Daphnia magna</i> , acute EC ₅₀ (48 h)	3.8 mg l ⁻¹	3.7 mg l ⁻¹	>189 mg l ⁻¹ (3 h)	3.6 mg l ⁻¹
Honeybee (oral and contact), acute LD ₅₀	>234 µg bee ⁻¹	>100 µg bee ⁻¹	>100 µg bee ⁻¹ (contact)	>100 µg bee ⁻¹
Earthworm, LC ₅₀ (14 days)	1000 mg kg ⁻¹	>1213 mg kg ⁻¹	>1000 mg kg ⁻¹	980 mg kg ⁻¹

beetle (*Exomala orientalis*) when applied at 1.1 kg active ingredient (a.i.) ha⁻¹ to a mixed field population on turf plots (Cowles and Villani, 1996; Cowles *et al.*, 1999). In the same study, higher rates (1.7–2.2 kg a.i. ha⁻¹) were required to control the European chafer, *Rhizotrogus (Amphimallon) majalis* (Razoumowsky), population. The Asiatic garden beetle, *Maladera castanea* (Arrow), was not sensitive to any of the doses of halofenozide tested.

The recommended rates for the control of lepidopteran larvae (such as cutworms, sod webworms, armyworms, and fall armyworms) and beetle grubs (Japanese beetle, northern and southern masked chafer, June beetle, Black turfgrass ataenius beetle, green June beetle, annual bluegrass weevil larvae, bill bugs, aphodius beetles, European chafer, and oriental beetle) are 12 kg a.i. ha⁻¹ and 18–24 kg a.i. ha⁻¹, respectively.

16.2.5.3. Tebufenozide (MIMIC; CONFIRM; ROMDAN; RH-5992) and methoxyfenozide (RUNNER; INTREPID; PRODIGY; RH-2485) Tebufenozide, at low use rates, shows remarkable insect selectivity for the control of lepidopteran larvae, including many members of the families Pyralidae, Pieridae, Tortricidae, and Noctuidae, which are pests of tree fruit, vegetables, row crops, and forests (Table 6; Le *et al.*, 1996; Dhadialla *et al.*, 1998; Carlson *et al.*, 2001). Laboratory studies have shown the following: tebufenozide administered through artificial diet or by leaves treated with tebufenozide is efficacious against *C. fumiferana* (Retnakaran *et al.*, 1999), corn earworm and fall armyworm (Chandler *et al.*, 1992), and beet armyworm (Chandler, 1994); both tebufenozide and methoxyfenozide are efficacious against laboratory reared and field populations of codling moth, *Cydia pomonella* (Pons *et al.*, 1999; Knight *et al.*, 2001), laboratory reared and multi-resistant cotton leafworm, *Spodoptera littoralis* (Smagghe *et al.*, 2000b), field populations of filbertworm, *Cydia latiferreana*, on hazelnut, and against *D. grandiosella* (Trisyono and Chippendale, 1998).

Tebufenozide has been used very successfully for the control of *C. fumiferana*, which is the most destructive insect defoliator of spruces, *Picea* sp., and balsam fir, *Abies balsamea* (L.) Mill, in North American forests (Sundaram *et al.*, 1996; Cadogan *et al.*, 1997, 1998, 2002). Cadogan *et al.* (1998) used a 240 g l⁻¹ formulation of commercial tebufenozide (MIMIC[®] 240LV) mixed with water and a tracer dye (Rhodamine WT), which was sprayed onto the trees using a light-wing aircraft, to determine the foliar deposit of the insecticide and its

efficacy against *C. fumiferana*. Single applications of 1 or 2 L ha⁻¹ resulted in a 61.4–93.6% or 85.6–98.3% reduction, respectively, of the spruce budworm populations. Following double applications, the mean population reductions ranged from 93.6 to 98.3% compared to mean population reductions of 61% in untreated control fields. Defoliation in untreated control plots was about 92% and in plots with single or double spray applications, defoliation was 40–62% or 31–62%, respectively. These results indicated that a double spray application was most efficacious.

MIMIC 240LV has also provided effective control of Jackpine budworm, *Choristaneura pinus*, and *Lymantria dispar* in North American forests, as well as control of processionary caterpillar, *Thaumetopomea pityocampa*, and European pine shoot moth, *Rhyaciona buoliana*, in forests in Spain and Chile, respectively (Lidert and Dhadialla, 1996; Butler *et al.*, 1997).

Two of the major lepidopteran pests of tree-fruits are codling moth and leaf rollers, which have been controlled by frequent applications of azinphosmethyl and other organophosphate insecticides (Riedl *et al.*, 1999). With increasing requirements to use more selective insecticides without effecting biological control of other pests, much attention has been paid to the use of IGRs like chitin synthesis inhibitors, JHAs, and agonists of 20E. The effectiveness of tebufenozide against larvae of the codling moth, *C. pomonella*, has been tested both under laboratory and field conditions (Pons *et al.*, 1999; Knight, 2000). Pons *et al.* (1999) found that tebufenozide has ovicidal and larvicidal but not adulticidal activity against *C. pomonella*. Tebufenozide was about 73 times more effective when presented to neonate larvae in artificial diet as compared to treated apples; possibly because once the neonates have burrowed their way into the apples they are no longer exposed to the insecticides. However, in tebufenozide treated artificial diets, the susceptibility to the insecticide decreased with increasing age. Because of the feeding behavior of codling moth larvae, which generally do not feed and do not ingest large amounts of foliage or fruit as they burrow into the core of the fruit, the exposure to the insecticides is very limited. However, the reproductive and ovicidal effects of tebufenozide may be more important than its larvicidal effects (Knight, 2000). Knight (2000) found that in 30 field trials, tebufenozide exhibited both residual and topical ovicidal effects for codling moth egg masses laid on leaves. Similar results were obtained by Pons *et al.* (1999).

These studies indicated that the timing of tebufenozide sprays was important in not only preventing

codling moth injury to the apples, but also in controlling oblique banded leaf rollers for which both tebufenozide and methoxyfenozide have been shown to have lethal and sublethal effects (Riedl and Brunner, 1996; Sun *et al.*, 2000). Knight (2000) proposed that delaying the first application of tebufenozide until after petal fall in apple for *Choristoneura rosaceana* (Harris) would correspond to an approximate codling moth timing of 30-degree days at which time this insect would be susceptible as well. This strategy, which targets the adults and eggs, was effective in the control of codling moth.

Both tebufenozide and methoxyfenozide are active primarily by ingestion, but also exhibit selective contact and ovicidal activity (Trisyono and Chippendale, 1997; Sun and Barrett, 1999; Sun *et al.*, 2000).

16.2.6. Ecotoxicology and Mammalian Reduced Risk Profiles

Both tebufenozide and methoxyfenozide have been classified by the US Environmental Agency (EPA) as reduced risk pesticides because of their low acute and chronic mammalian toxicity, low avian toxicity, and their safety to most beneficial arthropods and compatibility with integrated pest management programs. Both tebufenozide and methoxyfenozide are toxic primarily to lepidopteran pests, but they are also toxic to a few dipteran (*C. tentans*, mosquitos) pests. Of the 150 insect species tested, both tebufenozide and methoxyfenozide were found to be safe to members of the insect orders Hymenoptera, Coleoptera, Diptera, Heteroptera, Hemiptera, Homoptera, and Neuroptera (Glenn Carlson, unpublished data). When used at rates that are 18- to 1500-fold greater than that producing 90% mortality in lepidopteran larvae, both tebufenozide and methoxyfenozide had little or no effect on a panel of non-lepidopteran pests (Coleoptera, Homoptera, mites, and nematodes; Trisyono *et al.*, 2000; Carlson *et al.*, 2001; Medina *et al.*, 2001). Similarly, laboratory and/or field studies have shown that the two insecticides have little or

no adverse effect on a wide range of non-lepidopteran beneficial insects, like honeybees and predatory insects/mites, when applied at rates used for control of lepidopteran larvae.

In 1998, methoxyfenozide became one of the only four pesticide products to be awarded the “Presidential Green Chemistry Award,” which was established by the US Government during 1995 to recognize outstanding chemical processes and products that reduce negative impact on human health and the environment relative to the currently available technology.

The ecotoxicological and mammalian toxicity data for the four commercial ecdysone agonist insecticides are shown in Tables 7 and 8. These data clearly show the low mammalian, avian, and aquatic toxicity of these insecticides.

16.2.7. Resistance, Mechanism, and Resistance Potential

Tebufenozide is the first nonsteroidal ecdysone agonist with commercial application and has been used in Western Europe since the mid 1990s. In the USA, the first uses occurred concurrently in 1994 in Alabama and Mississippi under Section 18 exemptions (Walton *et al.*, 1995; Dhadialla *et al.*, 1998).

Resistance to tebufenozide was documented for the first time in the codling moth *C. pomonella* around Avignon in southern France (Sauphanor and Bouvier, 1995; Sauphanor *et al.*, 1998b) and in the greenheaded leafroller, *Planotortrix octo*, in New Zealand (Wearing, 1998). A small survey with the beet armyworm, *S. exigua*, collected in different greenhouses in Western Europe (Spain, Belgium, The Netherlands) showed lack of resistance development to tebufenozide (Smagghe *et al.*, 2003b). In this same period, Smagghe *et al.* (1999a) kept a laboratory strain of *S. exigua* under continuous pressure with sublethal concentrations of tebufenozide over 12 generations, and in the sixth generation a significantly lower toxicity (about five-fold) was observed. In addition, retention and

Table 8 Mammalian reduced risk profile of bisacylhydrazine insecticides

	<i>Tebufenozide</i>	<i>Methoxyfenozide</i>	<i>Chromofenozide</i>	<i>Halofenozide</i>
Acute oral LD ₅₀ (rat, mouse)	>5000 mg kg ⁻¹	>5000 mg kg ⁻¹	>5000 mg kg ⁻¹	2850 mg kg ⁻¹
Acute dermal LD ₅₀	>5000 mg kg ⁻¹	>2000 mg kg ⁻¹	>2000 mg kg ⁻¹	>2000 mg kg ⁻¹
Eye irritation (rabbit)	Nonirritating	Nonirritating	slightly irritating	Moderately irritating, positive for contact
Dermal sensitization (guinea pig)	Nonsensitizer	Negative	Mildly sensitizing	Allergy
Ames assay	Negative	Negative	Negative	Negative
Acute inhalation	>4.3 mg l ⁻¹	>4.3 mg l ⁻¹		>2.7 mg l ⁻¹
Reproduction (rat)	No effect	No effect	No effect	No effect

metabolic fate studies showed the importance of oxidative metabolism, leading to a rapid clearance from the body. Interestingly, a decrease in oviposition was noted with the lower toxicity indicating a possible fitness cost related to development of resistance (Smagghe and Degheele, 1997; Smagghe *et al.*, 1998). More recently, Moulton *et al.* (2002) reported resistance to tebufenozide in third instar larvae, which reached levels of 150-fold in a selected strain of *S. exigua* from Bangbua Thong (Thailand) as compared with a laboratory reference strain. In this region of Thailand, many insecticides including organophosphates (OPs), pyrethroids, benzoylphenylurea (BPUs), and *Bacillus Thuringiensis* (Bt) formulations, and even new IGRs have been rendered ineffective due to ill-advised agricultural practices, most notably dilution of insecticide residues on leaves by overhead drench irrigation (Moulton *et al.*, 2002). This practice is likely to be responsible for the high incidence of insecticide multiresistance in this area and the highly accelerated rate of resistance development. When this Thailand strain was dosed with methoxyfenozide, 120-fold lower toxicity was observed. These selection assays with tebufenozide and methoxyfenozide showed a reduction in toxicity for both compounds, suggesting at least some commonality of resistance mechanism (Moulton *et al.*, 2002). A greenhouse raised strain of *S. exigua*, in southern Spain was also found to be resistant to tebufenozide and methoxyfenozide. Although the level of resistance was lower in this second strain, it was high enough to allow studies on the mechanism(s) of resistance. In general, a higher breakdown activity leads to lower levels of the parent toxophore. For tebufenozide and methoxyfenozide the major first phase route of detoxification was through oxidation (Smagghe *et al.*, 1998, 2003). In addition, piperonyl butoxide, a P450 inhibitor, significantly synergized the toxicity of tebufenozide and methoxyfenozide, whereas DEF, an esterase inhibitor, was ineffective (Smagghe *et al.*, 1998, 1999), indicating that a lower toxicity was more likely from an increase in oxidative activity, rather than in esterase activity. At the cellular level, Retnakaran *et al.* (2001) reported that tebufenozide accumulated selectively in lepidopteran Cf-203 (*C. fumiferana*) cells in contrast to dipteran Dm-2 cells (*D. melanogaster*), which actively excluded the compound (see Section 16.2.4 for more details). Perhaps such exclusion systems may also account for the fact that older instars of the white-marked tussock moth (*Orgyia leucostigma*) are resistant to tebufenozide (Retnakaran *et al.*, 2001). The characterization of all such possible resistance processes is essential to provide information that is helpful

to prevent resistance from developing towards this valuable group of IGRs. But more information is needed with additional strains collected from the field, especially where growers have severe pest control problems, before a general interpretation can be formulated on resistance risks for this new type of IGRs in the field.

While the available data suggest oxidative metabolism as the primary reason for development of resistance to tebufenozide and methoxyfenozide, there is no evidence so far that suggests that target site modification could be involved as another route to resistance development in field or laboratory insect populations. However, at the cellular level, there is evidence that there could be alterations in the target site(s). Using insect cell lines, Cherbas and co-workers were the first to report that *in vitro* cultured Kc cells of *D. melanogaster* did not respond to 20E after continuous exposure (Cherbas, personal communication). Similarly, Spindler-Barth and Spindler (1998) reported that cells of another dipteran, *C. tentans*, maintained in the continuous presence of increasing concentrations of 20E or tebufenozide over a period of 2 years, developed resistance to both compounds. In these resistant subclones, all 20E regulated responses that are known to occur in sensitive cells were no longer detectable, suggesting that the hormone signalling pathway itself was interrupted (Spindler-Barth and Spindler, 1998; Grebe *et al.*, 2000). Further ligand binding experiments with extracts containing ecdysone receptors from susceptible and resistant cells indicated that ligand binding to EcR from resistant clones was significantly decreased. Moreover, an increase in 20E metabolism and a reduction in receptor concentration were noted in some clones. Similar effects have also been observed in another study using imaginal discs of *S. littoralis* selected for resistance to tebufenozide (Smagghe *et al.*, 2001).

While resistance to ecdysone agonist insecticides is inevitable, it can be prevented from occurring sooner with good resistance monitoring programs.

16.2.8. Other Chemistries and Potential for New Ecdysone Agonist Insecticides

After the discovery of the first three bisacylhydrazine insecticides by scientists at Rohm and Haas Company, Spring House, PA, USA, a number of laboratories initiated such efforts to discover new and novel chemistries with ecdysone mode of action and different pest selectivity. Mikitani (1996) at Sumitomo Company, Japan, discovered a benzamide, 3,5,-di-*t*-butyl-4-hydroxy-N-isobutyl-benzamide

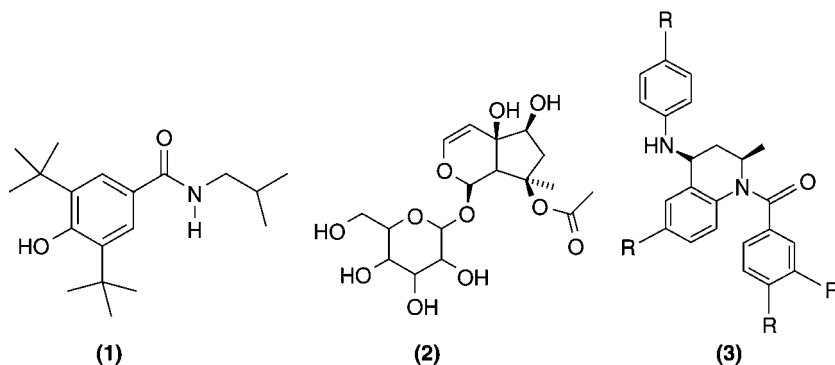


Figure 14 Chemical structures of new ecdysone agonists reported in literature: (1) 3,5-di-*t*-butyl-4-hydroxy-*N*-isobutyl-benzamide (DTBHIB) from Sumitomo; (2) 8-*O*-acetylharpagide from Merck Research Laboratories, Westpoint, PA, USA and (3) tetrahydroquinoline from FMC Corporation, Princeton, NJ, USA. In (3) R = halide.

(DTBHIB; **Figure 14**) and Elbrecht *et al.* (1996) at Merck Research Laboratories, Westpoint, PA, USA, reported the isolation of an iridoid glycoside, 8-*O*-acetylharpagide (**Figure 14**), from *Ajuga reptans*. Both these compounds were reported to induce 20E-like morphological changes in *Drosophila* Kc cells, as well as competitively displace tritiated ponasterone A from *Drosophila* ecdysteroid receptors with potencies similar to that of RH-5849, the unsubstituted bisacylhydrazine. However, the insecticidal activity of these compounds was not described. Attempts to replicate the results of Mikitani (1996) using DTBHIB and analogs failed to demonstrate that these compounds were competitive inhibitors of tritiated ponasterone A binding to DmEcR/DmUSP produced by *in vitro* transcription and translation (Dhadialla, unpublished observations). On the other hand, Dinan *et al.* (2001) demonstrated that the results obtained by Elbrecht *et al.* (1996) were due to co-purification of ecdysteroids in their 8-*O*-acetylharpagide preparation. When used as a highly purified preparation, Dinan *et al.* (2001) found that 8-*O*-acetylharpagide was not active as an agonist or an antagonist in *D. melanogaster* BII cell bioassay, and neither did it compete with tritiated ponasterone A for binding to the lepidopteran ecdysteroid receptor complex from *C. fumiferana*.

Finally, scientists at FMC discovered a new tetrahydroquinoline (THQ) class of compounds (**Figure 14**) that competitively displaced tritiated ponasterone A from both dipteran (*D. melanogaster*) and lepidopteran (*H. virescens*) EcR and USP heterodimers (unpublished data). Interestingly, the most active analogs of this class of compounds bound the DmEcR/DmUSP with much higher affinity than the HvEcR/HvUSP. This is the reverse of what was observed with bisacylhydrazines. When tested for ligand binding to EcR/USP proteins from

L. migratoria, *B. argentifoli*, and *T. molitor*, to which bisacylhydrazines show no measurable affinity, members of the THQ were found to bind with measurable affinity (μM range; Dhadialla and colleagues, unpublished results). Further work on this chemistry was continued at Rohm and Haas Company, Spring House, PA, USA, and its subsidiary, RheoGene LLC, Malvern, PA, USA, which resulted in the synthesis of a number of analogs that were active in transactivating reporter genes fused to different insect EcRs and heterodimeric partners (*L. migratoria* RXR, and human RXR) in mouse NIH3T3 cells (Michelotti *et al.*, 2003).

The discovery of THQs with ligand binding activities to various EcRs (Michelotti *et al.*, 2003), some of which do not interact with bisacylhydrazines, and the interpretation of X-ray crystal structure results of liganded HvEcR/HvUSP (Billas *et al.*, 2003) provides good evidence for the potential to discover new chemistries with ecdysone agonist activities. It should also be possible to design chemistries that specifically interact with EcR/USPs from a particular insect order.

16.2.9. Noninsecticide Applications of Nonsteroidal Ecdysone Agonists; Gene Switches in Animal and Plant Systems

A number of researchers started to explore the utilization of the ecdysone receptor as an inducible gene switch due to the knowledge that neither mammals nor plants have ecdysone receptors, and the discovery of bisacylhydrazine as true ecdysone agonists with reduced risk ecotoxicology and mammalian profiles. Gene switches are inducible gene regulation systems that can be used to control the expression of transgenes in cells, plants, or animals. There is a recognized need for tightly regulated eukaryotic molecular gene switch applications, such as for gene therapy, and in understanding the role played

by specific proteins in signaling pathways, cell differentiation, and development (Allgood and Eastman, 1997). Other applications include large-scale production of proteins in cells, cell-based high-throughput screening assays, and regulation of traits in both plants and animals. Unlike all other previous and current insecticides, commercial nonsteroidal ecdysone agonists are the first class of insect toxic chemistry that has led to such a high level of interest and utility outside the insecticide arena. The following is a brief overview of the use of bisacylhydrazine insecticides and EcRs as gene switches in mammalian and plant systems.

16.2.9.1. Gene switch in mammalian systems

Christopherson *et al.* (1992) demonstrated that DmEcR could function as an ecdysteroid-dependent transcription factor in human 293 cells cotransfected with an RSV-based expression vector that encoded DmEcR and a reporter gene, which contained four copies of *Drosophila* Hsp27 linked to a MTV promoter-CAT construct. They demonstrated that the activity of DmEcR could not be activated by any of the mammalian steroid hormones tested. Even amongst the ecdysteroids, the phytoecdysteroid muristerone A was the best transactivator of the reporter gene, while 20E was not as effective. These authors further demonstrated that chimeric receptors containing the LBD of DmEcR and the DBD of glucocorticoid receptor could also function when cotransfected with plasmids containing glucocorticoid receptor response elements fused to a reporter gene. Subsequently, No *et al.* (1996) demonstrated that an EcR-based gene switch consisting of DmEcR, *Homo sapiens* RXR, and appropriate response elements fused to a reporter gene in mammalian cells or transgenic mice could be transactivated with micromolar levels of muristerone A. Muristerone was shown to maintain its activity when injected into mice and was not found to be toxic, teratogenic, or inactivated by serum binding proteins. Moreover, overexpression of modified DmEcR and RXR did not appear to be toxic, at least in transfected cells. This study, like an earlier one (Yao *et al.*, 1995), demonstrated that HsRXR could substitute for its insect homolog and heterodimer partner for EcR, USP. However, in all these studies, the range of activating ligands was limited to ecdysteroids and, in particular, to muristerone A. In addition, RXR, which is present in almost all mammalian cells, was found not to be as good a partner for EcR as USP. Hence, very high endogenous levels were necessary for stimulation with muristerone A to occur.

With the availability of EcR and USPs from other insects, especially lepidopteran insects, and understanding the high affinity of nonsteroidal ecdysone agonists like tebufenozide and methoxyfenozide for lepidopteran EcRs/USPs, Suhr *et al.* (1998) demonstrated that the *B. mori* EcR (BmEcR), unlike DmEcR, could be transactivated to very high levels in mammalian cells with tebufenozide without adding an exogenous heterodimer partner like RXR. The endogenous levels of RXR were enough to provide the high transactivation levels. The work by Suhr *et al.* (1998) further demonstrated that while the D domain (hinge region) of EcR was necessary for high-affinity heterodimerization with USP, both D and E (LBD) domains were necessary for high-affinity interaction with RXR. By creating chimeric EcR using parts of E-domains from BmEcR and DmEcR, these investigators showed that a region in the middle of the E-domain (amino acids 402 to 508) in BmEcR constituted the region conferring high specificity for tebufenozide. Subsequent research by others has mainly focused on using EcRs and USPs from other insects as well as using different promoters for expression of the transfected genes (Kumar *et al.*, 2002; Dhadialla *et al.*, 2002; Palli and Kapitskaya, 2002; Palli *et al.*, 2002, 2003). Based on EcR homology modeling studies and site-directed mutagenesis of selected amino acid residues, Kumar *et al.* (2002) found that a CfEcR mutant, which had an A110P mutation in the LBD, was responsive only to nonsteroidal ecdysone agonists, including methoxyfenozide, but not to any of the ecdysteroids tested. This demonstrated that the affinity and functional specificity of an EcR for a ligand can be altered, thus offering the possibility of using multiplexed EcR-based gene switches that could regulate different traits with different ligands. Palli *et al.* (2003) developed a two-hybrid EcR based gene switch, which consisted of constructs containing DEF domains of CfEcR fused to the GAL4 DNA binding domain, and CfUSP or *Mus musculus* retinoid X receptor (MsRXR) EF domains fused to the VP16 activation domain. These constructs were tested in mammalian cells for their ability to drive a luciferase gene placed under the control of GAL4 response elements and synthetic TATAA promoter. This combination gave very low level basal activation of the reporter gene in the absence of the inducer. In the presence of the inducer, there was a rapid increase in the expression of the luciferase reporter gene, reaching levels as high as 8942-fold greater than basal level by 48 h. Withdrawal of the ligand resulted in 50% and 80% reduction of the reporter gene by 12 h and 24 h, respectively.

These studies clearly demonstrate the potential of utilizing EcR based gene switches that can be activated by ecdysteroids and nonsteroidal ecdysone agonists like tebufenozide, methoxyfenozide, and others. The fact that both tebufenozide and methoxyfenozide are registered as commercial insecticides, and have proven reduced risk mammalian and ecotoxicology profiles, makes them very attractive as inducers of the EcR based gene switches. The work of Kumar *et al.* (2002) clearly demonstrates the potential of mutating EcR to change its ligand specificity, thus opening additional possibilities of extending the use of the EcR gene switch in a multiplexed manner.

16.2.9.2. Gene switch for trait regulation in plants The reader is referred to very good recent reviews on this topic that not only describe the EcR-based chemically inducible gene regulation systems, but also other systems that have utility in plants (Jepson *et al.*, 1998; Zuo and Chua, 2000; Padidam, 2003). This section is restricted to descriptions of EcR-based gene switch systems.

In the mid-1990s, a number of agricultural companies initiated research to exploit the use of the EcR-based gene switch and nonsteroidal ecdysone agonists like tebufenozide as chemical inducers for regulation of traits (for example, fertility, flowering, etc.) in plants. Initial work was done using DmEcR and DmUSP as components of the gene switch (Goff *et al.*, 1996). In this case, the researchers used chimeric polypeptides (GAL4 DBD fused to LBD of DmEcR and VP16 activation domain fused to DmUSP) to activate the luciferase reporter gene fused to GAL4 response element in maize cells. In the presence of 10 μ M tebufenozide, about 20- to 50-fold activation of luciferase expression was obtained. Subsequently, a number of researchers developed variants of EcR gene switches using different chimeric combinations of heterologous DBD, LBDs from different lepidopteran EcRs, and transactivation domains that could interact with appropriate response elements to transactivate a reporter gene in a ligand-dependent manner (Martinez *et al.*, 1999a; Unger *et al.*, 2002; Padidam *et al.*, 2003). For example, Jepson *et al.* (1996) and Martinez *et al.* (1999b) used chimeric *H. virescens* EcR (HvEcR) composed of glucocorticoid receptor transactivation and DBD fused to LBD of HvEcR and *GUS* reporter gene fused to glucocorticoid receptor response element for transfection of maize and tobacco protoplast. In both cases, weak transactivation of the *GUS* reporter gene was obtained with tebufenozide and muristerone A, though the response with the latter was much

lower than with tebufenozide. However, 10 μ M and higher concentrations of the two ligands were required to transactivate the reporter genes.

Padidam *et al.* (2003) used a chimeric *C. fumiferana* EcR (CfEcR) composed of a CfEcR LBD, GAL4 and LexA DBDs, and VP16 activation domains that could be activated with methoxyfenozide in a dose-dependent manner from a GAL4- or LexA-response element to express a reporter gene. These researchers used *Arabidopsis* and tobacco plants for transformation with the gene switch components and obtained several transgenic plants that had little or no basal level of expression in the absence of methoxyfenozide. In the presence of methoxyfenozide, reporter expression was several fold higher than in the absence of methoxyfenozide. The above studies provided ample evidence of the utility of EcR gene switch, especially those that utilize EcR from a lepidopteran species, and tebufenozide and methoxyfenozide as chemical inducers for trait regulation in plants.

Demonstration of the utility of EcR-based gene switch for trait regulation in maize was demonstrated by Unger *et al.* (2002). A mutation in maize (MS45), which results in male-sterile phenotype, could be reversed by complementation to gain fertility using methoxyfenozide-dependent chimeric receptor gene switch to express the wild-type MS45 protein in tapetum and anthers. These researchers used the EcR LBD from, *O. nubilalis* to generate a chimeric receptor. The chimeric receptor was introduced into MS45 maize with the MS45 gene fused to the GAL4 response element, which in the absence of methoxyfenozide were male sterile. However, application of methoxyfenozide to plants containing either a constitutive promoter or anther specific promoter resulted in the restoration of fertility to MS45 plants grown in either the greenhouse or the field.

It is interesting to note that in all the above studies, except those by Goff *et al.* (1996), reporter transactivation response to tebufenozide, methoxyfenozide, or muristerone A via the EcR gene switch was obtained without the requirement of an exogenous heterodimeric partner (USP or RXR), suggesting that there may be other factor(s) in plants that can substitute for USP as a partner for EcR, or that EcR can function as a homodimer. However, as far as is known, there is no evidence of EcR binding an ecdysteroid or nonsteroid ligand in the absence of USP or RXR. Irrespective, these studies provide ample demonstration of the utility of EcR-based gene switch, which can be regulated with an ecdysteroid or a nonsteroidal ecdysone agonist. The use of nonsteroidal ecdysone agonists, like any of the commercialized products, is attractive because

their reduced risk for the environment and mammals, birds, aquatic animals, and beneficial arthropods has been found to be acceptable. The specificity and the high affinity with which the bisacylhydrazines bind ecdysteroid receptors also offers the opportunity to utilize different ligands and ecdysone receptors to regulate more than one trait in a plant or an animal system.

16.2.10. Conclusions and Future Prospects of Ecdysone Agonists

The discovery of bisacylhydrazine insecticides with a new mode of action, very high degree of selective insect toxicity and reduced risk to the environment, has spurred a lot of interest in this mode of action and chemistry. The biochemical and molecular information on insect EcRs from different orders of insects, crystal structures of liganded HvEcR/HvUSP, and of DmUSP and HvUSP, and the discovery of new non-bisacylhydrazine chemistries, offers potential for the discovery of other new and novel chemistries with ecdysone mode of action and those that would be selectively toxic to nonlepidopteran and coleopteran pests. Although the existing bisacylhydrazine insecticides are most efficacious when ingested, the use of this type of control method could be extended to act against other types of pests if ways could be found for these insecticides to be effective via topical or systemic applications. These bisacylhydrazine insecticides, because of their new mode of action, insect selectivity, and reduced risk ecotoxicology and mammalian profiles, are ideally suited for use in integrated insect management programs.

An altogether different benefit from the discovery of nonsteroidal ecdysone agonists as insecticides has been in the area of gene switch applications in plants and animals. In this particular application, it may be possible to alter the molecular structure of ecdysone receptor LBDs to accommodate new chemistries as gene switch ligands. This is an exciting active area of research that, if successfully applied, offers a number of opportunities in trait and gene regulation in both plant and animal systems. The search for new and novel chemistries that target ecdysone receptors will continue to be a fruitful area of research for several years.

16.3. Juvenile Hormone Analogs

Kopec (1922) extirpated the brain of a caterpillar and demonstrated that this prevented pupation; he attributed this phenomenon to a humoral factor produced by the brain. This discovery heralded the study of hormonal regulation of metamorphosis in

insects. Wigglesworth (1936) later described the secretion of a hormone that prevents metamorphosis from a pair of glands, the corpora allata (CA) attached to the base of the brain; he called it the “status quo” or juvenile hormone (JH). A few years later, Fukuda (1994) described the prothoracic glands as the source of the molting hormone or ecdysone, which was later characterized as a steroid hormone (Butenandt and Karlson, 1954). At about the same time, the physiology of JH was elegantly worked out by Williams (1952). The chemical characterization of its structure, however, eluded researchers until 1967 when Röller and co-workers finally showed that it was a sesquiterpene, epoxy farnesoic acid methyl ester. At this time, Williams (1967) made the now famous statement, “third generation pesticides” in describing the use of JHs as environmentally safe control agents to which the insect will be unable to develop resistance, the first and second generation pesticides being the inorganic and the chlorinated hydrocarbons, respectively. The development of highly potent synthetic analogs of JH, which were several fold more active than the native hormone, gave credence to William’s claim (Henrick *et al.*, 1973).

This section describes the biological activity of JH and its analogs as well as their modes of action as understood today. Some of the major uses of JHAs for pest management are cataloged and finally prospects for future development are mentioned.

16.3.1. Juvenile Hormone Action

Juvenile hormone has a unique terpenoid structure and is the methyl ester of epoxy farnesoic acid (see also Chapter 8). This sesquiterpene exists in at least six different forms (Figure 15). JH III is the most common type and is present in most insects. Five different JHs, JH 0, I, II, III, and 4-methyl-JH I, have been described in lepidopterans. The bis-epoxide of JH, JH B₃, is found along with JH III in higher Diptera (Cusson and Palli, 2000). Various studies show that JH III is the main JH in most insects whereas JH I and II are the principal ones in Lepidoptera. Biosynthesis of JH proceeds through the mevalonate pathway with acetyl coenzyme A (acetyl CoA) serving as the building block. Propionyl CoA is used wherever ethyl side chains occur. Epoxidation and esterification are the terminal steps in the biosynthetic process (Schooley and Baker, 1985; Brindle *et al.*, 1987).

JH secretion by the corpus allatum is regulated by two neurohormones, the allatotropins that stimulate secretion and the allatostatins that inhibit production. Severing the nerve connections or extirpating the neurosecretory cells results in the loss of

control (Tobe and Stay, 1985; Tobe *et al.*, 1985; Lee *et al.*, 2002). JH is cleared from the hemolymph by JH esterase, which selectively cleaves the methyl ester inactivating the hormone (Wroblewski *et al.*,

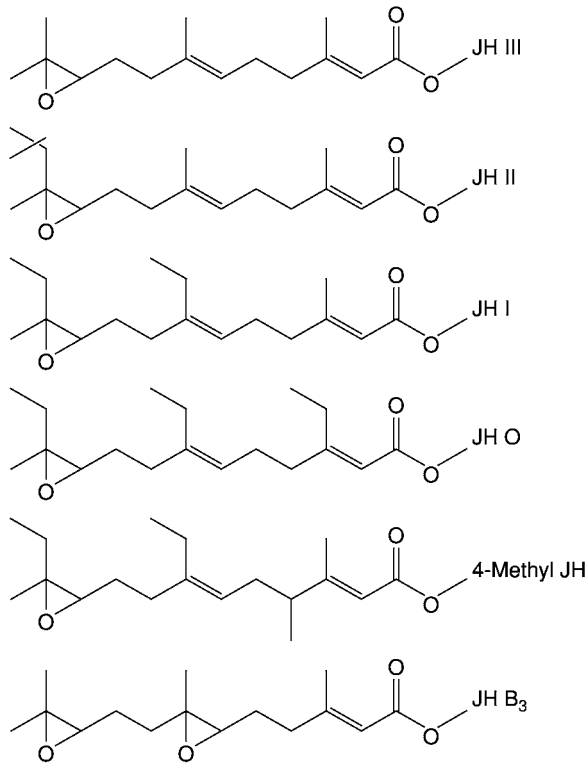


Figure 15 Chemical structure of naturally occurring JHs.

1990; Feng *et al.*, 1999). JH being highly lipophilic is made soluble by JH binding proteins (JHB) to facilitate transportation to the target sites as well as protect it from degradation from nonspecific esterases (Goodman, 1990). The receptor rich structure of the corpus allatum from the female cockroach, *Diploptera punctata*, is illustrated in **Figure 16** (Chiang *et al.*, 2002).

JH is perhaps one of the most pleiotropic hormones known and functions in various aspects of metamorphosis, reproduction, and behavior (Riddiford, 1994, 1996; Wyatt and Davey, 1996; Cusson and Palli, 2000; Palli and Retnakaran, 2000; Hiruma, 2003; Riddiford *et al.*, 2003).

The major function of JH is the maintenance of the larval status or the so-called juvenilizing effect. During the last larval instar in lepidoptera there is an absence of JH during the commitment peak of 20E, which results in the reprogramming of metamorphosis towards pupation (**Figure 17**). In the absence of JH, this ecdysone peak induces the expression of the broad complex gene (*BrC* or *Broad*), which is a transcription factor that initiates the larval–pupal transformation through several microRNAs (miRNAs) (Zhou and Riddiford, 2002; Sempere *et al.*, 2003). In adults, JH secretion resumes and is responsible for yolk protein (vitellogenin) synthesis and transport into the ovaries (Wyatt and Davey 1996). In addition, JH is also responsible for adult diapause where the ovaries do not develop due to the absence of JH and this effect

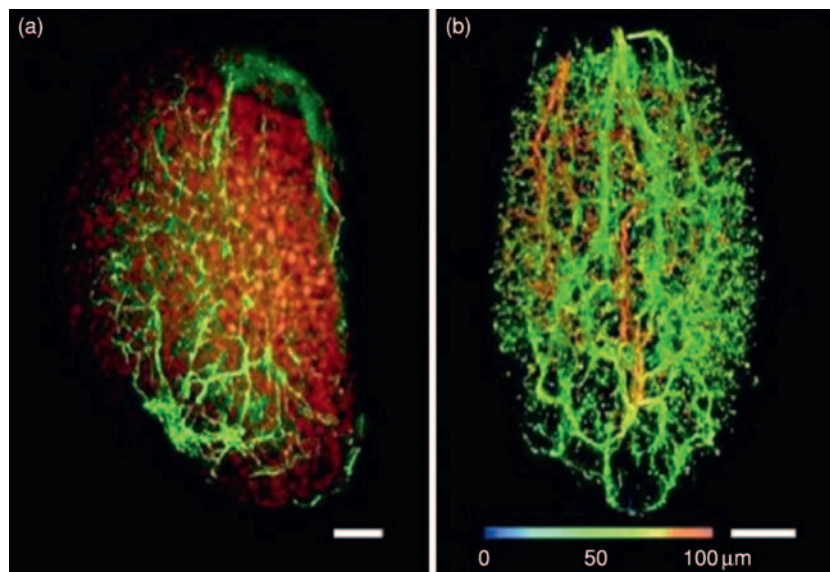


Figure 16 Corpus allatum of female cockroach, *Diploptera punctata*. (a) *N*-methyl-D-aspartate subtype of glutamate receptors (NMDAR) in the nerve fibers (green) involved in JH synthesis and nuclei (red) of parenchyma cells counterstained with propidium iodide. (b) Allatostatin-immunoreactive nerve fibers in the gland (depth code with different colors indicates distance of an object from the surface). Scale bar = 50 μm. (Reproduced with permission from Chiang, A.-S., *et al.*, 2002, Insect NMDA receptors mediate juvenile hormone biosynthesis. *Proc. Natl Acad. Sci. USA* 99, 37–42; © National Academy of Sciences, USA.)

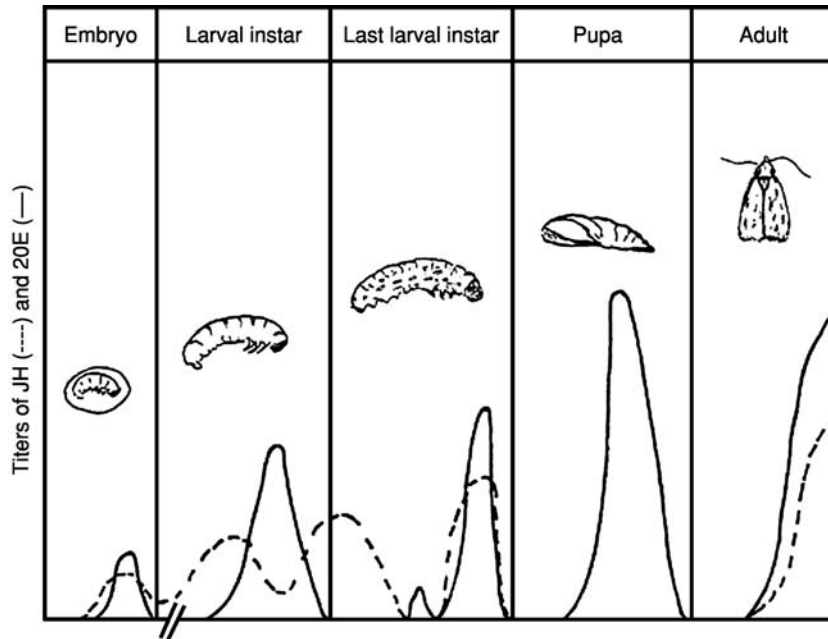


Figure 17 Juvenile hormone and 20-hydroxyecdysone (20E) titers in the various stages of a typical lepidopteran. MP, molt peak of 20E; CP, commitment peak of 20E; RD, reproduction peak of 20E.

can be reversed by applying JH. JH has also been shown to play a role in caste determination, pheromone production, polyphenism, migration, anti-freeze protein production, female sexual behavior, male accessory gland secretion, etc. (Wyatt and Davey, 1996).

16.3.2. Putative Molecular Mode of Action of JH

The mode of action at the molecular level has not been completely understood at present. Three types of action have been hypothesized and varying degrees of support for each of these modes have been reported. First is the direct action where it directly induces the secretion of a product (Iyengar and Kunkel, 1995; Feng *et al.*, 1999). Second is the indirect action where it modulates the action of 20E (Dubrovski *et al.*, 2000; Zhou and Riddiford, 2002). In these two instances, the action is at the genomic level where JH has to bind to nuclear receptors to evoke its action. The third involves the role of JH in the transport of vitellogenin into the oocytes through a membrane receptor (Sevala and Davey, 1989). A search for a nuclear as well as a membrane receptor by many investigators over several years has not yielded any concrete results. A nuclear protein isolated from *M. sexta* epidermis using a human retinoic acid receptor as a probe turned out to be an ecdysone-induced transcription factor, a homolog of *Drosophila* hormone receptor 3 (DHR3). A 29 kDa epidermal nuclear protein was found to be a low-affinity JHB (Palli *et al.*, 1994; Charles *et al.*, 1996).

A *D. melanogaster* mutant tolerant to the JHA, methoprene (Met) contained an 85 kDa protein that showed high affinity to JH III (Wilson and Fabian, 1986; Shemshedini *et al.*, 1990). The gene was cloned and was identified as a member of the basic helix-loop-helix (bHLH) PAS family of transcriptional regulators (Ashok *et al.*, 1998). Attempts to demonstrate the *Met* gene as the JH receptor have only added more confusion to the picture. The ultraspiracle (*USP*) gene, a homolog of the human retinoid-X receptor (RXR) and the heterodimeric partner of the EcR, was demonstrated to be involved in signal transduction of JH III in both yeast (Jones and Sharp, 1997) and Sf9 (Jones *et al.*, 2001; Xu *et al.*, 2002) cell based assays. In both cases, the cells were transformed with DNA sequences encoding DmUSP, in which JH III application resulted in transactivation. Isolated, recombinant DmUSP specifically bound JH III, and the authors report that *USP* homodimerizes and changes tertiary conformation, including the movement of the LBD α -helix 12 (Jones *et al.*, 2001; Xu *et al.*, 2002). Xu *et al.* (2002) also reported that ligand binding pocket point mutants of *USP* that did not bind JH III also acted as dominant negative suppressors of JH III activation of reporter promoter, and addition of wild-type *USP* rescued this activation. However, Hayward *et al.* (2003) were not able to demonstrate binding of tritiated JH III to *USP* from *L. migratoria*. In contrast to results obtained from Jones *et al.* (2001) and Xu *et al.* (2002), the crystal structure

of both HvUSP and DmUSP with a lipophilic ligand revealed the LBD α -helix 12 in an open, antagonist position (Billas *et al.*, 2001; Clayton *et al.*, 2001). While it is unclear whether USP is the true nuclear JH receptor, the evidence so far is at best equivocal.

Another approach to elucidating the molecular target(s) of JH action has been the use of JH responsive genes. Several have been identified, such as *jhp21* from *L. migratoria* (Zhang *et al.*, 1996). A response element in the promoter region of this gene binds to a protein, which is thought to be a transcription factor activated by JH (Zhou and Riddiford, 2002). Unfortunately, many of these JH responsive genes appear to be induced by JH at a slow rate, suggesting that they may not be the primary site of regulation. However, there are some genes, such as the JH esterase gene (*JHE*) from a *C. fumiferana* cell line, CF-203, that are induced by JH I within 1 h of administration (Feng *et al.*, 1999). This *Cf jhe* appears to be a primary JH responsive gene, which is induced by JH I and suppressed by 20E in a dose-dependent manner. Work in authors' laboratory has shown that a 30 bp minimal promoter region upstream of the *Cf jhe* transcription start site appears to bind to a nuclear protein isolated from

the CF-203 cells (Retnakaran *et al.*, unpublished data). Whether or not it needs to be a high-affinity, low-abundance protein to qualify as a putative JH nuclear receptor is currently under investigation. It has been suggested that JH could act through a membrane receptor as well, as shown by the work of Davey on vitellogenin transport into the oocytes (Wyatt and Davey, 1996). JH acts on the follicle cells of the ovary and makes them contract, causing large spaces to appear between the cells; this process has been termed "patency." This intercellular space formation (patency) permits the vitellogenin from the hemolymph to enter the ovary and gain access to the surface of the oocyte and subsequently enter these cells. This phenomenon was originally described in *R. prolixus* but was later shown in many other species including locusts (Wyatt and Davey, 1996). This membrane-bound receptor appears to be coupled to a G protein and the signal is transduced through a protein kinase system activating an ATPase that permits, among other things, the transport of vitellogenin into the cells (Figure 18).

And so the search goes on to unambiguously identify the nuclear and membrane receptors of JH. Characterizing such a receptor would open the

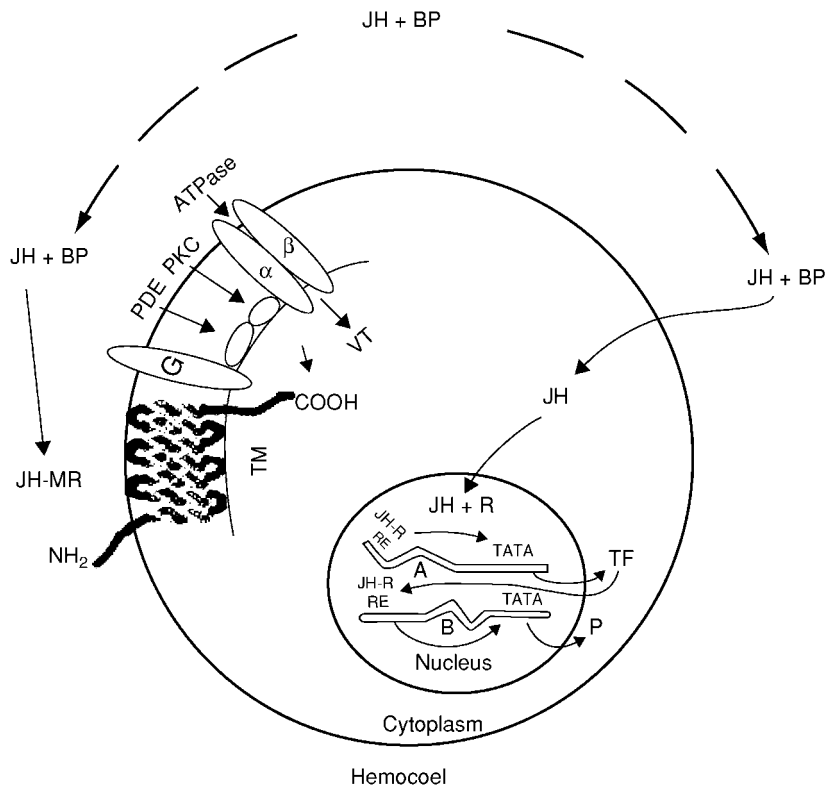


Figure 18 Putative dual receptor mechanism for JH action. A and B are two DNA transcripts; ATPase, $\text{Na}^+ \text{K}^+$ ATPase with α and β subunits; BP, JH binding protein; G, G protein; JH, juvenile hormone; JH-BP, JH bound to binding protein; JH-MR, JH bound to membrane receptor; PDE, phosphodiesterase; PKC, protein kinase C; RE, hormone response element; TATA, transcription initiation site; TF, transcription factor; TM, transmembrane region. (Reproduced with permission from Wyatt, G.R., Davey, K.G., 1996. Cellular and molecular actions of JH. II. Roles of JH in adult insects. *Adv. Insect Physiol.* 26, 1–155.)

door for a more rational approach to developing target specific control agents.

16.3.3. Pest Management with JHAs

The idea that insects would be unable to develop resistance against JH if it was used as a control agent was one of the driving principles behind the impetus to develop this hormone as an insecticide (Williams, 1967). The difficulty and cost of synthesizing such a complex molecule with a labile epoxide moiety and susceptibility to degradation delayed the realization of this concept. However, it soon became apparent that several synthetic analogs of JH, many of them several fold more active than the native hormone, could be used as control agents. The discovery that some synthetic synergists of insecticides have intense

JH activity (Bowers, 1968) was the harbinger for the development of various new analogs with diverse chemical structures. Consequently, the modes of action of these analogs may not necessarily be similar.

Over the years, numerous JHAs have been synthesized and their relative potencies, structure–activity relationships, and differential effects on various species have been studied (Slama *et al.*, 1974; Romanuk, 1981). Many naturally occurring JHAs, also called juvenoids, have been isolated from plants such as the “paper factor” from the balsam fir tree (*Abies balsamea*), and juvocimenes from the sweet basil plant (*Ocimum basilicum*) (Bowers and Nishida, 1980). During coevolution, the plants probably developed these JHAs to defend themselves against insects. Some representative examples of these compounds are shown in Figure 19.

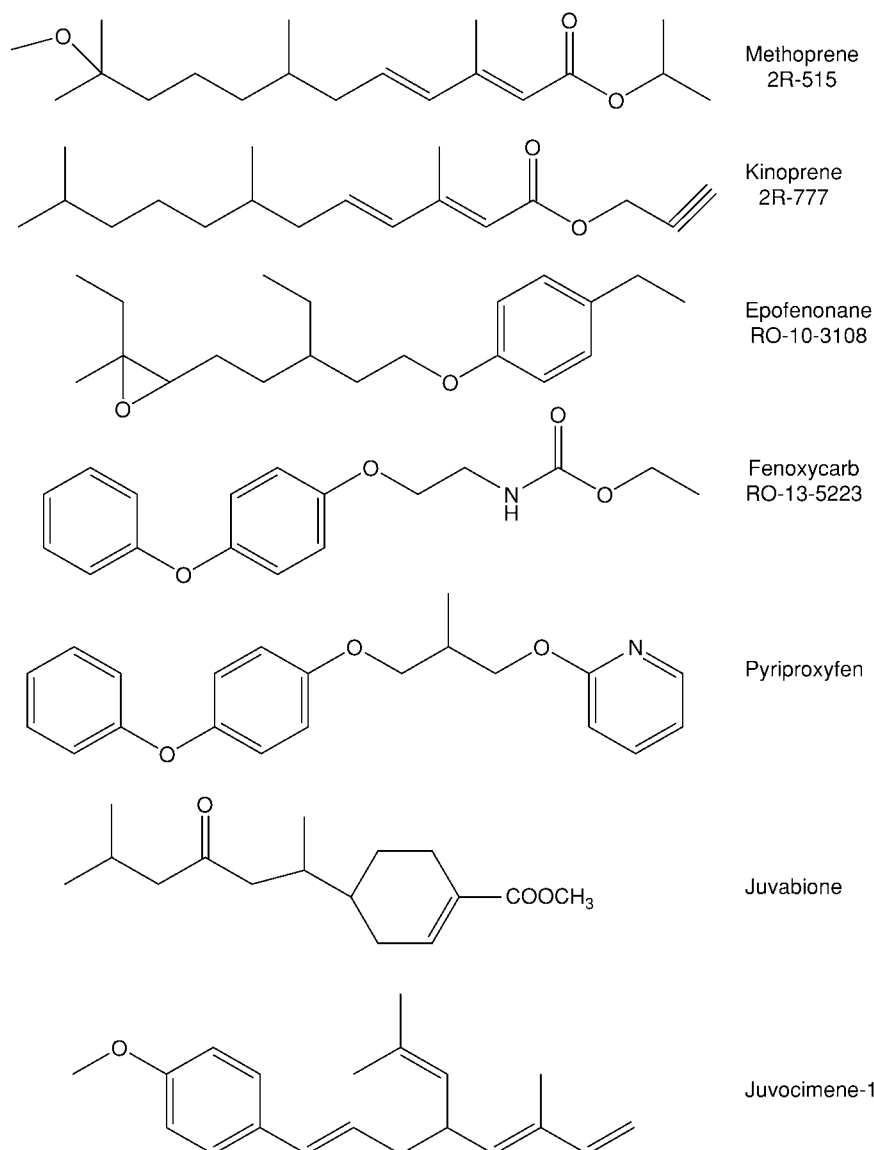


Figure 19 Some synthetic and naturally occurring JHAs.

JHAs can be broadly classified into two groups: the terpenoid JHAs such as methoprene and kinoprene and the phenoxy JHAs such as fenoxycarb and pyriproxyfen, with some such as epofenonane straddling the two (Figure 19).

16.3.3.1. Biological action of JHAs JHAs mimic the action of natural JH and, as such, can theoretically interfere with all the functions of JH. However, only a few such functions have been capitalized for control purposes (Retnakaran *et al.*, 1985). The organismal effects that have been exploited for control are relatively few, the most common being that seen on the last larval instar. Treatment of the larva in its last instar with JHA severely interferes with normal metamorphosis and results in various larval-pupal intermediates that do not survive (Figure 20). This effect, however, is not observed if the last instar larva is treated with the JHA methoprene during the first day or at the pharate stage (prepupal stage) of the stadium. If treated on day 1, the larva molts into a supernumerary larval instar and if it receives JHA at the pharate stage there is no effect (Retnakaran 1973a, 1973b; Retnakaran *et al.*, 1985). Morphogenetic control is ideally suited for controlling insects that are pests as adults, such as mosquitoes and biting flies.

JHAs block embryonic development at blastokinesis and act as ovicides (Riddiford and Williams, 1967; Retnakaran, 1970). In some instances, when older eggs are treated, there is a delayed effect that is

manifested at the last larval instar when it molts into a supernumerary instar (Riddiford, 1971). This type of ovicidal effect is useful especially in controlling fleas. In some cases where adults are treated, the material gets transferred to the eggs where they block embryonic development (Masner *et al.*, 1968). JHAs induce sterility in both sexes of adult tsetse flies (Langley *et al.*, 1990).

Adult or reproductive diapause is caused by the absence of JH secretion and this condition can be terminated by JHA treatment (De Wilde *et al.*, 1971; Retnakaran, 1974).

16.3.3.2. Properties and mode of action of selected JHAs

16.3.3.2.1. Methoprene (ALTOSID[®], APEXSE[®], DIANEX[®], PHARORID[®], PRECOR[®], VIODAT[®]) Methoprene is perhaps one of the best known terpenoid JHAs developed for pest control. A mutant strain of *D. melanogaster* that was tolerant to methoprene, the so-called *Met* flies, was generated by Wilson and Fabian (1986). These flies were also found to be tolerant to JH III, JH B₃, and several JHAs but not to many classes of insecticides. It was therefore tempting to speculate that this would be an elegant source to discover the JH receptor. An 85 kDa protein isolated from the fat body of wild flies was found to bind with high affinity to JH III. The same 85 kDa protein from *Met* flies showed a sixfold lower affinity for JH III (Shemshedini *et al.*, 1990). When the *Met* gene was cloned, it became

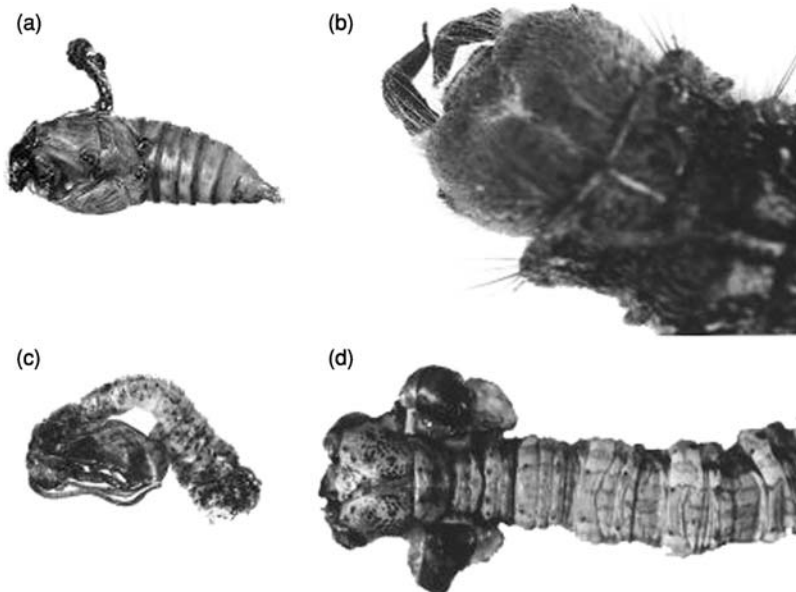


Figure 20 Morphogenetic effects of a JHA, methoprene, on the last larval instar of some lepidopteran insects. (a) A spruce budworm (*Choristoneura fumiferana*) pupa showing larval head and legs; (b) a forest tent caterpillar (*Malacosoma disstina*) showing aberrant mouthparts; (c) a pupa-like larva of the Eastern hemlock looper, *Lambdina fiscellaria*; (d) deformed Eastern hemlock looper larva with everted wing discs.

apparent that it was a bHLH-PAS and belonged to a family of transcriptional regulators, and this gene was not vital for the survival of the flies (Ashok *et al.*, 1998; Wilson and Ashok, 1998). This raises the possibility that JH activity could be exhibited by compounds that may interfere at any step during the synthesis, transportation, and target-site activity. Being extremely pleiotropic, the target-site activity could easily span a wide spectrum of functions. The methoprene-tolerant *Met* gene probably encodes a nonvital insecticide target protein of one type or another (Wilson and Ashok, 1998).

Methoprene is by far the most thoroughly studied JHA. Extensive EPA data collected over several years have shown that this JHA is relatively nontoxic to most nontarget organisms. Methoprene is rapidly broken down and excreted; its half-life in the soil is about 10 days. Mild toxicity to birds and some aquatic organisms has been observed. It has been used as a mosquito larvicide and for controlling many coleopterans, dipterans, homopterans, and siphonopterans (Harding, 1979).

16.3.3.2.2. Kinoprene (ENSTAR[®]) This is also an extremely mild JHA with little or no toxic effects. It breaks down in the environment and is relatively nonpersistent. It is relatively nonhazardous to bees and is nontoxic to birds, fish, and beneficial insects. It is decomposed by sunlight. It has been effectively used for controlling scales, mealy bugs, aphids, whiteflies, and fungal gnats. Its effects are morphological, ovicidal, and it acts as a sterilant (Harding, 1979).

16.3.3.2.3. Fenoxycarb (INSEGAR[®], LOGIC[®], TORUS[®], PICTYL[®], VARIKILL[®]) Fenoxycarb was the first phenoxy JHA found to be effective (Grenier and Grenier, 1993). It is unusual in having a carbamate moiety but does not show any carbamate-like toxicity. This JHA is considered slightly toxic to nontarget species, especially aquatic crustaceans, and carries the signal word “caution” on its label. It has been used as an effective control agent against fire ants (as bait), fleas, mosquitos, cockroaches, scale insects, and sucking insects. It is considered a general use insecticide. Unlike methoprene and kinoprene, fenoxycarb can be toxic to some of the beneficial insects such as neuropterans (Liu and Chen, 2001).

16.3.3.2.4. Pyriproxifen (KNACK[®], SUMILARV[®], ADMIRAL[®]) This JHA is a phenoxy analog similar to fenoxycarb. It is by far one of the most potent JHAs currently available. It is mildly toxic to some aquatic organisms but is nontoxic to bees. Its effects

are similar to the other JHAs and causes morphogenetic effects as well as sterility. It has been used effectively in the form of bait for controlling the red imported fire ant (*Solenopsis invicta*) in California. It has been used for controlling aphids, scales, whiteflies, and the Pear psylla, and the tsetse fly (*Glossina morsitans*) in Zimbabwe. In the latter, treatment of both sexes results in pyriproxifen being transferred to the female uterus where the embryo is killed (Langley *et al.*, 1990).

16.3.4. Use of JHAs for Pest Management

JHAs have been successfully used for controlling certain types of pests, especially where the pest is the adult stage. It has not been very effective against most lepidopteran agricultural pests because the larval stage is responsible for crop destruction. While some of the control measures tested since the previous edition of this series (Retnakaran *et al.*, 1985) are described below, **Table 9** summarizes the effects of the various JHA insecticides on agricultural and stored insect pests.

16.3.4.1. Control of public health and veterinary insects In most instances, the adult form is the active pest and, therefore, these species can be managed by JHA insecticides. Mosquitos, fleas, cockroaches, fire ants, and tsetse flies are some of the major pests that are vulnerable to JHAs (**Table 10**).

16.3.4.2. Anti-JH for pest management Compounds that prevent JH production, facilitate JH degradation, or destroy the corpus allatum all belong to this group. It is a catchall of various compounds that negate the activity of JH. Ideally, such compounds should be very effective pest control agents. Treatment of newly emerged larvae with such a compound would theoretically create miniature pupae, thus abbreviating the destructive part of the insect's life history. Many of the anti-JH compounds turned out to be highly toxic and did not pan out as good control agents. Once the JH receptor is characterized, it can be a target for control. Some of the effects that have been studied with anti-JH compounds are shown in **Table 11**.

16.3.5. Effects on Nontarget Invertebrates and Vertebrates

The effects of JHA insecticides have been studied and extensively reviewed (Grenier and Grenier, 1993; Miyamoto *et al.*, 1993; Palli *et al.*, 1995). The phenoxy JHAs, fenoxycarb, and pyriproxifen have been shown to be toxic to a number of dipteran, coleopteran, and hemipteran predators and parasitoids of scale insects (Mendel *et al.*, 1994).

Table 9 Control of agricultural and stored product insects with JHA insecticides

<i>Insect species</i>	<i>Host</i>	<i>Hormone analog</i>	<i>Activity</i>	<i>Reference</i>
<i>Nephotettix cincticeps</i> <i>N. nigropictus</i> <i>N. virescens Recilia dorsalis</i> (leafhoppers)	Rice	NC-170 4-chloro-5-(6-chloro-3-pyridylmethoxy)-2-(3,4-dichlorophenyl)-pyridazin-3(2H)-one	Good activity, 100 ppm suppressed population for more than 6 weeks	Miyake <i>et al.</i> (1991)
<i>Adoxophyes orana</i> <i>Pandemis heparana</i> <i>Archips podana</i> , <i>Archips rosana</i> (leafrollers)	Apple Orchards	Fenoxycarb and epofenonane	Adequate reduction, reinfestation never resulted in an increase to a harmful population level; good activity	de Reede <i>et al.</i> (1985)
<i>Lycoriella mali</i> (mushroom pest)	Compost	Methoprene	Resistance to this compound does not seem to be developing rapidly	Keil <i>et al.</i> (1988)
<i>Heliothis virescens</i> (tobacco budworm)	Cotton	Fenoxycarb	Good activity, opens new aspects for the control of lepidopterous pests with this compound	Masner <i>et al.</i> (1986)
<i>Rhagoletis pomonella</i> (apple maggot)	Apple	Pyriproxyfen	Good activity	Duan <i>et al.</i> (1995)
<i>Ceratitis capitata</i> (Mediterranean fruit fly)	Fruits in general	Methoprene	Good activity prevents adult eclosion	Saul <i>et al.</i> (1987)
<i>Adoxophyes orana</i> <i>Pandemis heparana</i> (leafrollers)	Apple orchards	Epofenonane and fenoxycarb	Good activity, the foliar residue remained active for at least 4 weeks and parasites seem to be less susceptible to it than the host itself	de Reede <i>et al.</i> (1984)
<i>Manduca sexta</i> (tobacco hornworm)	Tobacco plants	JHAs such as 1,3- and 1,4-bisNCS	Good activity, can disrupt insect growth and development	Ujváry <i>et al.</i> (1989)
<i>Ceroplastes floridensis</i> (Florida wax scale)	Guava plants	Fenoxycarb	Insects on treated plants deposited only a small amount of wax	Eisa <i>et al.</i> (1991)
<i>Cacopsylla pyricola</i> (Pear psylla)	Pear	Fenoxycarb	May still be a viable strategy for managing this	Krysan (1990)
<i>Melanoplus sanaguinipes</i> , <i>M. differentialis</i> (grasshopper)	Rangelands and crops	Fenoxycarb	Good activity; eliminates egg production and reduces oviposition	Capinera <i>et al.</i> (1991)
<i>Spodoptera littura</i> (tobacco cutworm)	Vegetable crops	Pyriproxyfen	Good activity, reduces the number of eggs	Hastakoshi (1992)
<i>Christoneura rosaceana</i> (leafroller)	Fruit trees	Fenoxycarb	Good activity, abnormal development is noted in all treatments	Aliniazee and Arshad (1998)
<i>Cydia pomonella</i> (codling moth)	Apple and pear	Phenoxyphenol pyridine and pyrazine carboxamides <i>N</i> -(4-phenoxyphenol) pyridinecarboxamides and <i>N</i> -(4-phenoxyphenyl) pyrazinecarboxamides	Good activity, prolongs development	Balcells <i>et al.</i> (2000)
<i>Laodelphax striatellus</i> (brown planthopper)	Rice	NC-170 4-chloro-5-(6-chloro-3-pyridylmethoxy)-2-(3,4-dichlorophenyl)-pyridazine-3(H)-one	Good activity, 100 ppm prevents larvae from entering diapause	Miyake <i>et al.</i> (1991)
<i>Dacus dorsalis</i> (oriental fruit fly)	Papayas	Methoprene	Good activity, higher doses showed no survival	Saul <i>et al.</i> (1987)
<i>Trichoplusia ni</i> (cabbage looper)	Cabbage	Methoprene	Good activity, amount of eggs is reduced	Campero and Haynes (1990)

Table 9 Continued

<i>Insect species</i>	<i>Host</i>	<i>Hormone analog</i>	<i>Activity</i>	<i>Reference</i>
<i>Bemisia tabaci</i> (sweet potato whitefly)	Cotton and vegetable crops	Pyriproxyfen	Good activity, strong translaminar effect and acts on all stages	Ishaaya and Horowitz (1992)
<i>Bemisia tabaci</i> (sweet potato whitefly)	Cotton and vegetable crops	insect growth regulators such as buprofezin and pyriproxyfen	activity not well seen	Palumbo <i>et al.</i> (2001)
<i>Trialeurodes vaporariorum</i> (greenhouse whitefly)	Greenhouse crops	Pyriproxyfen	Good activity, affects all stages	Ishaaya <i>et al.</i> (1994)
<i>Reticulitermes santonensis</i> <i>Reticulitermes flaviceps</i> <i>Coptotermes formosanus</i> (termites)	Wood	Carbamate derivative of 2-(4-hydroxybenzyl) cyclohexanone (W-328)	Good activity, 500 ppm concentration results in significant soldier differentiation	Hrdý <i>et al.</i> (2001)
<i>Reticulitermes speratus</i> (termites)		Ethyl(2-(<i>p</i> -phenoxyphenoxy)ethyl)carbamate	Good activity, results in the collapse of the whole termite colony	Tsunoda <i>et al.</i> (1986)
<i>Ips paraconfusus</i> Lanier (California beetle)	Logs of ponderosa pine	Fenoxycarb	Good activity, acts as an effective chemosterilant	Chen and Border (1989)
<i>Lipaphis erysimi</i> (mustard aphid)	Leafy vegetables	Pyriproxyfen	Good activity, causes direct mortality, reduces longevity and inhibits progeny formation	Liu and Chen (2000)
<i>Cacopsylla pyricola</i> (Pear psylla)	Pear	Fenoxycarb	Good activity	Lyoussoufi <i>et al.</i> (1994)
<i>Reticulitermes speratus</i> (Japanese subterranean termite)	Wood in general	Ethyl (2-(<i>p</i> -phenoxyphenoxy)ethyl)carbamate	Good activity, disturbance of caste differentiation results in collapse of the colony	Tsunoda <i>et al.</i> (1986)
<i>Rhyzopertha dominica</i> (lesser grain borer)	Wheat grain	2',7',-epoxy-3,7'-dimethylundec-2'-enyl-6-thyl-3-pyridyl ether	Good activity, 4 ppm resulted in insect mortality	Mkhize (1991)
<i>Tribolium castaneum</i> (red flour beetle), <i>Rhyzopertha dominica</i> (lesser grain borer), <i>Sitophilus oryzae</i> (rice weevil)—red flour beetle	Wheat flour	Methoprene and pyriproxyfen	Good activity, 20 ppm reduced the population by 80–99%	Kostyukovsky <i>et al.</i> (2000)

However, the ectoparasite of the California red scale and Florida wax scale, *Aphytis holoxanthus*, was insensitive to pyriproxifen (Peleg, 1988). Fenoxycarb was also found to be toxic to *Colpoclypeus florus*, a parasitoid of *Adoxophyes orana* and *Pandemis heparana*. Pupation in the predatory coccinellid, *Chilocorus bipustulatus* L., was inhibited when larvae fed on the scale insect, *Chrysomphalus aonidum*, were dipped into a 0.025% solution of fenoxycarb (cited in Grenier and Grenier, 1993). Additional effects of JHA insecticides have been summarized in Table 12.

The toxicological profiles of JHA insecticides for mammalian (rat), aquatic (rainbow trout and Daphnia), predatory, and parasitoid insects have been

summarized in Table 13. In general, JHA insecticides have low acute toxicity to fish, birds, and mammals. Both fenoxycarb and pyriproxifen have very low toxicity to adult bees. However, effects on brood development have been observed as a result of worker bees feeding pollen containing fenoxycarb residues to larvae (Wildboltz, 1988).

Last instars of certain aquatic insects are susceptible to JHA insecticides. Morphogenetic abnormalities have been observed in the heteropteran, *Notonecta unifaciata*, and in the dragon flies, *Anax junius* and *Pantala hymenaea*, with applications of fenoxycarb (Miura and Takahashi, 1987). Similarly, pyriproxifen also produced morphogenetic effects when applied to last instars of the

Table 10 Control of some insect pests of public health and veterinary importance with JHA insecticides

<i>Insect species</i>	<i>Host</i>	<i>Hormone analog</i>	<i>Activity</i>	<i>Reference</i>
<i>Monomorium pharaonis</i> (pharaoh ant)	Large office buildings, houses, apartment buildings, food establishments and hospitals	Pyriproxyfen	Good activity when used at concentrations of 0.25%, 0.5%, and 1%	Vail <i>et al.</i> (1995)
<i>Culex quinquefasciatus</i> <i>Aedes aegypti</i> (mosquitos)	Humans	VCRC/INS/A-23	Activity was good and the compound was found cost effective	Tyagi <i>et al.</i> (1985)
<i>Chironomus fusciceps</i> (midge)	Sulfur containing pods near residential areas	Pyriproxyfen	Good activity, at 0.00177 and 0.05369 ppm caused 50 to 90% emergence inhibition	Takagi <i>et al.</i> (1995)
<i>Glossian morsitans</i> , <i>G. pallidipes</i> (testse flies)	Cattle and humans	Pyriproxyfen	Good activity, it can replace pesticides	Hargrove and Langley (1993)
<i>Blattella germanica</i> (german cockroach)	Residences	Fenoxycarb and hydroprene	It can reduce the population	King and Bennett (1988)
<i>Anopheles balabacensis</i> (mosquitos)	Humans	Pyriproxyfen	Good activity, 0.005 ppb reduce the egg and sperm production and blood feeding and copulating activity	Iwanaga and Kanda (1988)
<i>Ctenocephalides felis</i> (cat flea)	Cat	Methoprene	Activity is 12.7 and 127 ng cm ⁻² , resulted in 15–5.2% adult emergence. Good control	Moser <i>et al.</i> (1992)
<i>Solenopsis invicta</i> (red imported fire ant)		Pyriproxyfen	Good activity, cause 80–85% reduction in colony size	Banks and Lofgren (1991)
<i>Blattella orientalis</i> (oriental cockroach)	Industrial, food manufacturing, and domestic premises	S-hydroprene	Good activity	Edwards and Short (1993)
<i>Ctenocephalides felis</i> (cat flea)	Cat	Methoprene	Good activity when cat fleas less than 24 h old were treated	Olsen (1985)
<i>Culex pipiens pallens</i> <i>Culex tritaeniorhynchus</i>	Humans	Pyriproxyfen	Activity remains unclear	Kamimura and Arakawa (1991)
<i>Ctenocephalides felis</i> and <i>Ctenocephalides canis</i> (dog and cat fleas)	Cat and dog	Methoprene and pyriproxifen	Good activity	Taylor (2001)
<i>Culex pipiens</i> (mosquitos)	Humans	4-alkoxyphenoxy- and 4-(alkylphenoxy) alkanaloxime o-ethers	Good activity	Hayashi <i>et al.</i> (1989)
<i>Culex pipiens</i>	Humans	(Phenoxyphenoxy) and (benzylphenoxy) propyl ethers	Good activity	Niwa <i>et al.</i> (1989)
<i>Alphitobius diaperinus</i> (mealworm)	Poultry production	Methoprene and fenoxycarb	Good activity	Edwards and Abraham (1985)
<i>Ctenocephalides felis</i> (cat fleas)	Cats	CGA-2545/728	Good activity	Rasa <i>et al.</i> (2000)
<i>Ctenocephalides felis</i> (cat fleas)	Cats	Methoprene, pyriproxifen and fenoxycarb	Good activity	Miller <i>et al.</i> (1999)

Table 11 Insect control with some anti-JH compounds

<i>Insect species</i>	<i>Host</i>	<i>Hormone analog</i>	<i>Activity</i>	<i>Reference</i>
<i>Manduca sexta</i> (tobacco hornworm)	Tobacco, tomato	ETB (ethyl 4-[2- <i>tert</i> -butyl carbonyloxy} butoxy]benzoate)	Some activity, 50 µg of ETB caused formation of larval-pupal intermediates after the fourth instar	Beckage and Riddiford (1983)
<i>Bombyx mori</i> (silkworm)	Mulberry	1-Citronellyl-5-phenylimidazole (KK-22)	Some activity, induction of precocious metamorphosis seems to correlate with prolongation of the larval developmental period in the third instar	Kuwano and Eto (1984)
<i>Blattella germanica</i> (German cockroach)	Household pest	Precocenes	Short-term activity seems to be effective and long-term activity shows that damage to CA is irreversible	Belles <i>et al.</i> (1985)
<i>Bombyx mori</i> (silkworm)	Mulberry	Ethoxy (KK-110) and 4-chlorophenyl (KK-135)	Activity can be inhibited by simultaneous application of methoprene	Kuwano <i>et al.</i> (1990)
<i>Nilaparvata lugens</i> (brown planthopper)	Rice	Precocene II	Activity with an increase in good ovarian growth	Ayoade <i>et al.</i> (1995)
<i>Spodoptera littoralis</i> (Egyptian cotton worm)	Cotton	Precocene I and II	Good activity but should be undertaken with care in order to minimize disruptive effects on parasitoids	Hagazi <i>et al.</i> (1998)
<i>Hyphantria cunea</i> (Fall webworm)	Hardwood trees	Fluoromevalonate (Fmev, ZR-3516)	Activity was shown as evoking varying degrees of ecdysial disturbance, which resulted in death of the insect	Farag and Varjas (1983)
<i>Oxycareus lavaterae</i> (Lygaeid)	European forests	Precocenes	Activity shows a high degree of uniformity	Belles and Baldellou (1983)
<i>Bombyx mori</i> (silkworm)	Mulberry	1-Benzyl-5-[(E)-2,6-dimethyl-1,5-heptadienyl]imidazole (KK-42)	Good activity, 100% precocious pupation	Kuwano <i>et al.</i> (1985)
<i>Locusta migratoria migratorioides</i> ; <i>Oncopeltus fasciatus</i> ; <i>Schistocerca gergaria</i> (locusts)	Various plants	Precocene II	Good activity, ovipositor protrusion, and death at higher concentrations	Degheele <i>et al.</i> (1986)
<i>Nilaparvata lugens</i> (Brown planthopper)	Rice	Precocene II (anti-allatin)	Good activity with high rate of mortality	Pradeep and Nair (1989)
<i>Spodoptera mauritia</i> (lawn army worm)	Agricultural crops	Fluoromevalonate (Fmev)	Good activity, induced various morphogenetic abnormalities, death before pupation occurred	Nair and Rajalekshmi (1989)
<i>Drosophila melanogaster</i> (fruit fly)	Various fruits	5-Ethoxy-6-(4-methoxyphenyl)methyl-1,3-benzodioxole J2581	Activity can be reversed by application of JH of methoprene	Song <i>et al.</i> (1990)
<i>Diploptera punctata</i> (cockroach)	Various types of diet	1,5-disubstituted imidaxoles	Good activity, powerful inhibitors of the last step of juvenile synthesis	Unnithan <i>et al.</i> (1995)
<i>Manduca sexta</i> (tobacco hornworm)	Tomato and tobacco	Farnesol dehydrogenase	Good activity, unique dehydrogenase that should be examined further	Sen and Garvin (1995)
<i>Oncopeltus fasciatus</i> (milkweed bug)	Milkwood	6,7-Dimethoxy-2,2-dimethyl 2 <i>H</i> -chromene	Activity shows deficiency of JH	Bowers and Unnithan (1995)

Continued

Table 11 Continued

<i>Insect species</i>	<i>Host</i>	<i>Hormone analog</i>	<i>Activity</i>	<i>Reference</i>
<i>Heliothis virescens</i> and <i>Trichoplusia ni</i> (cabbage looper)	Field crops and vegetables	Virus AcJHE-SG (transgenic virus with JH esterase gene)	Good activity, death occurred from contraction-paralysis and disruption of the normal sequence of events at the molt	Bonning <i>et al.</i> (1995)
<i>Sogatella furcifera</i> (whitebacked rice planthopper)	Rice	Precocene II	Activitiy showed inductio metathetely by itself occurred	Miyake and Mitsui (1995)
<i>Zaprius parvittiger</i> (banana fruitfly)	Bananas	Precocene	Good activity, with 0.076, 0.1 ppm in 1 μ L of acetone, adult longevity and fecundity were reduced	Rup Pushpinder and Baniwal (1985)
<i>Musca domestica</i> (housefly) <i>Diploptera</i> <i>punctata</i> (cockroach)	Various diets	2-(1-Imidazolyl)-2-methyl-1- phenyl-1-propanone and 2-methyl-1-phenyl-2- (1,2,4,-triazol-1-yl)-1- propanone	Activity was seen at 0.2 mM	Bélai <i>et al.</i> (1988)
<i>Aphis craccivora</i> (ground nut aphid)	Legumes	Precocene II	Good activity, with 2.0 μ g/ aphid, precocious metamorphosis was seen	Srivastava and Jaiswl (1989)
<i>Pieris brassicae</i> (cabbage butterfly) and <i>Leptimotarsa</i> <i>decemlineata</i> (Colorado potato beetle)	Vegetables and potato	2,2-Dimethylchromene derivatives	Good activity	Darvas <i>et al.</i> (1989)
<i>Heliothis zea</i> (corn earworm)	Corn	Precocene II	Good activity, growth and development were inhibited	Binder and Bowers (1991)

Table 12 Effects of JH analog insecticides on beneficial and aquatic species

<i>Parasite/predator/aquatic</i>	<i>Host/habitat</i>	<i>Hormone analog</i>	<i>Activity</i>	<i>Reference</i>
<i>Chilocorus nigritus</i>	Citrus red scale	Pyriproxyfen	Harmful to nontargeted species	Magaula and Samways (2000)
<i>Daphnia pulex</i>	Aquatic	Methoprene	Decrease in the incidence of all-male broods and an increase in the incidence of all-female broods	Peterson <i>et al.</i> (2000)
<i>Aphanteles congregatus</i>	Tobacco hornworm	Methoprene	Low dose would allow the insect to pupate normally	Beckage and Riddiford (1981)
<i>Copidosoma floridanum</i>	Cabbage looper	Methoprene	Slight activity, inhibited morphogenesis	Strand <i>et al.</i> (1991)
<i>Encarsia pergandiella</i> <i>E.transvena</i> , <i>E.formosa</i>	White fly	Pyriproxyfen	Relatively safe to parasitoids	Liu and Stansly (1997)
<i>Moina macrocopa</i>	Aquatic	Methoprene	Low activity on reproduction	Chu <i>et al.</i> (1997)
<i>Ceroplastes destructor</i>	Citrus orchards	Pyriproxyfen and fenoxycarb	Arrested the first and second instar	Wakgari and Giliomee (2001)
<i>Chrysoperla rufilabris</i>	Aphids	Fenoxycarb and pyriproxyfen	Ovicidal effect and delay in development	Liu and Chen (2001)
<i>Rhithropanopeus harrishii</i> (mud crab)	Aquatic	Methoprene	No activity was noticed	Celestial and McKenney (1993)

Table 13 Ecotoxicological profile of JHA insecticides (from various EPA reports)

Compound	Mammals (rat) LD_{50}	Fish (rainbow trout) 96h LC_{50}	Crustacea (Daphnia) 48h LC_{50}	Honey bees LC_{50}	Predators	Parasitoids
Methoprene ^a	>34 g kg ⁻¹	>3.3. mg l ⁻¹	>0.51 mg l ⁻¹	>1000 mg bee ⁻¹	Minimal effects	Minimal effects
Kinoprene ^b	>5 g kg ⁻¹	>20 mg l ⁻¹	>0.113 mg l ⁻¹	Nontoxic	Minimal effects	Minimal effects
Fenoxycarb ^c	>10 g kg ⁻¹	>1.6 mg l ⁻¹	>1.6 mg l ⁻¹	>1000 ppm	Low toxicity	Low toxicity
Pyriproxifen ^d	>5 g kg ⁻¹	>325 mg l ⁻¹	>400 mg l ⁻¹	>100 mg bee ⁻¹	Low toxicity	Low toxicity

^aALTOSID[®], APEXSE[®], DIANEX[®], PHARORID[®], VIODAT[®].

^bENSTAR[®].

^cINSEGAR[®], LOGIC[®], TORUS[®], PICTYL[®], VARIKILL[®].

^dKNACK[®], SUMILARV[®], ADMIRAL[®].

dragonfly, *Orthetrum albistrum* and the midge, *Chironomus yoshimatsui* (Miyamoto *et al.*, 1993).

16.3.6. Conclusions and Future Research of JHAs

JHAs have not proven to be the wonderful control agents they were purported to be. However, they have advantages for controlling pests of public health. Many of them are environmentally attractive. The anti-JH compounds have, for the most part, remained at the experimental stage. Interfering with JH action will become an attractive option, once the JH receptor is characterized.

16.4. Insecticides with Chitin Synthesis Inhibitory Activity

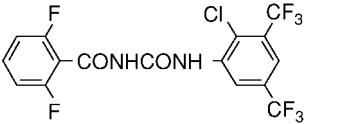
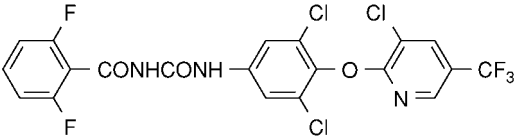
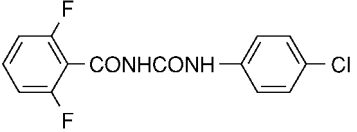
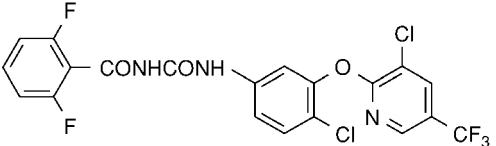
If the insect cuticle, which provides the exoskeletal structure, is disrupted during its formation, it is lethal to the insect. The cuticle needs to be waterproof for protection, soft and flexible to allow movement, extensible in between segments to allow for increase during feeding and growth, and also rigid to provide firm points of muscle attachment as well as for mandibles and claws. The cuticle is the product of a single layer of epidermal cells, and consists of different layers of which the procuticle contains chitin, a major component (30–60%) of insect cuticle. Chitin is a β -1,4-linked aminopolysaccharide homopolymer of *N*-acetylglucosamine (GlcNAc), and is by far one of the most abundant biological materials found on earth. This nitrogenous amino sugar polysaccharide is cross-linked to proteins via biphenyl linkages to form a protective matrix, which consists of a chitin microfibers–protein complex. Chitin biosynthesis, which is not fully understood, is a complex process consisting of a series of enzymatic steps beginning with a glucose unit, which is converted to GlcNAc that is linked with UTP, transported within the cell in combination with dolichol phosphate, and polymerized into

chitin. The newly polymerized chitin is covalently linked to proteins to form chitin microfibrils in the cuticle. Polymerization to form chitin is catalyzed by the enzyme chitin synthase (CHS or chitin-UDP-glucosamine-transferase), which occurs in several forms (CHS 1, 2 and 3; EC 2.4.1.16). This enzyme has probably undergone sequential gene duplication and divergence during evolution, resulting in its expression in different forms in diverse species, one of which is the human hyaluronan synthase. It has a conserved amino acid sequence essential for chitin biosynthesis in yeasts, and it has evolved into two types: the fungal form, which occurs as an inactive zymogen requiring proteolysis for activation, and the arthropod form, which is membrane bound. For more details on chitin biosynthesis and cuticle formation, the reader should refer to **Chapter 12**. Over the past three decades, the chitin biosynthetic pathway has proven to be important for developing insect control agents that selectively inhibit any of the chitin synthetic steps in insects. Two types of insect regulatory chitin synthesis inhibitors (CSI) have been developed and used as commercial compounds for controlling agricultural pests: the benzoylphenyl ureas (BPUs), and buprofezin and cyromazine (Spindler *et al.*, 1990; Retnakaran and Oberlander, 1993; Londerhausen, 1996; Palli and Retnakaran, 1999).

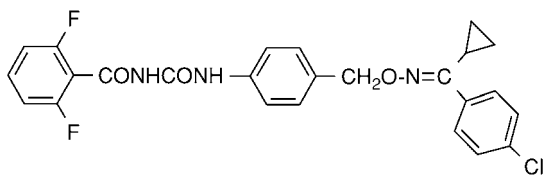
16.4.1. Brief Review of Old Chitin Synthesis Inhibitors

The insecticidal activity of the first BPUs was discovered around 1970 by scientists at Philips-Duphar BV, (now Crompton Corp., Weesp, The Netherlands) and the first commercial compound of this series was diflubenzuron, Dimilin[®] (Van Daalen *et al.*, 1972; Grosscurt, 1978) marketed by Uniroyal Chemical (**Table 14**). Diflubenzuron and older CSIs have already been extensively reviewed by Retnakaran *et al.* (1985), Grosscurt *et al.* (1987), and Retnakaran and Wright (1987).

Table 14 Chemical structures of the chitin synthesis inhibitors (CSI), 11 benzoylephenyl ureas (BPUs), and buprofezin and cyromazine, their biological effects and their target pest species

Compound	Chemical structure	Biological effects	Uses to control pest species
Bistrifluron		Inhibition of larval molting leading to death	Whiteflies in tomato (<i>Trialeurodes vaporariorum</i> and <i>Bemisia tabaci</i>), and lepidopterous insects in vegetables, cabbage, persimmon, apples, and other fruits (e.g., <i>Spodoptera exigua</i> , <i>Plutella xylostella</i> , <i>Stathmopoda masinissa</i> , and <i>Phyllonorycter ringoniella</i>) at 75–400 g ha ⁻¹
Chlorfluazuron		Acts as an antimolting agent, leading to death of the larvae and pupae	<i>Heliothis</i> , <i>Spodoptera</i> , <i>Bemisia tabaci</i> , and other chewing insects on cotton, and <i>Plutella</i> , <i>Thrips</i> and other chewing insects on vegetables. Also used on fruit, potatoes, ornamentals, and tea. Applied at 2.5 g hl ⁻¹
Diflubenzuron		Nonsystemic insect growth regulator with contact and stomach action. Acts at time of insect molting, or at hatching of eggs	Wide range of leaf-eating insects in forestry, woody ornamentals, and fruit. Controls certain major pests in cotton, soybeans, citrus, tea, vegetables, and mushrooms. Also controls larvae of flies, mosquitos, grasshoppers, and migratory locusts. Used as an ectoparasiticide on sheep for control of lice, fleas, and blowfly larvae. Is suitable for inclusion in integrated control programs. Effective at 25–75 g a.i./ha against most leaf-feeding insects in forestry; in concentrations of 0.01–0.015% a.i. against codling moth, leaf miners, and other leaf-eating insects in top fruit; in concentrations of 0.0075–0.0125% a.i. against citrus rust mite in citrus; and at a dosage of 50–150 g a.i./ha against a number of pests in cotton (cotton boll weevil, armyworms, leafworms), soya beans (soya bean looper complex) and maize (armyworms). Also for control of larvae of mushroom flies in mushroom casing (1 g a.i. m ⁻²); mosquito larvae (25–100 g a.i. ha ⁻¹); fly larvae in animal housings (0.5–1 g a.i. m ⁻² surface); and locusts and grasshoppers (60–67.5 g a.i. ha ⁻¹)
Fluazuron		Systemic acarine growth regulator with contact and stomach action inhibiting chitin formation	Ixodicide for strategic control of the cattle tick <i>Boophilus microplus</i> (including all known resistant strains) on beef cattle

Flucycloxuron

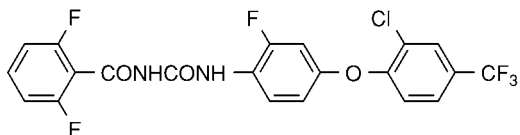


Nonsystemic acaricide and insecticide inhibiting the molting process in mites and insects. It is only active against eggs and larval stages. Adult mites and insects are not affected

Eggs and larval stages of Eriophyid and Tetranychid mite species on a variety of crops, including fruit crops, vegetables, and ornamentals. Also controls larvae of a number of insects. Because of its relative selectivity, it is well-suited for integrated control programs.

Recommended concentration for control of mites on fruit crops is 0.01–0.015% a.i. On insects, good activity has been found against codling moth, leaf miners, and some leaf rollers in pome fruit, at the same concentration. In ornamentals grown under glass/plastic, lower concentrations can be used. In grapes, the recommended dosage is 125–150 g a.i. ha⁻¹

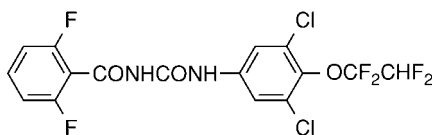
Flufenoxuron



Insect and acarid growth regulator with contact and stomach action. Treated larvae die at the next molt or during the ensuing instar. Treated adults lay nonviable eggs

Control of immature stages of many phytophagous mites (*Aculus*, *Brevipalpus*, *Panonychus*, *Phyllocoptura*, *Tetranychus* spp.) and insect pests on pome fruit, vines, citrus fruit, tea, cotton, maize, soybeans, vegetables, and ornamentals

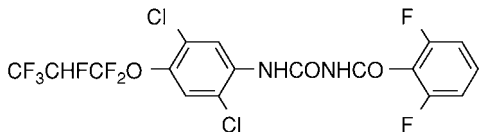
Hexaflumuron



Ingested, systemic insecticide

In agriculture for control of larvae of Lepidoptera, Coleoptera, Homoptera, and Diptera on top fruit, cotton, and potatoes. Major use now is in bait, for control of subterranean termites

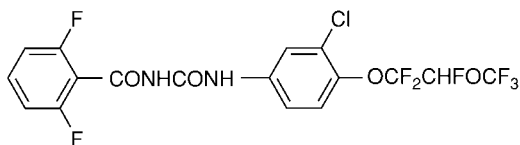
Lufenuron



Insecticide/acaricide acting mostly by ingestion; larvae are unable to molt, and also cease feeding

Lepidoptera and Coleoptera larvae on cotton, maize, and vegetables; and citrus whitefly and rust mites on citrus fruit, at 10–50 g ha⁻¹. Also for the prevention and control of flea infestations on pets

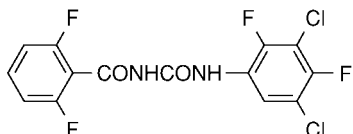
Novaluron



Insecticide acts by ingestion and contact, affecting molting. Causes abnormal endocuticular deposition and abortive molting

Under development by Makhteshim Chemical Works for control of Lepidoptera, whitefly, and agromyzid leaf miners in top fruit, vegetables, cotton, and maize

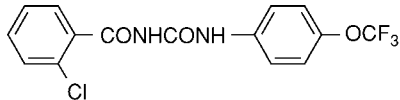
Teflubenzuron



Antimolting agent, leading to death of the larvae and pupae

Heliothis, *Spodoptera*, *Bemisia tabaci* and other chewing insects on cotton; and *Plutella*, *Thrips* and other chewing insects on vegetables. Also used on fruit, potatoes, ornamentals, and tea. Applied at 2.5 g hl⁻¹.

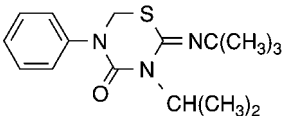
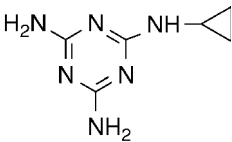
Triflumuron



Ingested insecticide, acting by inhibition of molting

Lepidoptera, Psyllidae, Diptera, and Coleoptera on fruit, soybeans, vegetables, forest trees, and cotton. Also used against larvae of flies, fleas, and cockroaches in public and animal health

Table 14 Continued

Compound	Chemical structure	Biological effects	Uses to control pest species
Buprofezin		Probable chitin synthesis and prostaglandin inhibitor. Hormone disturbing effect, leading to suppression of ecdysis. Persistent insecticide and acaricide with contact and stomach action; not translocated in the plant. Inhibits molting of nymphs and larvae, leading to death. Also suppresses oviposition by adults; treated insects lay sterile eggs	Insecticide with persistent larvicidal action against Homoptera, some Coleoptera, and also Acarina. Effective against Cicadellidae, Deltocephalinae (leafhoppers), and Delphacidae (planthoppers) in rice, at 50–250 g ha ⁻¹ ; Cicadellidae (lady beetle) in potatoes; Aleyrodidae (whitefly) in citrus, cotton, and vegetables, at 0.025–0.075 g ha ⁻¹ ; Coccidae, Diaspididae (scale insects) and Pseudococcidae (mealybugs) in citrus and top fruit, at 25–50 g hl ⁻¹ ; Tarsonemidae in vegetables, at 250–500 g ha ⁻¹ . Suitable for IPM programs
Cyromazine		Contact action, interfering with molting and pupation. When used on plants, action is systemic: applied to the leaves, it exhibits a strong translaminar effect; applied to the soil, it is taken up by the roots and translocated acropetally	Control of Diptera larvae in chicken manure by feeding to poultry or treating the breeding sites. Also used to control flies on animals. Used as a foliar spray to control leaf miners (<i>Liriomyza</i> spp.) in vegetables (e.g., celery, melons, tomatoes, lettuce), mushrooms, potatoes, and ornamentals, at 75–225 g ha ⁻¹ ; also used at 190–450 g ha ⁻¹ in drench or drip irrigation

Data compiled from Palli, S.R., Retnakaran, A., **1999**. Molecular and biochemical aspects of chitin synthesis inhibition. In: Jollès, P., Muzzarelli, R.A.A. (Eds.), Chitin and Chitinases. Birkhäuser-Verlag, pp. 85–98; Tomlin, C.D.S., Ed., **2000**. The Pesticide Manual, 12th edn. British Crop Protection Council Publications; and Ishaaya, I., **2001**. Biochemical processes related to insecticide actions: an overview. In: Ishaaya, I. (Ed.), Biochemical Sites of Insecticide Action and Resistance. Springer, pp. 1–16.

The discovery of diflubenzuron spawned the discovery and development of a whole array of new analogs by different agricultural companies (Table 14). These include: chlorfluazuron (AIM[®], ATARON[®], HELIX[®], JUPITER[®]; Ishihara Sangyo), flucycloxuron (ANDALIN[®]; Uniroyal Chemical), flufenoxuron (CASCADE[®]; BASF), hexaflumuron (CONSULT[®], RECRUIT[®], TRUENO[®]; Dow AgroSciences LLC), lufenuron (MATCH[®]; Syngenta AG), teflubenzuron (NOMOLT[®], DART[®], DIARACT[®]; BASF) and triflumuron (ALSYSTIN[®], BAYCIDAL[®]; Bayer AG) (Zoebelein *et al.*, 1980; Haga *et al.*, 1982, 1992; Becher *et al.*, 1983; Sbragia *et al.*, 1983; Retnakaran *et al.*, 1985; Anderson *et al.*, 1986; Retnakaran and Wright, 1987; Grosscurt *et al.*, 1987; Sheets *et al.*, 2000; Tomlin, 2000).

16.4.2. New Chemistries and Products

In the last decade, some newer BPU analogs were discovered: novaluron (RIMON[®]), bistrifluron, fluazuron (ACATAK[®]), and noviflumuron (RECRUIT[®] III) by Makhteshim Chemicals, Dongbu Hannong Chemical Co., Syngenta AG, and Dow AgroSciences LLC, respectively (Bull *et al.*, 1996; Ishaaya *et al.*, 1996; Kim *et al.*, 2000; Tomlin, 2000; Karr *et al.*, 2004) (Table 14).

Two other IGRs, buprofezin (APPLAUD[®]) and cyromazine (NEOPREX[®], TRIGARD[®], VETRAZIN[®]), with chemistries different from BPUs, but which also interfere with molting and chitin biosynthesis were developed by Nihon Nohyaku and Ciba-Geigy AG (now Syngenta AG), respectively (Table 14; Hall and Foehse 1980; Williams and Berry, 1980; Kanno *et al.*, 1981; Reynolds and Blakey, 1989; Tomlin, 2000).

16.4.3. Mode of Action and SAR

Typically, the chemistry and symptoms of the CSIs are unique, and not similar to other insecticides. Studies with diflubenzuron, the most thoroughly investigated compound of the BPUs, revealed that it alters cuticle composition, especially inhibition of chitin, resulting in abnormal endocuticular deposition that affects cuticular elasticity and firmness, and causes abortive molting. The reduced chitin levels in the cuticle seem to result from inhibition of biochemical processes leading to chitin formation. To date, it is not clear whether inhibition of chitin synthetase is the primary biochemical site for the reduced level of chitin, since in some studies BPUs do not inhibit chitin synthetase in cell-free systems (Retnakaran *et al.*, 1985; Grosscurt and Jongsma, 1987; Retnakaran and Wright, 1987; Londerhausen, 1996; Oberlander and Silhacek, 1998; Palli and Retnakaran, 1999). In addition to

chitin synthetase inhibition, other modes of action have been suggested for BPUs, such as: (1) inhibit the transport of UDP-GlcNAc across biomembranes; (2) block the binding of chitin to cuticular proteins resulting in inhibition of cuticle deposition and fibrillogenesis; (3) inhibit the formation of chitin due to an inhibition of the protease that activates chitin synthase, and activation of chitinases and phenoloxidases, which are both connected with chitin catabolism; (4) affect ecdysone metabolism, resulting in ecdysone accumulation that stimulates chitinase, which in turn digests nascent chitin; (5) block the conversion of glucose to fructose-6-phosphate; and (6) inhibit the DNA synthesis (Soltani *et al.*, 1984; Retnakaran *et al.*, 1985; Cohen, 1985; Retnakaran and Wright, 1987; Retnakaran and Oberlander, 1993; Mikólajczyk *et al.*, 1994; Zimowska *et al.*, 1994; Oberlander and Silhacek, 1998; Palli and Retnakaran, 1999; Oberlander and Smagghe, 2001). In addition, recent studies using imaginal discs and cell-free systems indicated that BPUs inhibit the 20-hydroxecdysone dependent GlcNAc incorporation into chitin, suggesting that BPUs affect ecdysone dependent biochemical sites, which lead to chitin inhibition (Mikólajczyk *et al.*, 1994; Zimowska *et al.*, 1994; Oberlander and Silhacek, 1998). BPUs act mainly by ingestion, and for the most part they are effective as larvicides, but in some species they also suppress oviposition. They act as ovicides, reducing the egg-laying rate or hindering the hatching process by inhibiting embryonic development. In most cases, the embryo was fully developed in the egg but the larva failed to hatch (Retnakaran and Wright, 1987; Ishaaya, 2001). BPUs were also found to reduce cyst and egg production in two free-living plant nematodes (Veech, 1978; Evans, 1985). In addition to being toxic by ingestion, some BPUs exhibit contact toxicity. Moreover, most BPUs, except hexaflumuron, have no systemic or translaminar activity (Retnakaran and Wright, 1987; Tomlin, 2000; Ishaaya, 2001).

The mode of action of buprofezin resembles that of the BPUs, although its structure is not analogous (Uchida *et al.*, 1985; De Cock and Degheele, 1998; Ishaaya, 2001). The compound strongly inhibits the incorporation of ³H-glucose and GlcNAc into chitin. As a result of chitin deficiency, the procuticle of treated larval stages loses its elasticity and the insect is unable to molt. In addition, buprofezin may work as a prostaglandin inhibitor that may lead to suppression of ecdysis and slightly reduced DNA synthesis. It also exerts its effect on egg-hatch and on the larval stages in the rice planthopper, *Nilaparvata lugens*, and the whiteflies, *Bemisia tabaci* and

Trialeurodes vaporariorum (De Cock *et al.*, 1995; De Cock and Degheele, 1998; Ishaaya, 2001). It has no ovicidal activity but suppresses embryogenesis through adults (Table 14).

Although the exact mode of action of the IGR cyromazine, which is predominantly active against dipteran larvae, is not known, evidence has been presented to suggest that its target site for interference with sclerotization is different from that of BPU (Biddington, 1985). In larvae intoxicated with cyromazine, the cuticle rapidly becomes less extensible and unable to expand compared with the cuticle of untreated larvae. The cuticle may be stiffer because of increased cross-linking between the various cuticle components, the nature of which remains unknown. As summarized in Table 14, cyromazine is an IGR with contact action interfering with molting and pupation. It has good systemic activity. When applied to the leaves, it exhibits a strong translaminar activity, and when applied to the soil it is taken up by the roots and translocated acropetally (Hall and Foehse, 1980; Awad and Mulla, 1984; Reynolds and Blakey, 1989; Viñuela and Budia, 1994; Tomlin, 2000).

With classical SAR (Hansch-Fujita), the effects of different substituents on the benzene rings in BPUs were analyzed for larvicidal activity against *Chilo suppressalis*, *S. littoralis*, and *B. mori* (Nakagawa *et al.*, 1987, 1989a, 1989b). For the benzoyl moiety, the toxicity was higher with a higher total hydrophobicity, a higher electron withdrawal from the side chain, and a lower steric bulkiness of *ortho*-substituents. In addition, introduction of electron-withdrawing and hydrophobic substituents at the *para*-position of the phenyl (aniline) moiety enhanced the larvicidal activity, whereas bulkier groups were unfavorable. Stacking did not occur between the two aromatic moieties along the urea moiety (Sotomatsu *et al.*, 1987). To ascertain the above results, the relative activity of BPUs was determined by measuring the incorporation of [¹⁴C]-GlcNAc in larval integuments cultured *in vitro* (Nakagawa *et al.*, 1989b). There was a colinear relationship between *in vitro* activities and *in vivo* larvicidal toxicities if the hydrophobic factor(s) participating in transport were considered separately. In addition, integuments of the three Lepidoptera were incubated in conditions with and without the synergists piperonylbutoxide (PB) and *S,S,S*-tributylphosphorotrithioate (DEF). In the Qualitative Structure–activity Relation (QSAR) equation measured without synergist, an electron-withdrawing effect was favorable to the activity, but an electron-donating group was favorable to the activity in the presence of PB. These results mean

that electron-withdrawing groups are playing a role in suppressing the oxidative metabolism. DEF had no significant effect, suggesting that hydrolytic degradation of the phenyl moiety was not of significant consequence as compared to its oxidative degradation. In summary, the SAR results suggested that for BPUs the specific larvicidal spectrum is due to inherent differences in metabolism in addition to differences in the physicochemical substituent effects (Nakagawa *et al.*, 1987, 1989a, 1989b; Sotomatsu *et al.*, 1987).

16.4.4. Spectrum of Activity

Most CSI compounds are very potent against a variety of different pests with the highest activity towards lepidopterous insects and whiteflies. The main commercial applications are in field crops/agriculture, forestry, horticulture, and in the home as summarized in Table 14 (Retnakaran and Wright, 1987; Tomlin, 2000; Ishaaya, 2001). Novaluron acts by ingestion and contact against lepidopterans (*S. littoralis*, *S. exigua*, *S. frugiperda*, *Tuta absoluta* and *Helicoverpa armigera*), whitefly, *B. tabaci*, eggs and larvae, and different stages of the leafminer, *Liriomyza huidobrensis*. Bistrifluron is active against various lepidopteran pests and whiteflies on apple, Brassica leafy vegetables, tomato, persimmon, and other fruits (Kim *et al.*, 2000). Hexaflumuron and the newer noviflumuron are now used in bait for control of subterranean termites (Sheets *et al.*, 2000; Karr *et al.*, 2004). As a specialty in the series of CSIs, fluazuron is the only ixodicide with a strong activity against cattle ticks (Kim *et al.*, 2000) (Table 14).

Buprofezin has a persistent larvicidal action against sucking Homoptera, such as the greenhouse whitefly, *T. vaporariorum*, the sweet potato whitefly, *B. tabaci*, both of which are important pests of cotton and vegetables, the brown planthopper, *N. lugens* in rice, the citrus scale insects, *Aonidiella aurantii* and *Sassetia oleae*, and some Coleoptera and Acarina. In contrast, cyromazine is used for control of dipteran larvae in chicken manure. It is also used as a foliar spray to control leafminers (*Liriomyza* sp.) in vegetables and ornamentals, and to control flies on animals (Hall and Foehse, 1980; Williams *et al.*, 1980; Kanno *et al.*, 1981; Reynolds and Blakey, 1989; Tomlin, 2000) (Table 14).

16.4.5. Ecotoxicology and Mammalian Safety

Overall, CSIs have selective insect toxicities and as such are considered “soft insecticides.” For diflubenzuron, the harbinger of all BPUs, its environmental fate has been extensively investigated and was

Table 15 Environmental effects of some CSIs

Compound	Mammals	Fish	Crustacea	Bees	Predators	Parasitoids
Bistrifluron	Rat LD ₅₀ > 5 g kg ⁻¹	Carp LC ₅₀ (48 h) > 0.5 mg l ⁻¹		LD ₅₀ > 100 µg bee ⁻¹		
Chlorfluazuron	Rat LD ₅₀ > 8.5 g kg ⁻¹	Carp LC ₅₀ (48 h) > 300 mg l ⁻¹	<i>Daphnia</i> LC ₅₀ (48 h) 0.9 µg l ⁻¹	LD ₅₀ > 100 µg bee ⁻¹	Highly toxic	No – very little effects
Diflubenzuron	Rat LD ₅₀ > 4.6 g kg ⁻¹	Zebra fish, rainbow trout LC ₅₀ (96 h) > 0.2 mg l ⁻¹	<i>Daphnia</i> LC ₅₀ (48 h) 7.1 µg l ⁻¹	Not hazardous LD ₅₀ > 100 µg bee ⁻¹	Safe – little effects	Safe-little adverse effects
Flucyclohexuron	Rat LD ₅₀ > 5 g kg ⁻¹	Rainbow trout, sunfish LC ₅₀ (96 h) > 0.1 mg l ⁻¹	<i>Daphnia</i> LC ₅₀ (48 h) 4.4 µg l ⁻¹	LD ₅₀ > 100 µg bee ⁻¹	No – moderate effects	No – harmful effects
Flufenoxuron	Rat LD ₅₀ > 3 g kg ⁻¹	Rainbow trout LC ₅₀ (96 h) > 100 mg l ⁻¹			No adverse effects – harmful	No – little adverse effects
Hexaflumuron	Rat LD ₅₀ > 5 g kg ⁻¹	rainbow trout: not lethal, <i>Tilapia</i> LC ₅₀ > 3 µg l ⁻¹	<i>Daphnia</i> LC ₅₀ (96 h) 0.1 µg l ⁻¹	LD ₅₀ > 100 µg bee ⁻¹	Very little effects	Harmless, but ectoparasites strongly affected
Lufenuron	Rat LD ₅₀ > 2 g kg ⁻¹	Carp, rainbow trout, sunfish LC ₅₀ (96 h) > 30–70 mg l ⁻¹		LC ₅₀ > 38 µg/bee ⁻¹ LD ₅₀ > 8 µg/bee ⁻¹	Safe – harmful	Moderately harmful
Novaluron	Rat LD ₅₀ > 5 g kg ⁻¹	Rainbow trout, sunfish LC ₅₀ (96 h) > 1 mg l ⁻¹	<i>Daphnia</i> LC ₅₀ (48 h) 58 µg l ⁻¹	Nontoxic at recommended rates; LC ₅₀ > 100 µg bee ⁻¹ LD ₅₀ > 1 mg bee ⁻¹	Low toxicity – harmful	Little–harmful effects
Teflubenzuron	Rat LD ₅₀ > 5 g kg ⁻¹	Trout, carp LC ₅₀ (96 h) > 0.5 mg l ⁻¹		LD ₅₀ > 1 mg bee ⁻¹	Harmless – highly toxic	No – very little effects
Triflumuron	Rat LD ₅₀ > 5 g kg ⁻¹	Rainbow trout LC ₅₀ (96 h) > 320 mg l ⁻¹	<i>Daphnia</i> LC ₅₀ (48 h) 225 µg l ⁻¹	Toxic	Harmless – moderately toxic	Moderately harmful
Buprofezin	Rat LD ₅₀ 2.4 g kg ⁻¹	Carp, rainbow trout LC ₅₀ (48 h) > 2.7–1.4 mg l ⁻¹	<i>Daphnia</i> LC ₅₀ (3 h) 50.6 mg l ⁻¹	No effect at 2 g l ⁻¹	Harmless – moderately toxic	Very little effects
Cyromazine	Rat LD ₅₀ 3.4 mg kg ⁻¹	Carp, rainbow trout, sunfish LC ₅₀ (96 h) > 90 mg l ⁻¹	<i>Daphnia</i> LC ₅₀ (48 h) > 9.1 mg l ⁻¹	No effect up to 5 µg	Harmless – moderately toxic	Harmless – moderate effects

Data compiled from Darvas, B., Polgár, L.A., **1998**. Novel-type insecticides: specificity and effects on non-target organisms. In: Ishaaya, I., Degheele, D. (Eds.), *Insecticides with Novel Modes of Action: Mechanism and Application*. Springer, pp. 188–259; Tomlin, C.D.S. (Ed.), **2000**. *The Pesticide Manual*, 12th edn. British Crop Protection Council Publications; and Ishaaya, I., **2001**. Biochemical processes related to insecticide actions: an overview. In: Ishaaya, I. (Ed.), *Biochemical Sites of Insecticide Action and Resistance*. Springer, pp. 1–16.

shown to be broken down by various microbial agents without accumulation in soil and water. The fear that spray run-off into streams may cause widespread mortality of nontarget species has not been realized. Moreover, its chitin synthesis inhibitory action is quite specific, and related biochemical processes, such as chitin synthesis in fungi and biosynthesis of hyaluronic acid and other mucopolysaccharides in chickens, mice, and rats, are not affected. A representative list of the salient environmental effects of CSIs against mammals (rat), vertebrates (fish), crustaceans, bees, predators, and parasitoids is given in **Table 15**. However, owing to selective toxicity towards arthropods, BPUs have, varying degrees of effects on crustaceans as well as beneficial insects, which requires their use with care. In this case, the IOBC/WPRS (International Organization for Biological and Integrated control of Noxious Animals and Plants, West Palaearctic Regional Section, 2004) sequential testing scheme has proven its value for testing the side effects on a species-by-species basis and under (semi-)field conditions (Hassan, 1992). However, since most of the BPU formulations need to be ingested to be effective, topical effects on parasites, predators, and pollinators are minimal. Retnakaran and Wright (1987), Perez-Farinos *et al.* (1998), and Medina *et al.* (2002) claimed that the selectivity of BPUs may result from a relatively low cuticular absorption in residual and direct contact assays. As summarized in **Table 15**, the majority of the CSIs are harmless or exert little adverse effect on bees, predators, or parasitoids, which renders these BPUs acceptable for inclusion in integrated pest management (IPM) programs (Elzen, 1989; Hassan *et al.*, 1991, 1994; Vogt, 1994; Van de Veire *et al.*, 1996; Darvas and Polgar, 1998; Sterk *et al.*, 1999; Tomlin, 2000).

16.4.6. Resistance, Mechanism for Resistance and Resistance Potential

Pimprikar and Georghiou (1979) were the first to report very high levels of resistance (>1,000-fold) to diflubenzuron in a housefly population stressed with the compound. Since then, several research groups in different parts of the world have reported resistance to CSIs in Lepidoptera, Diptera, and whiteflies.

In Southeast Asia, selection of diamondback moth, *Plutella xylostella*, from Thailand during six generations with chlorfluazuron and teflubenzuron resulted in resistance levels of 109-fold and 315-fold, respectively (Ismail and Wright, 1991, 1992). While marked cross-resistance between chlorfluazuron and teflubenzuron was demonstrated, there was no evidence of cross-resistance to diflubenzuron, and little or no cross-resistance to flufenoxuron and

hexaflumuron. Pretreatment with the synergists PB and DEF increased the toxicity of chlorfluazuron and teflubenzuron up to 34-fold and 28-fold, respectively, suggesting that microsomal monooxygenases and esterases are involved in resistance. In another study with *P. xylostella* from Malaysia, selection with diflubenzuron increased resistance to teflubenzuron, but had no effect on chlorfluazuron (Furlong and Wright, 1994). In this study, while use of PB and DEF suggested the involvement of microsomal monooxygenases and esterases in teflubenzuron resistance, glutathion-S-transferase (GSTs) had limited involvement.

As documented by Moffit *et al.* (1988) and Sauphanor *et al.* (1998), the codling moth *C. pomonella* shows very high levels of resistance to diflubenzuron in the USA and France. In southern France, failure in *C. pomonella* control was observed for several years, revealing a 370-fold resistance for diflubenzuron and cross-resistance with two other BPUs, teflubenzuron (7-fold) and triflumuron (102-fold), as well as to the ecdysone agonist, tebufenozide (26-fold) (Sauphanor and Bouvier, 1995). Interestingly, resistance to diflubenzuron was linked to cross-resistance to deltamethrin. In both cases, enhanced mixed-function oxidase and GST activities are involved in resistance, rather than target site modification. In addition, a fitness cost described in both resistant strains was mainly associated with metabolic resistance (Boivin *et al.*, 2001). Finally, a lack of relationship between ovidical and larvicidal resistance for diflubenzuron in *C. pomonella* may be due to different transport properties as well as differential enzymatic metabolism (Sauphanor *et al.*, 1998).

In some strains of *S. littoralis*, resistance to diflubenzuron was also increased by a factor of about 300 (El-Guindy *et al.*, 1983). El Saïdy *et al.* (1989) reported that diflubenzuron and teflubenzuron were hydrolyzed rapidly by all tissues tested, and the gut wall was the most active tissue reaching 61% hydrolytic breakdown for diflubenzuron and 16% for teflubenzuron. Interestingly, profenofos and DEF could inhibit degradation of both BPUs tested under optimal conditions. Ishaaya and Casida (1980) had earlier reported that these organophosphorous compounds can inhibit insect esterases in larval gut integument. The strong synergism activity of DEF and profenofos indicated that the major route of detoxification in *S. littoralis* was through hydrolysis, while oxidative metabolism was found to be of minor importance for resistance.

In the Australian sheep blowfly, *Lucilia cuprina*, resistance to diflubenzuron was inherited in a codominant (S male × R female) or incompletely

recessive (R male \times S female) manner, and there was also some maternal influence on inheritance of monooxygenase activities, suggesting that diflubenzuron resistance is polygenic and involves mechanisms additional to monooxygenases (Kotze and Sales, 2001).

McKenzie and Batterman (1998) reported the development of resistance to cyromazine in populations of *L. cuprina*, which, however, had remained susceptible for almost 20 years of exposure. This resistance in *L. cuprina* was controlled by a single gene in each variant and two resistance loci were identified: one (Cyr4) closely linked to the marker "reduced eyes" on chromosome IV, and the other, Cyr5, closely linked to the "stubby bristles" marker on chromosome V (Yen *et al.*, 1996). Similarly, in *D. melanogaster*, cyromazine resistance was due to a mutation in a single, but different gene than the one identified in resistant *L. cuprina*. The resistance genes, designated *Rst(2)Cyr* and *Rst(3)Cyr*, were localized to puff positions 64 on chromosome II and 47 on chromosome III, respectively (Adcock *et al.*, 1993).

With respect to resistance in *Musca domestica*, Keiding (1999) reviewed the period 1977 to 1994. In this period, only two IGRs, diflubenzuron and cyromazine, were widely used for fly control, either by direct contact application to breeding sites, manure, garbage, etc., or as an admixture in feed for poultry or pigs. Widespread use of diflubenzuron or cyromazine by direct treatment of manure has not led to resistance of practical importance except for resistance to diflubenzuron on a few farms in the Netherlands. However, there are reports showing that the use of either compound mixed with feed has resulted in the development of moderate to high resistance to these compounds. High resistance in adult flies to organophosphates and pyrethroids does not usually confer cross-resistance to the larvicidal effects of diflubenzuron or cyromazine, but cross-resistance between organophosphorous compounds and JHAs (methoprene) has been reported. In Denmark, Keiding *et al.* (1992) came to the same conclusions where housefly control with both compounds was carried out on farms for 1–9 years. On none of the farms was any general increase of tolerance to diflubenzuron found. In the same study, two strains were selected with diflubenzuron and cyromazine where moderate to high resistance developed if the selection pressure was strong, especially when used as feed-through applications on poultry farms where all feed contains diflubenzuron or cyromazine. If the treatment of fly breeding sources is less complete, resistance problems may not develop.

More recently, Kristensen and Jespersen (2003) observed resistance in houseflies to diflubenzuron for the first time in Denmark, and some field populations with resistance to cyromazine. A fivefold cyromazine resistant strain was established and this was 3-, 5-, and 90-fold resistant to diflubenzuron, triflumuron, and methoprene, respectively. In reviewing the mechanism of resistance in houseflies to diflubenzuron and cyromazine, Ishaaya (1993) indicated that resistance was due to increased levels of detoxifying enzymes, decreased penetration, and enhanced excretion of the compounds. Cross-resistance between cyromazine and diflubenzuron was observed but the mechanism seems more target site related than metabolic.

For the control of whiteflies, novel compounds like buprofezin were introduced at the beginning of the 1990s (Horowitz *et al.*, 1994; De Cock and Degheele, 1998). In Israel, a survey in cotton fields over 4 years (1989–1992) with two or three applications of buprofezin per season indicated a fivefold increase in tolerance in the *B. tabaci*, and the resistance ratio for resistance to pyriproxifen was >500-fold (Horowitz and Ishaaya, 1994). Based on the success of the program in Israel, the usefulness of buprofezin was once again demonstrated in a resistance management program in Arizona to control resistance to pyriproxifen in populations of the whitefly, *Bermisia argentifolii* in cotton (Dennehy and Williams, 1997). In another study in southern Spain where highly multiresistant strains of *B. tabaci* showed lower efficacy to buprofezin, pyriproxifen resistance was not obvious (Elbert and Nauen, 2000). For the greenhouse whitefly, *T. vaporariorum*, which is a major pest problem in protected crops in Western Europe, a >300-fold resistance was scored in a strain collected in northern Belgium (De Cock *et al.*, 1995). No cross-resistance was recorded for pyriproxifen and diafenthiuron, indicating their potential in a resistance management program. In UK greenhouses, Gorman *et al.* (2002) also found very strong resistance to buprofezin in *T. vaporariorum*, and these strains were cross-resistant to teflubenzuron.

The chitin synthesis inhibition site has proven to be important for developing control agents that act against important groups of insect pests and many of these CSIs are environmentally safe. However, details of the exact mode of action of CSIs have not been elucidated so far. Although there were already some incidences of resistance to CSIs in the last two decades in different pest insects, these IGR compounds remain suitable for use as rotation partners in integrated and resistance

management programs based on their new and selective mode of action in contrast to broad-spectrum neurotoxins.

At present, it is believed that chitin synthesis has by no means been fully exploited as an attractive target and that opportunities exist to further discover new CSIs. A better understanding of the biosynthetic pathway for chitin synthesis and cuticle deposition, as well as precise mode of action of CSIs would allow for development of better and more efficient high throughput assays for discovery of new and novel CSIs.

16.5. Conclusions and Future Prospects of Insect Growth- and Development-Disrupting Insecticides

The three classes of insecticides reviewed in this chapter are much slower acting than those acting on neural target sites. The end user has, of course, been used to seeing insects die within a very short time following application of neuroactive insecticides. The discovery and availability of insecticides that inhibit growth and development of insects brought a paradigm shift from the faster acting neurotoxic insecticides. This change has necessitated educating the distributors and the users on the mode of action of these new insecticides. The bisacylhydrazine insecticides are generally faster acting than the JHA and CSI insecticides. Moreover, an attractive feature of the bisacylhydrazine insecticides is their ability to prevent crop damage by inhibition of feeding within 3–12 h after application.

Of the three classes of insecticides that disrupt growth and development in insects, the mode of action of nonsteroidal ecdysone agonist bisacylhydrazine insecticides is the best understood at the molecular level. This detailed understanding has been possible, both with the cloning and expression of cDNAs encoding EcR and USPs from several insects, and the availability of stable and easy to synthesize bisacylhydrazines. Unlike the JHAs and CSI, the molecular targets for bisacylhydrazine insecticides are not only known but the interaction of some of these insecticides with specific amino acid residues in the ligand binding pocket of the target ecdysone receptor are also known. Moreover, reasons for the selective insect toxicity of bisacylhydrazine insecticides are also well understood. Publication of the crystal structure of unliganded and liganded (with ecdysteroid and bisacylhydrazine) HvEcR/HvUSP, and the discovery of new nonsteroidal ecdysone agonist chemistries like tetrahydroquinolines, provides new tools, and suggests possibilities of discovering new ecdysone agonist

insecticides. It should now be possible to use combinatorial chemistry approaches, around leads generated either via *in silico* screening or rational design (based on the three-dimensional structures and interactions of ligand in ligand binding pocket of EcR), to discover new and novel chemistries that target EcRs from specific insect orders. The discovery of additional new, novel, selective, and potent nonsteroidal ecdysone agonists will create opportunities to extend their applications in individually or simultaneously regulating different ecdysone receptor gene switches in plants and animals. Further, the discovery of non steroidal ecdysone agonist bisacylhydrazine insecticides has opened tremendous opportunities to explore both basic and applied biology.

The molecular basis of action of JHA and CSI insecticides is not well understood, although both classes of chemistries were discovered long before the bisacylhydrazine insecticides. The discovery of the JH receptor(s) has been elusive (see **Chapter 8**). To complicate matters further, it is not clear if the JHAs use the same molecular target/site as the natural JHs do (Dhadialla *et al.*, 1998). Dhadialla *et al.* (1998) alluded to the possibility that the JH receptor may be a complex of two or more proteins, and that JH and JHAs could manifest their action by interacting either with different proteins in the complex or by interacting at different, but effective, sites. Consequently, the research to discover new JH agonist or antagonist chemistries has been slow. An antagonist of JH (different from a precocene type of mode of action) that acts either by inhibiting one of the JH biosynthesis steps or antagonizes the action of JH at the receptor level would be useful. With that, it may be possible to have agonists/antagonists of JH with insect selective toxicity, as has been possible for the ecdysone agonists.

Even though the precise mode of action of CSI insecticides is unknown, many analogs and variants of the original diflubenzuron, Dimilin[®], have been synthesized and registered as insecticides. A better understanding of the biosynthetic pathway for chitin synthesis and cuticle deposition, and molecular characterization of the various enzymes involved, as well as the precise mode of action of CSIs, would allow for development of better and more efficient high-throughput assays for discovery of new and novel CSIs.

Finally, in spite of their slower speed of kill of insect pests than the faster acting neurotoxic insecticides, the three classes of insect growth and development disrupting insecticides are well suited for use in insect pest control. They are also suited for

resistance management programs due to their novel and different modes of action. The bisacylhydrazine insecticides are particularly attractive, due to their selective insect toxicity and high degree of mammalian and ecotoxicological reduced risk profiles.

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References

- Adcock, G.J., Batterham, P., Kelly, L.E., McKenzie, J.A., 1993. Cyromazine resistance in *Drosophila melanogaster* (Diptera, Drosophilidae) generated by ethyl methanesulfonate mutagenesis. *J. Econ. Entomol.* 86, 1001–1008.
- Aliniaze, M.T., Arshad, M., 1998. Susceptibility of immature stages of the oblique banded leafroller, *Choristoneura rosaceana* (Lepidoptera: Tortricidae) to fenoxycarb. *J. Entomol. Soc. Br. Columbia* 95, 59–63.
- Allgood, V.E., Eastman, E.M., 1997. Chimeric receptors as gene switches. *Curr. Opin. Biotechnol.* 8, 474–479.
- Aller, H.E., Ramsay, J.R., 1988. RH-5849 – A novel insect growth regulator with a new mode of action. *Brighton Crop Prot. Conf.* 2, 511–518.
- Anderson, M., Fisher, J.P., Robinson, J., Debray, P.H., 1986. Flufenoxuron – an acylurea acaricide/insecticide with novel properties. *Brighton Crop Prot. Conf.* 1, 89–96.
- Arbeitman, M.N., Hogness, D.S., 2000. Molecular chaperones activate the *Drosophila* ecdysone receptor, an RXR heterodimer. *Cell* 101, 67–77.
- Asano, S., Kuwano, E., Eto, M., 1986. Precocious metamorphosis induced by an anti-juvenile hormone compound applied to 3rd instar silkworm larvae, *Bombyx mori* L. (Lepidoptera: Bombycidae). *App. Entomol. Zool.* 21(2), 305–312.
- Ashburner, M., 1973. Sequential gene activation by ecdysone in polytene chromosomes of *Drosophila melanogaster*. I. Dependence upon ecdysone concentration. *Devel. Biol.* 35, 47–61.
- Ashburner, M., Chiara, C., Meltzer, P., Richards, G., 1974. Temporal control of puffing activity in polytene chromosomes. *Cold Spring Harbor Symp. Quant. Biol.* 38, 655–662.
- Ashok, M., Turner, C., Wilson, T.G., 1998. Insect juvenile hormone resistance gene homology with the bHLH-PAS family of transcriptional regulators. *Proc. Natl. Acad. Sci. USA* 95(6), 2761–2766.
- Awad, T.I., Mulla, M.S., 1984. Morphogenetic and histopathological effects induced by the insect growth regulator cyromazine in *Musca domestica* (Diptera, Muscidae). *J. Med. Entomol.* 21, 419–426.
- Ayoade, O., Morooka, S., Tojo, S., 1995. Induction of macroptery, precocious metamorphosis, and retarded ovarian growth by topical application of precocene II, with evidence for its non-systemic allatocidal effects in the brown planthopper, *Nilaparvata lugens*. *J. Insect Physiol.* 42(6), 529–540.
- Balcells, M., Avilla, J., Profitos, J., Canela, R., 2000. Synthesis of phenoxyphenyl pyridine and pyrazine carboxamides activity against *Cydia pomonella* (L.) eggs. *J. Agric. Food Chem.* 48, 83–87.
- Banks, W.A., Lofgren, C.S., 1991. Effectiveness of the insect growth regulator pyriproxyfen against the red imported fire ant *Hymenoptera formicidae*. *J. Entomol. Sci.* 26(3), 331–338.
- Becher, H.M., Becker, P., Prokic-Immel, R., Wirtz, W., 1983. CME, a new chitin synthesis inhibiting insecticide. *Brighton Crop Prot. Conf.* 1, 408–415.
- Beckage, N.E., Riddiford, L.M., 1981. Effects of methoprene and juvenile hormone on larval ecdysis, emergence, and metamorphosis of the endoparasitic wasp, *Apanteles congregatus*. *J. Insect Physiol.* 28(4), 329–334.
- Beckage, N.E., Riddiford, L.M., 1983. Lepidopteran anti-juvenile hormones: effects on development of *Apanteles congregatus* in *Manduca sexta*. *J. Insect Physiol.* 29(8), 633–637.
- Bélai, I., Matolcsy, G., Farnsworth, D.E., Feyereisen, R., 1988. Inhibition of insect cytochrome P-450 by some metyrapone analogues and compounds containing a cyclopropylamine moiety and their evaluation as inhibitors of juvenile hormone biosynthesis. *Pestic. Sci.* 24, 205–219.
- Bellés, X., Baldellou, M.I., 1983. Precocious metamorphosis induced by precocenes on *Oxycarenus lavaterae*. *Entomol. Exp. Appl.* 34(2), 129–133.
- Bellés, X., Messegues, A., Piulachs, M.B., 1985. Sterilization induced by precocenes on females of *Blattella germanica*(L): short and long term effects. *Zeitschrift für Angewandte Entomologie* 100, 409–417.
- Biddington, K.C., 1985. Ultrastructural changes in the cuticle of the sheep blowfly, *Lucilia cuprina*, induced by certain insecticides and biological inhibitors. *Tissue Cell* 17, 131–140.
- Billas, I.M.L., Moulinier, L., Rochel, N., Moras, D., 2001. Crystal structure of the ligand-binding domain of the ultraspiracle protein USP, the ortholog of retinoid X receptors in insects. *J. Biol. Chem.* 276, 7465–7474.
- Billas, I.M.L., Twema, T., Garnier, J.-M., Mitschler, A., Rochel, N., et al., 2003. Structural adaptability in the ligand-binding pocket of the ecdysone receptor. *Nature* 426, 91–96.

- Binder, B.F., Bowers, W.S., 1991. Behavioral changes and growth inhibition in last instar larvae of *Heliothis zea* induced by oral and topical application of precocene II. *Entomol. Exp. Appl.* 59, 207–217.
- Blackford, M., Dinan, L., 1997. The tomato moth *Lacnobia oleracea* (Lepidoptera: Noctuidae) detoxifies ingested 20-hydroxyecdysone, but is susceptible to the ecdysteroid agonists RH-5849 and RH-5992. *Insect Biochem. Mol. Biol.* 27, 167–177.
- Boivin, T., Chabert d'Hieres, C., Bouvier, J.C., Beslay, D., Sauphanor, B., 2001. Pleiotropy of insecticide resistance in the codling moth, *Cydia pomonella*. *Entomol. Exp. Appl.* 99, 381–386.
- Bonning, B.C., Hoover, K., Booth, T.F., Duffey, S., Hammock, B.D., 1995. Development of a recombinant baculovirus expressing a modified juvenile hormone esterase with potential for insect control. *Arch. Insect Biochem. Physiol.* 30, 177–194.
- Bourne, P.C., Whiting, P., Dhadialla, T.S., Hormann, R.E., Girault, J.-P., et al., 2002. Ecdysteroid 7,9(11)-dien-6-ones as potential photoaffinity labels for ecdysteroid binding proteins. *J. Insect Sci.* 2, 1–11 (available online at <http://www.insectscience.org/2.11>).
- Bouvier, J.C., Boivin, T., Beslay, D., Sauphanor, B., 2002. Age-dependent response to insecticides and enzymatic variation in susceptible and resistant codling moth larvae. *Arch. Insect Biochem. Physiol.* 51, 55–66.
- Bowers, W.S., 1968. Juvenile hormone: activity of natural and synthetic synergists. *Science* 161, 895–897.
- Bowers, W.S., Nishida, R., 1980. Juvocimences L potent juvenile hormone mimics from sweet basil. *Science* 209, 1030–1032.
- Bowers, W.S., Unnithan, G.C., Fukushima, J., Toda, J., Sugiyama, T., 1995. Synthesis and biological activity of furanyl anti-juvenile hormone compounds. *Pestic. Sci.* 43, 1–11.
- Brindle, P.A., Baker, F.C., Tsai, L.W., Reuter, C.C., Schooley, D.A., 1987. Sources of propionate for the biogenesis of ethyl-branched insect juvenile hormones: role of isoleucine and valine. *Proc. Natl Acad. Sci. USA* 84, 7906–7910.
- Bull, M.S., Swindale, S., Overend, D., Hess, E.A., 1996. Suppression of *Boophilus microplus* populations with fluzaron – an acarine growth regulator. *Austral. Vet. J.* 74, 468–470.
- Butenandt, A., Karlson, P., 1954. Über die Isolierung eines metamorphose-hormons des Insekten in kristallisiertem Form. *Z. Naturforsch. Teil B* 9, 389–391.
- Butler, L., Kondo, V., Blue, D., 1997. Effects of tebufenozide (RH-5992) for gypsy-moth (Lepidoptera, Lymantriidae) suppression on nontarget canopy arthropods. *Env. Entomol.* 26(5), 1009–1015.
- Cadogan, B.L., Retnakaran, A., Meating, J.H., 1997. Efficacy of RH5992, a new insect growth regulator against spruce budworm (Lepidoptera: Tortricidae) in a boreal forest. *J. Econ. Entomol.* 90, 551–559.
- Cadogan, B.L., Thompson, D., Retnakaran, A., Scharbach, R.D., Robinson, A., et al., 1998. Deposition of aerially applied tebufenozide (RH5992) on balsam fir (*Abies balsamea*) and its control of spruce budworm (*Choristoneura fumiferana* [Clem.]). *Pestic. Sci.* 53, 80–90.
- Cadogan, B.L., Scharbach, R.D., Krause, R.E., Knowles, K.R., 2002. Evaluation of tebufenozide carry-over and residual effects on spruce budworm (Lepidoptera: Tortricidae). *J. Econ. Entomol.* 95, 578–586.
- Campero, D.M., Haynes, K.F., 1990. Effects of methoprene on chemical communication, courtship, and oviposition in the cabbage looper (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 83(6), 2263–2268.
- Cao, S., Qian, X., Song, G., 2001. *N'*-tert-butyl-*N'*-aroyl-*N*-(alkoxycarbonylmethyl)-*N*-aroylhydrazines, a novel nonsteroidal ecdysone agonist: synthesis, insecticidal activity, conformational, and crystal structure analysis. *Can. J. Chem.* 79, 272–278.
- Capinera, J.L., Epsky, N.D., Turick, L.L., 1991. Responses of *Malonoplus sanguinipes* and *M. differentialis* (Orthoptera: Acrididae) to fenoxycarb. *J. Econ. Entomol.* 84(4), 1163–1168.
- Carlson, G.R., Dhadialla, T.S., Hunter, R., Jansson, R.K., Jany, C.S., et al., 2001. The chemical and biological properties of methoxyfenozide, a new insecticidal ecdysteroid agonist. *Pest Mgt. Sci.* 57, 115–119.
- Carton, B., Heirman, A., Smaghe, G., Tirry, L., 2000. Relationship between toxicity, kinetics and *in vitro* binding of nonsteroidal ecdysone agonists in the cotton leafworm and the Colorado potato beetle. *Med. Fac. Landbouww. Univ. Gent.* 65(2a), 311–322.
- Celestial, D.M., McKenney, C.L.Jr., 1993. The influence of an insect growth regulator on the larval development of the mud crab *Rhithropanopeus harrisi*. *Environ. Pollut.* 85(2), 169–173.
- Chan, T.H., Ali, A., Britten, J.F., Thomas, A.W., Strunz, G.M., et al., 1990. The crystal structure of 1,2-dibenzoyl-1-tert-butylhydrazine, a non-steroidal ecdysone agonist, and its effects on spruce bud worm (*Choristoneura fumiferana*). *Can. J. Chem.* 68, 1178–1181.
- Chandler, L.D., Pair, S.D., Harrison, W.E., 1992. RH-5992, a new insect growth regulator active against corn earworm and fall armyworm (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 85, 1099–1103.
- Chandler, L.D., 1994. Comparative effects of insect growth regulators on longevity and mortality of beet armyworm (Lepidoptera: Noctuidae) larvae. *J. Entomol. Sci.* 29, 357–366.
- Charles, J.P., Wojtasek, H., Lentz, A.J., Thomas, B.A., Bonning, B.C., et al., 1996. Purification and reassessment of ligand binding by the recombinant, putative juvenile hormone receptor of the tobacco hornworm, *Manduca sexta*. *Arch. Insect. Biochem. Physiol.* 31, 371–393.
- Cherbas, P., Cherbas, L., Lee, S.-S., Nakanishi, K., 1988. [125I]iodo-ponasterone A is a potent ecdysone and a sensitive radioligand for ecdysone receptors. *Proc. Natl Acad. Sci., USA* 85, 2096–2100.
- Chen, N.M., Borden, J.H., 1989. Adverse effect of fenoxycarb on reproduction by the California five-spined IPS,

- IPS *Paraconfusus lanier* (Coleoptera: Scolytidae). *Can. Entomol.* 121, 1059–1068.
- Chiang, A.-S., Lin, W.-Y., Liu, H.-P., Pszszalkowski, A., Fu, T.-F., *et al.*, 2002. Insect NMDA receptors mediate juvenile hormone biosynthesis. *Proc. Natl Acad. Sci. USA* 99, 37–42.
- Cho, W.-L., Kapitskaya, M.Z., Raikhel, A.S., 1995. Mosquito ecdysteroid receptor: analysis of the cDNA and expression during vitellogenesis. *Insect Biochem. Mol. Biol.* 25, 19–27.
- Christopherson, K.S., Mark, M.R., Bajaj, V., Godowski, P.J., 1992. Ecdysteroid-dependent regulation of genes in mammalian cells by a *Drosophila* ecdysone receptor and chimeric transactivators. *Proc. Natl Acad. Sci. USA* 89, 6314–6318.
- Chu, K.H., Wong, C.K., Chiu, K.C., 1997. Effects of the insect growth regulator (S)-methoprene on survival and reproduction of the freshwater cladoceran, *Moina macrocopa*. *Environ. Pollut.* 96(2), 173–178.
- Chung, A.C.-K., Durica D.S., Clifton, W., Roe, A., Hopkins, P.M., 1998. Cloning of crustacean ecdysteroid receptor and retinoid-X-receptor gene homologs and elevation of retinoid-X-receptor mRNA by retinoic acid. *Mol. Cell. Endocrinol.* 139, 209–227.
- Clayton, G.M., Peak-Chew, S.Y., Evans, R.M., Schwabe, J.W.R., 2001. The structure of the ultraspiracle ligand-binding domain reveals a nuclear receptor locked in an inactive conformation. *Proc. Natl Acad. Sci. USA* 98, 1549–1554.
- Clement, C.Y., Bradbrook, D.A., Lafont, R., Dinan, L., 1993. Assessment of a microplate-based bioassay for the detection of ecdysteroid-like or antiecdysteroid activities. *Insect Biochem. Mol. Biol.* 23, 187–193.
- Clever, U., Karlson, P., 1960. Induktion von puff-veränderungen in den speicheldriisenchromosomen von *Chironomus tentans* durch ecdysone. *Exp. Cell. Res.* 20, 623–662.
- Cohen, E., 1985. Chitin synthetase activity and inhibition in different insect microsomal preparations. *Experientia* 41, 470–472.
- Cowles, R.S., Villani, M.G., 1996. Susceptibility of Japanese beetle, oriental beetle, and european chafer (Coleoptera: Scarabaeidae) to halofenozide, an insect growth regulator. *J. Econ. Entomol.* 89(6), 1556–1565.
- Cowles, R.S., Alm, S.R., Villani, M.G., 1999. Selective toxicity of halofenozide to exotic white grubs (Coleoptera: Scarabaeidae). *J. Econ. Entomol.* 92(2), 427–434.
- Cusson, M., Palli, S.R., 2000. Can juvenile hormone research help rejuvenate integrated pest management? *Can. Entomol.* 132, 263–280.
- Darvas, B., Pap, L., Kelemen, M., Laszlo, P., 1998. Synergistic effects of verbutin with dibenzoylhydrazine-type ecdysteroid agonists on larvae of *Aedes aegypti* (Diptera: Culicidae). *J. Econ. Entomol.* 91, 1260–1264.
- Darvas, B., Polgár, L.A., 1998. Novel-type insecticides: specificity and effects on non-target organisms. In: Ishaaya, I., Degheele, D. (Eds.), *Insecticides with Novel Modes of Action: Mechanism and Application*. Springer, Berlin, pp. 188–259.
- Darvas, B., Timár, T., Varjas, L., Kulcsár, P., Hosztafi, S., Bordás, B., 1989. Synthesis of novel 2,2-dimethylchromene derivatives and their toxic activity on larvae of *Pieris brassicae* (Lep., Pieridae), and *Leptinotarsa decemlineata* (Col., Chrysomelidae). *Acta Phytopathol. Entomol. Hungarica* 24(3–4), 455–472.
- De Cock, A., Degheele, D., 1998. Buprofezin: a novel chitin synthesis inhibitor affecting specifically planthoppers, whiteflies and scale insects. In: Ishaaya, I., Degheele, D. (Eds.), *Insecticides with Novel Modes of Action: Mechanism and Application*. Springer, pp. 74–91.
- De Cock, A., Ishaaya, I., Van de Veire, M., Degheele, D., 1995. Response of buprofezin-susceptible and -resistant strains of *Trialeurodes vaporariorum* (Homoptera: Aleyrodidae) to pyriproxyfen and diafenthiuron. *J. Econ. Entomol.* 88, 763–767.
- De Reede, R.H., Alkema, P., Blommers, L.H.M., 1985. The use of the insect growth regulator fenoxycarb and epofenonane against leafrollers in integrated pest management in apple orchards. *Entomol. Exp. Appl.* 39, 265–272.
- De Reede, R.H., Groendijk, R.F., Wit, A.K.M., 1984. Field tests with the insect growth regulators, epofenonane and fenoxycarb, in apple orchards against leafrollers and side-effects on some leafroller parasites. *Entomol. Exp. Appl.* 35, 275–281.
- Dedos, S.G., Asahina, M., Fugo, H., 1993. Effect of fenoxycarb application on the pupal–adult development of the silkworm, *Bombyx mori*. *J. Sericult. Sci. Japan* 62(4), 276–285.
- Degheele, D., Fontier, H., Auda, M., De Loof, A., 1986. Preliminary study on the mode of action of precocene II on *Musca domestica* L. *Lucilia caesar* L. *Med. Fac. Landbouww. Univ. Gent.* 51(1), 101–108.
- Dennehy, T.J., Williams, L., 1997. Management of resistance in *Bemisia* in Arizona cotton. *Pestic. Sci.* 51, 398–406.
- DeWilde, J., De Kort, C.A.D., DeLoof, A., 1971. The significance of juvenile hormone titers. *Mitt. Schweiz. Ent. Ges.* 44, 79–86.
- Dhadialla, T.S., Carlson, G.R., Le, D.P., 1998. New insecticides with ecdysteroidal and juvenile hormone activity. *Annu. Rev. Entomol.* 43, 545–569.
- Dhadialla, T.S., Cress, D.E., Carlson, G.R., Hormann, R.E., Palli, S.R., *et al.*, 2002. Ecdysone receptor, retinoid X receptor and ultraspiracle protein based dual switch inducible gene expression modulation system. *PCT Int. Appl. WO 2002029075*. pp. 79.
- Dhadialla, T.S., Tzertzinis, G., 1997. Characterization and partial cloning of ecdysteroid receptor from a cotton boll weevil embryonic cell line. *Arch. Insect Biochem. Physiol.* 35, 45–57.
- Dinan, L., Whiting, P., Bourne, P., Coll, J., 2001. 8-O-Acetylharpagide is not an ecdysteroid agonist. *Insect Biochem. Mol. Biol.* 31, 1077–1082.
- Duan, J.J., Prokopy, R.J., Yin, C.-M., Bergwiler, C., Oouchi, H., 1995. Effects of pyriproxyfen on ovarian

- development and fecundity of *Rhagoletis pomonella* flies. *Entomol. Exp. Appl.* 77, 17–21.
- Dubrovski, E.B., Dubrovskaya, V.A., Bilderback, A.L., Berger, E.M., 2000. The isolation of two juvenile hormone-inducible genes in *Drosophila melanogaster*. *Devel. Biol.* 224(2), 486–495.
- Edwards, J.P., Abraham, L., 1985. Laboratory evaluation of two insect juvenile hormone analogues against *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae). *J. Stored Prod. Res.* 21(4), 189–194.
- Edwards, J.P., Short, J.E., 1993. Elimination of a population of the oriental cockroach (Dictyoptera: Blattidae) in a simulated domestic environment with the insect juvenile hormone analog (S)-hydropene. *J. Econ. Entomol.* 86(2), 436–443.
- Eisa, A.A., El-Fatah, M.A., El-Nabawi, A., El-Dash, A.A., 1991. Inhibitory effects of some insect growth regulators on developmental stages, fecundity and fertility of the florida wax scale, *Ceroplastes floridensis*. *Phytoparasitica* 19(1), 49–55.
- El Saidy, M.F., Auda, M., Degheele, D., 1989. Detoxification mechanism of diflubenzuron and teflubenzuron in the larvae of *Spodoptera littoralis* (Boisd.). *Pestic. Biochem. Physiol.* 35, 211–222.
- Elbert, A., Nauen, R., 2000. Resistance of *Bemisia tabaci* (Homoptera: Aleyrodidae) to insecticides in southern Spain with special reference to neonicotinoids. *Pest Manag. Sci.* 56, 60–64.
- Elbrecht, A., Chen, Y., Jurgens, T., Hensens, O.D., Zink, D.L., et al., 1996. 8-O-acetyl-harpagide is a nonsteroidal ecdysteroid agonist. *Insect Biochem. Mol. Biol.* 26, 519–523.
- El-Guindy, M.A., El-Rafai, A.R.M., Abdel-Satter, M.M., 1983. The pattern of cross-resistance to insecticides and juvenile hormone analogues in a diflubenzuron-resistant strain of the cotton leafworm, *Spodoptera littoralis*. *Pestic. Sci.* 14, 235–245.
- Elzen, G.W., 1989. Sublethal effects of pesticides on beneficial parasitoids. In: Jepson, P.C. (Ed.), *Pesticides and Non-target Invertebrates*. Intercept, pp. 29–159.
- Evans, K., 1985. An approach to control of *Globodera rostochiensis* using inhibitors of collagen and chitin synthesis. *Nematologica* 30, 247–250.
- Farag, A.I., Varjas, L., 1983. Precocious metamorphosis and moulting deficiencies induced by an anti-JH compound FMEV in the fall webworm, *Hyphantria cunea*. *Entomol. Exp. Appl.* 34, 65–70.
- Farinos, G.P., Smaghe, G., Tirry, L., Castañera, P., 1999. Action and pharmacokinetics of a novel insect growth regulator, halofenozide, in adult beetles of *Aubeonymus mariaefrancisciae* and *Leptinotarsa decemlineata*. *Arch. Insect Biochem. Physiol.* 41, 201–213.
- Farkas, R., Slama, K., 1999. Effect of bisacylhydrazine ecdysteroid mimics (RH-5849 and RH-5992) on chromosomal puffing, imaginal disc proliferation and pupariation in larvae of *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* 29, 1015–1027.
- Feng, Q.L., Ladd, T.R., Tomkins, B.L., Sundaram, M., Sohi, S.S., et al., 1999. Spruce budworm (*Choristoneura fumiferana*) juvenile hormone esterase: hormonal regulation, developmental expression and cDNA cloning. *Mol. Cell. Endocrinol.* 148, 95–108.
- Fukuda, S., 1994. The hormonal mechanism of larval moulting and metamorphosis in the silkworm. *J. Fac. Sci. Imp. Univ. Tokyo* 4, 477–532.
- Furlong, M.J., Wright, D.J., 1994. Examination of stability of resistance and cross-resistance patterns to acylurea insect growth regulators in field populations of the diamondback moth, *Plutella xylostella*, from Malaysia. *Pestic. Sci.* 42, 315–326.
- Goff, S.A., Crossland, L.D., Privalle, L.S., 1996. Control of gene expression in plants by receptor mediated transactivation in the presence of a chemical ligand. *PCT Int. Appl. WO 96/27673*. pp. 59.
- Goodman, W.G., 1990. A simplified method for synthesizing juvenile hormone-protein conjugates. *J. Lipid Res.* 31(2), 354–357.
- Gordon, R., 1995. Toxic effects of a commercial formulation of fenoxycarb against adult and egg stages of the eastern spruce budworm, *Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae). *Can. Entomol.* 127, 1–5.
- Gorman, K., Hewitt, F., Denholm, I., Devine, G.J., 2002. New developments in insecticide resistance in the glasshouse whitefly (*Trialeurodes vaporariorum*) and the two-spotted spider mite (*Tetranychus urticae*) in the UK. *Pest Manag. Sci.* 58, 123–130.
- Grebe, M., Rauch, P., Spindler-Barth, M., 2000. Characterization of subclones of the epithelial cell line from *Chironomus tentans* resistant to the insecticide RH 5992, a non-steroidal moulting hormone agonist. *Insect Biochem. Mol. Biol.* 30, 591–600.
- Grenier, S., Grenier, A.-M., 1993. Fenoxycarb, a fairly new insect growth regulator: a review of its effects on insects. *Ann. Appl. Biol.* 122, 369–403.
- Grosscurt, A.C., 1978. Diflubenzuron: some aspects of its ovicidal and larvicidal mode of action and an evaluation of its practical possibilities. *Pestic. Sci.* 9, 373–386.
- Grosscurt, A.C., ter Haar, M., Jongsma, B., Stoker, A., 1987. PH 70–23: a new acaricide and insecticide interfering with chitin deposition. *Pestic. Sci.* 22, 51–59.
- Guo, X., Harmon, M.A., Laudet, V., Manglesdorf, D.J., Palmer, M.J., 1997. Isolation of a functional ecdysteroid receptor homologue from the ixodid tick *Amblyomma americanum* (L.). *Insect Biochem. Mol. Biol.* 27, 945–962.
- Haga, T., Toki, T., Koyanagi, T., Nishiyama, R., 1982. Structure-activity relationships of a series of benzoylpyridyloxyphenyl-urea derivatives. *Int. Congr. Pestic. Chem.* 2, 7.
- Haga, T., Toki, T., Tsujii, Y., Nishaymar, R., 1992. Development of an insect growth regulator, chlorfluzuron. *J. Pestic. Sci.* 17, S103–S113.
- Hagazi, E.M., El-Singaby, N.R., Khafagi, W.E., 1998. Effects of precocenes (I and II) and juvenile hormone I on *Spodoptera littoralis* (Boisd.) (Lep., Noctuidae)

- larvae parasitized by *Microplitis rufiventris* Kok. (Hym., Braconidae). *J. Appl. Entomol.* 122(8), 453–456.
- Hall, R.D., Foehse, M.C., 1980. Laboratory and field tests of CGA-72662 for control of the house fly and face fly in poultry, bovine, or swine manure. *J. Econ. Entomol.* 73, 564–569.
- Hannan, G.N., Hill, R.J., 1997. Cloning and characterization of LcEcR: a functional ecdysone receptor from the sheep blowfly, *Lucilia cupirina*. *Insect Biochem. Mol. Biol.* 27, 479–488.
- Hannan, G.N., Hill, R.J., 2001. *Lcusp*, an ultraspiracle gene from the sheep blowfly, *Lucilia cupirina*: cDNA cloning, developmental expression of RNA and confirmation of function. *Insect Biochem. Mol. Biol.* 31, 771–781.
- Harding, W.C., 1979. Pesticide profiles, part one: insecticides and miticides. *Univ. Maryland, Coop. Ext. Serv. Bull.* 267, 1–30.
- Hargrove, J.W., Langley, P.A., 1993. A field trial of pyriproxyfen-treated targets as an alternative method for controlling tsetse (Diptera: Glossinidae). *Bull. Entomol. Res.* 83, 361–368.
- Hassan, S.A., 1992. Guidelines for testing the effects of pesticides on beneficial organisms: description of test methods. *IOBC/WPRS Bull.* 15, 1–186.
- Hassan, S.A., Bigler, F., Bogenschultz, H., Boller, E., Brun, J., et al., 1991. Results of the 5th joint pesticide testing program carried out by the IOBC/WPRS working group “Pesticides and beneficial organisms.” *Entomophaga* 36, 55–67.
- Hassan, S.A., Bigler, F., Bogenschultz, H., Boller, E., Brun, J., et al., 1994. Results of the 6th joint pesticide testing program of the IOBC/WPRS working group “Pesticides and beneficial organisms.” *Entomophaga* 39, 107–119.
- Hastakoshi, M., 1992. An inhibitor mechanism for oviposition in the tobacco cutworm: *Spodoptera litura* by juvenile hormone analogue pyriproxyfen. *J. Insect Physiol.* 38(10), 793–801.
- Hayashi, T., Iwamura, H., Nakagawa, Y., Fujita, T., 1989. Development of (4-alkoxyphenoxy) alkanaloxime O-ethers as potent insect juvenile hormone mimics and their structure-activity relationships. *J. Agric. Food Chem.* 37, 467–472.
- Hayward, D.C., Bastiani, M.J., Truman, J.W.H., Riddiford, L.M., Ball, E.E., 1999. The sequence of *Locusta* RXR, homologous to *Drosophila* ultraspiracle, and its evolutionary implications. *Devel. Genes Evol.* 209, 564–571.
- Hayward, D.C., Dhadialla, T.S., Zhou, S., Kuiper, M.J., Ball, E.E., et al., 2003. Ligand specificity and developmental expression of RXR and ecdysone receptor in the migratory locust. *J. Insect Physiol.* 49, 1135–1144.
- Henrick, C.A., Staal, G.B., Siddal, J.B., 1973. Alkyl 3,7,1,1-trimethyl-2,4-dodecadienoates, a new class of potent insect growth regulators with juvenile hormone activity. *J. Agric. Food Chem.* 21, 354–359.
- Hicks, B.J., Gordon, R., 1992. Effects of the juvenile hormone analog fenoxycarb on various developmental stages of the eastern spruce budworm, *Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae). *Can. Entomol.* 124, 117–123.
- Hicks, B.J., Gordon, R., 1994. Effect of the juvenile hormone analog fenoxycarb on post-embryonic development of the eastern spruce budworm, *Choristoneura fumiferana*, following treatment of the egg stage. *Entomol. Exp. Appl.* 71, 181–184.
- Hill, T.A., Foster, R.E., 2000. Effect of insecticides on the diamondback moth (Lepidoptera: Plutellidae) and its parasitoid *Diadegma insulare* (Hymenoptera: Ichneumonidae). *J. Econ. Entomol.* 93(3), 763–768.
- Hiruma, K., 2003. Juvenile hormone action in insect development. In: Henry, H.L., Norman, A.W. (Eds.), *Encyclopedia of Hormones*. Elsevier Science, Amsterdam, pp. 528–535.
- Horowitz, A.R., Ishaaya, I., 1994. Managing resistance in insect growth-regulators in the sweet-potato whitefly (Homoptera, Aleyrodidae). *J. Econ. Entomol.* 87, 866–871.
- Horowitz, A.R., Forer, G., Ishaaya, I., 1994. Managing resistance in *Bemisia tabaci* in Israel with emphasis on cotton. *Pestic. Sci.* 42, 113–122.
- Hrdý, I., Kuldová, J., Wimmer, Z., 2001. A juvenile hormone analogue with potential for termite control: laboratory test with *Reticulitermes santonensis*, *Reticulitermes flaviceps* and *Coptotermes formosanus* (Isopt., Rhinotermitidae). *J. Appl. Entomol.* 125(7), 403–411.
- Hsu, A.C.-T., Fujimoto, T.T., Dhadialla, T.S., 1997. Structure-activity study and conformational analysis of RH-5992, the first commercialized nonsteroidal ecdysone agonist. In: Hedin, P.A., Hollingworth, R.M., Masler, E.P., Miyamoto, J., Thompson, D.G. (Eds.), *Phytochemicals for Pest Control*, ACS Symposium Series, vol. 658. American Chemical Society, pp. 206–219.
- Hsu, A.C.-T., 1991. 1,2-Diacyl-1-alkyl-hydrazines; a novel class of insect growth regulators. In: Baker, D.R., Fenyves, J.G., Moberg, W.K. (Eds.), *Synthesis and Chemistry of Agrochemicals, II*. ACS Symposium Series, vol. 443. American Chemical Society, pp. 478–490.
- Hu, W., Feng, Q., Palli, S.R., Krell, P.J., Arif, B.M., et al., 2001. The ABC transporter Pdr5p mediates the efflux of nonsteroidal ecdysone agonists in *Saccharomyces cerevisiae*. *Eur. J. Biochem.* 268, 3416–3422.
- Imhof, M.O., Rusconi, S., Lezzi, M., 1993. Cloning of a *Chironomus tentans* cDNA encoding a protein c(EcRH) homologous to the *Drosophila melanogaster* ecdysone receptor (dEcR). *Insect Biochem. Mol. Biol.* 23, 115–124.
- Ishaaya, I., 1993. Insect detoxifying enzymes – their importance in pesticide synergism and resistance. *Arch. Insect Biochem. Physiol.* 22, 263–276.
- Ishaaya, I., 2001. Biochemical processes related to insecticide actions: an overview. In: Ishaaya, I. (Ed.), *Biochemical Sites of Insecticide Action and Resistance*. Springer, Berlin, pp. 1–16.
- Ishaaya, I., Casida, J.E., 1980. Properties and toxicological significance of esterases hydrolyzing permethrin and

- cypermethrin in *Trichoplusia ni* larval gut integument. *Pestic. Biochem. Physiol.* 14, 178–184.
- Ishaaya, I., De Cock, A., Degheele, D., 1994. Pyriproxyfen, a potent suppressor of egg hatch and adult formation of the greenhouse whitefly (Homoptera: Aleyrodidae). *J. Econ. Entomol.* 87(5), 1185–1189.
- Ishaaya, I., Horowitz, A.R., 1992. Novel phenoxy juvenile hormone analog (pyriproxyfen) suppresses embryogenesis and adult emergence of sweetpotato whitefly (Homoptera: Aleyrodidae). *J. Econ. Entomol.* 85(6), 2113–2117.
- Ishaaya, I., Yablonski, S., Horowitz, A.R., 1995. Comparative toxicology of two ecdysteroid agonists, RH-2485 and RH-5992, on susceptible and pyrethroid resistant strains of the Egyptian cotton leafworm, *Spodoptera littoralis*. *Phytoparasitica* 23, 139–145.
- Ishaaya, I., Yablonski, S., Mendelson, Z., Mansour, Y., Horowitz, A.R., 1996. Novaluron (MCW-275), a novel benzoylphenyl urea, suppressing developing stages of lepidopteran, whitefly and leafminer pests. *Brighton Crop Prot. Conf.* 2, 1013–1020.
- Ismail, F., Wright, D.J., 1991. Cross-resistance between acylurea insect growth regulators in a strain of *Plutella xylostella* L. (Lepidoptera, Yponomeutidae). *Pestic. Sci.* 33, 359–370.
- Ismail, F., Wright, D.J., 1992. Synergism of teflubenzuron and chlorfluazuron in an acylurea-resistant field strain of *Plutella xylostella* L. (Lepidoptera, Yponomeutidae). *Pestic. Sci.* 34, 221–226.
- Iwanaga, K., Kanda, T., 1988. The effects of a juvenile hormone active oxime ether compound on the metamorphosis and reproduction of an anopheline vector, *Anopheles balabacensis* (Diptera: Culicidae). *Appl. Entomol. Zool.* 23(2), 186–193.
- Iyengar, A.R., Kunkel, J.G., 1955. Follicle cell calmodulin in *Blattella germanica*: transcript accumulation during vitellogenesis is regulated by juvenile hormone. *Devel. Biol.* 170, 314–320.
- Jepson, I., Martinez, A., Greenland, A.J., 1996. A gene switch comprising an ecdysone receptor or fusion product allows gene control by external chemical inducer and has agricultural and pharmaceutical applications. *PCT Int. Appl. WO 9637609*. pp. 121.
- Jepson, I., Martinez, A., Sweetman, J.P., 1998. Chemical inducible gene expression systems for plants. A review. *Pestic. Sci.* 54, 360–367.
- Jiang, R.-J., Koolman, J., 1999. Feedback inhibition of ecdysteroids: evidence for a short feedback loop repressing steroidogenesis. *Arch. Insect Biochem. Physiol.* 41, 54–59.
- Jones, G., Sharp, P.A., 1997. Ultraspiracle: an invertebrate nuclear receptor for juvenile hormones. *Proc. Natl Acad. Sci. USA* 94, 13499–13503.
- Jones, G., Wozniak, M., Chu, Y.-X., Dhar, S., Jones, D., 2001. Juvenile hormone III-dependent conformational changes of the receptor ultraspiracle. *Insect Biochem. Mol. Biol.* 32, 33–49.
- Kamimura, K., Arakawa, R., 1991. Field evaluation of an insect growth regulator, pyriproxyfen, against *Culex pipiens* and *Culex tritaeniorhynchus*. *Jap. J. San. Zool.* 42(3), 249–254.
- Kanno, H., Ikeda, K., Asai, T., Maekawa, S., 1981. 2-Tert-butylimino-3-isopropyl-5-perhydro-1,3,5-thiadiazin 4-one (NNI 750), a new insecticide. *Brighton Crop Prot. Conf.* 1, 59–69.
- Kapitskaya, M., Wang, S., Cress, D.E., Dhadialla, T.S., Raikhel, A.S., 1996. The mosquito *ultraspiracle* homologue, a partner of ecdysteroid receptor heterodimer: cloning and characterization of isoforms expressed during vitellogenesis. *Mol. Cell. Endocrinol.* 121, 119–132.
- Karr, L.I., Sheets, J.J., King, J.E., Dripps, J.E., 2004. Laboratory performance and pharmacokinetics of the benzoylphenylurea noviflumuron in eastern subterranean termites (Isoptera: Rhinotermitidae). *J. Econ. Entomol.* 97, 593–600.
- Kasuya, A., Sawada, Y., Tsukamoto, Y., Tanaka, K., Toya, T., et al., 2003. Binding mode of ecdysone agonists to the receptor: comparative modeling and docking studies. *J. Mol. Model.* 9, 58–65.
- Katzenellenbogen, J.A., Katzellenbogen, B.S., 1984. Affinity labeling of receptors for steroid and thyroid hormones. *Vit. Horm.* 41, 213–274.
- Keiding, J., 1999. Review of the global status and recent development of insecticide resistance in field populations of the housefly, *Musca domestica* (Diptera: Muscidae). *Bull. Entomol. Res.* 89, S9–S67.
- Keiding, J., Elkhodary, A.S., Jespersen, J.B., 1992. Resistance risk assessment of 2 insect development inhibitors, diflubenzuron and cyromazine, for control of the housefly *Musca domestica* L. 2. Effect of selection pressure in laboratory and field populations. *Pestic. Sci.* 35, 27–37.
- Keil, C.B., Othman, M.H., 1988. Effects of methoprene on *Lycoriella mali* (Diptera: Sciaridae). *J. Econ. Entomol.* 81(6), 1592–1597.
- Kim, K.S., Chung, B.J., Kim, H.K., 2000. DBI-3204: A new benzoylphenyl urea insecticide with a particular activity against whitefly. *Brighton Crop Prot. Conf.* 1, 41–46.
- King, J.E., Bennett, G.W., 1988. Mortality and developmental abnormalities induced by two juvenile hormone analogs on nymphal german cockroaches (Diptera: Blattellidae). *J. Econ. Entomol.* 81(1), 225–227.
- Knight, A.L., 2000. Tebufenozide targeted against codling moth (Lepidoptera: Tortricidae) adults, eggs, and larvae. *J. Econ. Entomol.* 93(6), 1760–1767.
- Knight, A.L., Dunley, J.E., Jansson, R.K., 2001. Baseline monitoring of codling moth (Lepidoptera: Tortricidae) larval response to benzoylhydrazine insecticides. *J. Econ. Entomol.* 94, 264–270.
- Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P., et al., 1991. The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* 67, 59–77.
- Kopec, S., 1922. Studies on the necessity of the brain for the inception of insect metamorphosis. *Biol. Bull.* 42, 323–341.

- Kostyukovsky, M., Chen, B., Atsmi, S., Shaaya, E., 2000. Biological activity of two juvenoids and two ecdysteroids against three stored product insects. *Insect Biochem. Mol. Biol.* 30, 891–897.
- Kothapalli, R., Palli, S.R., Ladd, T.R., Sohi, S.S., Cress, D., *et al.*, 1995. Cloning and developmental expression of the ecdysone receptor gene from the spruce budworm, *Choristoneura fumiferana*. *Devel. Genet.* 17, 319–330.
- Kotze, A.C., Sales, N., 2001. Inheritance of diflubenzuron resistance and monooxygenase activities in a laboratory-selected strain of *Lucilia cuprina* (Diptera: Calliphoridae). *J. Econ. Entomol.* 94, 1243–1248.
- Kristensen, M., Jespersen, J.B., 2003. Larvicidal resistance in *Musca domestica* (Diptera: Muscidae) populations in Denmark and establishment of resistant laboratory strains. *J. Econ. Entomol.* 96, 1300–1306.
- Krysan, J.L., 1990. Fenoxycarb and diapause: a possible method of control for pear psylla (Homoptera: Psyllidae). *J. Econ. Entomol.* 83(2), 293–299.
- Kumar, M.B., Fujimoto, T., Potter, D.W., Deng, Q., Palli, S.R., 2002. A single point mutation in ecdysone receptor leads to increased ligand specificity: implications for gene switch applications. *Proc. Natl Acad. Sci. USA* 99, 14710–14715.
- Kuwano, E., Kikuchi, M., Eto, M., 1990. Synthesis and insect growth regulatory activity of 1-neopentyl-5-substituted imidazoles. *J. Fac. Agric. Kyushu Univ.* 35(1–2), 35–41.
- Kuwano, E., Takeya, R., Eto, M., 1985. Synthesis and anti-juvenile hormone activity of 1-substituted-5-[(E)-2,6-dimethyl-1,5-heptadienyl]imidazoles. *Agric. Biol. Chem.* 49(2), 483–486.
- Kuwano, E., Takeya, R., Eto, M., 1984. Synthesis and anti-juvenile hormone activity of 1-citronellyl-5-substituted imidazoles. *Agric. Biol. Chem.* 48(12), 3115–3119.
- Langley, P.A., Felton, T., Stafford, K., Oouchi, H., 1990. Formulation of pyriproxyfen, a juvenile hormone mimic, for tsetse control. *Med. Vet. Entomol.* 4, 127–133.
- Le, D.P., Thirugnanam, M., Lidert, Z., Carlson, G.R., Ryan, J.B., 1996. RH-2485: A new selective insecticide for caterpillar control. *Proc. Brighton Crop Prot. Conf.* 2, 481–486.
- Lee, K.-Y., Denlinger, D.L., 1997. A role for ecdysteroids in the induction and maintenance of the pharate first instar diapause of the gypsy moth, *Lymantria dispar*. *J. Insect Physiol.* 43, 289–296.
- Lee, K.-Y., Chamberlin, M.E., Horodyski, F.M., 2002. Biological activity of *Manduca sexta* allatotropin-like peptides, predicted products of tissue-specific and developmentally regulated alternatively spliced mRNAs. *Peptides* 23, 1933–1941.
- Legaspi, J.C., Legaspi, B.C., Jr., Saldana, R.R., 1999. Laboratory and field evaluations of biorational insecticides against the Mexican rice borer (Lepidoptera: Pyralidae) and a parasitoid (Hymenoptera: Braconidae). *J. Econ. Entomol.* 92(4), 804–810.
- Lidert, Z., Dhadialla, T.S., 1996. MIMIC insecticide an ecdysone agonist for the global forestry market. In: Proceedings of the International Conference on Integrated Management of Forestry Lymantridae, Warsaw-Sekocin, pp. 59–68.
- Liu, T.-X., Chen, T.-Y., 2000. Effects of a juvenile hormone analog, pyriproxyfen, on the apterous form of *Lipaphis erysimi*. *Entomol. Exp. Appl.* 98, 295–301.
- Liu, T.-X., Chen, T.-Y., 2001. Effects of the insect growth regulator fenoxycarb on immature *Chrysoperla rufilabris* (Neuroptera: Chrysopidae). *Fl. Entomol.* 84(4), 628–633.
- Liu, T.-X., Stansly, P.A., 1997. Effects of pyriproxyfen on three species of *Encarsia* (Hymenoptera: Aphelinidae), endoparasitoids of *Bemisia argentifolii* (Homoptera: Aleyrodidae). *Biol. Microbiol. Cont.* 90(2), 404–411.
- Locke, M., 1998. Epidermis. In: Harrison, F.W., Locke, M. (Eds.), *Microscopic Anatomy of Invertebrates*, vol. 11A: Insecta. Wiley-Liss, New York, pp. 75–138.
- Londerhausen, M., 1996. Approaches to new parasitocides. *Pestic. Sci.* 48, 269–292.
- Lopez, J.D., Jr., Lathief, M.A., Meola, R.W., 1999. Effect of insect growth regulators on feeding response and reproduction of adult bollworm. *Proc. Beltwide Cotton Conf.* 2, 1214–1221.
- Lyousseoufi, A., Gadenne, C., Rieus, R., d'Arcier, F.F., 1994. Effects of an insect growth regulator, fenoxycarb, on the diapause of the pear psylla, *Cacopsylla pyri*. *Entomol. Exp. Appl.* 72(3), 239–244.
- Magaula, C.N., Samways, M.J., 2000. Effects of insect growth regulators on *Chilocorus nigritus* (Fabricius) (Coleoptera: Coccinellidae), a non-target natural enemy of citrus red scale, *Aonidiella aurantii* (Maskell) (Homoptera: Diaspididae), in southern Africa: evidence from laboratory and field trials. *African Entomol.* 8(1), 47–56.
- Martinez, A., Sparks, C., Drayton, P., Thompson, J., Greenland, A., Jepson, I., 1999a. Creation of ecdysone receptor chimeras in plants for controlled regulation of gene expression. *Mol. Gen. Genet.* 261, 546–552.
- Martinez, A., Sparks, C., Hart, C.A., Thompson, J., Jepson, I., 1999b. Ecdysone agonist inducible transcription in transgenic tobacco plants. *Plant J.* 19, 97–106.
- Mascarenhas, V.J., Leonard, B.R., Burris, E., Graves, J.B., 1996. Beet armyworm (Lepidoptera: Noctuidae) control on cotton in Louisiana. *Fl. Entomol.* 79, 336–343.
- Masner, P., Angst, M., Dorn, S., 1986. Fenoxycarb, an insect growth regulator with juvenile hormone activity: a candidate for *Heliothis virescens* (F.) control on cotton. *Pestic. Sci.* 18, 89–94.
- Masner, P., Salama, K., Landa, V., 1968. Natural and synthetic materials with insect hormone activity. IV. Specific female sterility effects produced by a juvenile hormone analogue. *J. Embryol. Exp. Morphol.* 20, 25–31.
- McKenzie, J.A., Batterham, P., 1998. Predicting insecticide resistance: mutagenesis, selection and response. *Phil. Trans. Roy. Soc. Lond. B Biol. Sci.* 353, 1729–1734.

- Medina, P., Budia, F., Tirry, L., Smagghe, G., Viñuela, E., 2001. Compatibility of spinosad, tebufenozide and azadirachtin with eggs and pupae of the predator *Chrysoperla carnea* (Stephens) under laboratory conditions. *Biocontrol Sci. Technol.* 11, 597–610.
- Medina, P., Smagghe, G., Budia, F., del Estal, F., Tirry, L., et al., 2002. Significance of penetration, excretion, transovarial uptake to toxicity of three insect growth regulators in predatory lacewing adults. *Arch. Insect Biochem. Physiol.* 51, 91–101.
- Mendel, Z., Blumberg, D., Ishaaya, I., 1994. Effects of some insect growth regulators on natural enemies of scale insects (Hom.: Coccoidea). *Entomophaga* 39, 199–209.
- Michelotti, E.L., Tice, C.M., Palli, S.R., Thompson, C.S., Dhadialla, T.S., 2003. Tetrahydroquinolines for modulating the expression of exogenous genes via an ecdysone receptor complex. *PCT Int. Appl. WO 2003105849*. pp. 129.
- Mikitani, K., 1996a. A new nonsteroidal class of ligand for the ecdysteroid receptor 3,5-di-*tert*-butyl-4-hydroxy-N-isobutyl-benzamide. *Biochem. Biophys. Res. Commun.* 227, 427–432.
- Mikitani, K., 1996b. Ecdysteroid receptor binding activity and ecdysteroid agonist activity at the level of gene expression are correlated with the activity of dibenzoyl hydrazines in larvae of *Bombyx mori*. *J. Insect Physiol.* 42, 937–941.
- Mikólajczyk, P., Oberlander, H., Silhacek, D.L., Ishaaya, I., Shaaya, E., 1994. Chitin synthesis in *Spodoptera frugiperda* wing imaginal discs. I. Chlorfluazuron, diflubenzuron, and teflubenzuron inhibit incorporation but not uptake of [¹⁴C]-N-acetyl-D-glucosamine. *Arch. Insect Biochem. Physiol.* 25, 245–258.
- Miller, R.J., Broce, A.B., Dryden, M.W., Hopkins, T., 1999. Susceptibility of insect growth regulators and cuticle deposition of the cat flea (Siphonaptera: Pulicidae) as a function of age. *J. Med. Entomol.* 36(6), 780–787.
- Miura, T., Takahashi, R.M., 1987. Impact of fenoxycarb, a carbamate insect growth regulator, on some aquatic invertebrates abundant in mosquito breeding habitats. *J. Am. Mosq. Control Assoc.* 3, 476–480.
- Miyake, T., Mitsui, T., 1995. Multiple physiological activity of an anti-juvenile hormone, precocene 2 on the whitebacked rice planthopper. *J. Pestic. Sci.* 20, 17–24.
- Miyake, T., Haruyama, H., Mitsui, T., Sakurai, A., 1991. Effects of a new juvenile hormone mimic, NC-170, on metamorphosis and diapause of the small brown planthopper, *Laodelphax striatellus*. *J. Pestic. Sci.* 17, 75–82.
- Miyake, T., Haruyama, H., Ogura, T., Mitsui, T., Sakurai, A., 1990. Effects of insect juvenile hormone active NC-170 on metamorphosis, oviposition and embryogenesis in leafhoppers. *J. Pest. Sci.* 16, 441–448.
- Miyamoto, J., Hirano, M., Takimoto, Y., Hatakoshi, M., 1993. Insect growth regulators for insect control, with emphasis on juvenile hormone analogs: present and future prospects. In: Duke, S.O., Menn, J.J., Plimmer, J.R. (Eds.), *Pest Control with Enhanced Environmental Safety*, ACS Symposium Series, vol. 524. American Chemical Society, Washington, DC, pp. 144–168.
- Mkhize, J.N., 1991. Activity of a juvenile hormone analog as a protectant against the lesser grain borer, *Rhizopertha dominica* (F.) (Coleoptera: Bostrichidae). *Insect Sci. Appl.* 13(2), 183–187.
- Moffit, H.R., Westgrad, P.H., Mantey, K.D., Van de Baan, H.E., 1988. Resistance to diflubenzuron in codling moth (Lepidoptera: Tortricidae). *J. Econ. Entomol.* 81, 1511–1515.
- Mohammed-Ali, A.D., Chan, T.-H., Thomas, A.W., Strunz, G.M., Jewett, B., 1995. Structure–activity relationship study of synthetic hydrazines as ecdysone agonists in the control of spruce budworm (*Choristoneura fumiferana*). *Can. J. Chem.* 73, 550.
- Moser, B.A., Koehler, P.G., Patterson, R.S., 1992. Effect of methoprene and diflubenzuron on larval development of the cat flea (Siphonaptera: Pulicidae). *J. Econ. Entomol.* 85(1), 112–116.
- Mouillet, J.F., Delbecque, J.P., Quenedey, B., Delachambre, J., 1997. Cloning of two putative ecdysteroid receptor isoforms from *Tenebrio molitor* and their developmental expression in the epidermis during metamorphosis. *Eur. J. Biochem.* 248, 856–863.
- Moulton, J.K., Pepper, D.A., Jansson, R.K., Dennehy, T.J., 2002. Pro-active management of beet armyworm (Lepidoptera: Noctuidae) resistance to tebufenozide and methoxyfenozide: baseline monitoring, risk assessment, and isolation of resistance. *J. Econ. Entomol.* 95, 414–424.
- Muyle, H., Gordon, R., 1989. Effects of selected juvenile hormone analogs on sixth instar larvae of the eastern spruce budworm, *Choristoneura fumiferana* Clemens (Lepidoptera: Tortricidae). *Can. Entomol.* 121, 1111–1116.
- Nair, V.S.K., Rajalekshmi, E., 1989. Effects of fluoromevalonate on penultimate and last instar larvae of *Spodoptera mauritia* Boisduval. *Indian J. Expt. Biol.* 27(2), 170–173.
- Nakagawa, Y., Akagi, T., Iwamura, H., Fujita, T., 1989a. Quantitative structure-activity studies of benzoylphe-nylurea larvicides. VI. Comparison of substituent effects among activities against different insect species. *Pestic. Biochem. Physiol.* 33, 144–157.
- Nakagawa, Y., Hattori, K., Minakuchi, C., Kugimiya, S., Ueno, T., 2000a. Relationships between structure and molting hormonal activity of tebufenozide, methoxyfenozide, and their analogs in cultured integument system of *Chilo suppressalis* Walker. *Steroids* 65, 117–123.
- Nakagawa, Y., Hattori, K., Shimizu, B., Akamatsu, M., Miyagawa, H., et al., 1998. Quantitative structure-activity studies of insect growth regulators XIV.

- Three-dimensional quantitative structure-activity relationship of ecdysone agonists including dibenzoylhydrazine analogs. *Pestic. Sci.* 53, 267–277.
- Nakagawa, Y., Minakuchi, C., Takahashi, K., Ueno, T., 2002. Inhibition of [³H]ponasterone A binding by ecdysone agonists in the intact Kc cell line. *Insect Biochem. Mol. Biol.* 32, 175–180.
- Nakagawa, Y., Minakuchi, C., Ueno, T., 2000b. Inhibition of [³H]ponasterone A binding by ecdysone agonists in the intact Sf-9 cell line. *Steroids* 65, 537–542.
- Nakagawa, Y., Matsutani, M., Kurihara, N., Nishimura, K., Fujita, T., 1989b. Quantitative structure-activity studies of benzoylphenylurea larvicides. VIII. Inhibition of N-acetylglucosamine incorporation into the cultured integument of *Chilo suppressalis* Walker. *Pestic. Biochem. Physiol.* 43, 141–151.
- Nakagawa, Y., Shimuzu, B., Oikawa, N., et al., 1995a. Three-dimensional quantitative structure-activity analysis of steroidal and dibenzoylhydrazine-type ecdysone agonists. In: Hanch, C., Fujita, T. (Eds.), Classical and Three-Dimensional QSAR in Agrobiocchemistry ACS Symposium, vol. 606. American Chemical Society, Washington, DC, pp. 288–301.
- Nakagawa, Y., Smagghe, G., Kugimiya, S., et al., 1999. Quantitative structure-activity studies of insect growth regulators: XVI. Substituent effects of dibenzoylhydrazines on the insecticidal activity to Colorado potato beetle *Leptinotarsa decemlineata*. *Pestic. Sci.* 55, 909–918.
- Nakagawa, Y., Smagghe, G., Tirry, L., Fujita, T., 2001a. Quantitative structure-activity studies of insect growth regulators: XIX. Effects of substituents on the aromatic moiety of dibenzoylhydrazines on larvicidal activity against the beet armyworm *Spodoptera exigua*. *Pest Manag. Sci.* 58, 131–138.
- Nakagawa, Y., Smagghe, G., Van Paemel, M., Tirry, L., Fujita, T., 2001b. Quantitative structure-activity studies of insect growth regulators: XVIII. Effects of substituents on the aromatic moiety of dibenzoylhydrazines on larvicidal activity against the Colorado potato beetle *Leptinotarsa decemlineata*. *Pest Mgt. Sci.* 57, 858–865.
- Nakagawa, Y., Sotomatsu, T., Irie, K., Kitahara, K., Iwamura, H., et al., 1987. Quantitative structure-activity studies of benzoylphenylurea larvicides. III. Effects of substituents at the benzoyl moiety. *Pestic. Biochem. Physiol.* 27, 143–155.
- Nakagawa, Y., Soya, Y., Nakai, K., et al., 1995b. Quantitative structure-activity studies of insect growth regulators. XI. Stimulation and inhibition of N-acetylglucosamine incorporation in a cultured integument system by substituted *N-tert-butyl-N,N'*-dibenzoylhydrazines. *Pestic. Sci.* 43, 339–345.
- Niwa, A., Iwamura, H., Nakagawa, Y., Fujita, T., 1989. Development of (phenoxyphenoxy)- and (benzylphenoxy) propyl ethers as potent insect juvenile hormone mimetics. *J. Agric. Food Chem.* 37, 462–467.
- No, D., Yao, T.-P., Evans, M.E., 1996. Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proc. Natl Acad. Sci. USA* 93, 3346–3351.
- Oberlander, H., Silhacek, D.L., Porcheron, P., 1995. Non-steroidal ecdysteroid agonists: Tools for the study of hormonal action. *Arch. Insect Biochem. Physiol.* 28, 209–223.
- Oberlander, H., Silhacek, D.L., 1998. New perspectives on the mode of action of benzoylphenyl urea insecticides. In: Ishaaya, I., Degheele, D. (Eds.), Insecticides with Novel Modes of Action: Mechanism and Application. Springer, pp. 92–105.
- Oberlander, H., Smagghe, G., 2001. Imaginal discs and tissue cultures as targets for insecticide action. In: Ishaaya, I. (Ed.), Biochemical Sites of Insecticide Action and Resistance. Springer, Berlin, pp. 133–150.
- Oikawa, N., Nakagawa, Y., Nishimura, K., Ueno, T., Fujita, T., 1994a. Quantitative structure-activity analysis of larvicidal 1-(substituted benzoyl)-2-benzoyl-1-*tert*-butylhydrazines against *Chilo suppressalis*. *Pestic. Sci.* 41, 139–148.
- Oikawa, N., Nakagawa, Y., Nishimura, K., Ueno, T., Fujita, T., 1994b. Quantitative structure-activity studies of insect growth regulators. X. Substituent effects on larvicidal activity of 1-*tert*-butyl-1-(2-chlorobenzoyl)-2-(substituted benzoyl)hydrazines against *Chilo suppressalis* and design synthesis of potent derivatives. *Pestic. Biochem. Physiol.* 48, 135–144.
- Olsen, A., 1985. Ovicidal effect on the cat flea, *Ctenocephalides felis* (Bouché), of treating fur of cats and dogs with methoprene. *Int. Pest Control*. January/February, 10–14.
- Padidam, M., 2003. Chemically regulated gene expression in plants. *Curr. Opin. Plant Biol.* 6, 169–177.
- Padidam, M., Gore, M., Lu, D.L., Smirnova, O., 2003. Chemical-inducible, ecdysone receptor-based gene expression system for plants. *Transgenic Res.* 12, 101–109.
- Palli, S.R., Kapitskaya, M.Z., 2002. Novel substitution variants of nuclear receptors and their use in a dual switch inducible system for regulation of gene expression. *PCT Int. Appl.* WO 2002066615. pp. 110.
- Palli, S.R., Kapitskaya, M.Z., Kumar, M.B., Cress, D.E., 2003. Improved ecdysone receptor-based inducible gene regulation system. *Eur. J. Biochem.* 270, 1308–1315.
- Palli, S.R., Kumar, M.B., Cress, D.E., Fujimoto, T.T., 2002. Substitution variants of nuclear receptors and their use in a dual switch inducible system for regulation of gene expression. *PCT Int. Appl.* WO 2002066612. pp. 148.
- Palli, S.R., Ladd, T.R., Sohi, S.S., Cook, B.J., Retnakaran, A., 1996. Cloning and developmental expression of *Choristoneura* hormone receptor 3, an ecdysone-inducible gene and a member of the steroid receptor superfamily. *Insect Biochem. Mol. Biol.* 26, 485–499.
- Palli, S.R., Ladd, T.R., Tomkins, W., Primavera, M., Sundaram, M.S., et al., 1999. Biochemical and biological modes of action of ecdysone agonists on the spruce budworm. *Pestic. Sci.* 55, 656–657.

- Palli, S.R., Primavera, M., Tomkins, W., Lambert, D., Retnakaran, A., 1995. Age-specific effects of a non-steroidal ecdysteroid agonist, RH-5992, on the spruce budworm, *Choristoneura fumiferana* (Lepidoptera: Tortricidae). *Eur. J. Entomol.* 92, 325–332.
- Palli, S.R., Retnakaran, A., 1999. Molecular and biochemical aspects of chitin synthesis inhibition. In: Jollès, P., Muzzarelli, R.A.A. (Eds.), *Chitin and Chitinases*. Birkhäuser Verlag, pp. 85–98.
- Palli, S.R., Retnakaran, A., 2000. Ecdysteroid and juvenile hormone receptors: properties and importance in developing novel insecticides. In: Ishaaya, I. (Ed.), *Biochemical Sites of Insecticide Action and Resistance*. Springer, pp. 107–132.
- Palli, S.R., Touchara, K., Charles, J., Bonning, B.C., Atkinson, J.K., et al., 1994. A nuclear juvenile hormone-binding protein from larvae of *Manduca sexta*: A putative receptor for the metamorphic action of juvenile hormone. *Proc. Natl Acad. Sci. USA* 91(13), 6191–6195.
- Palumbo, J.C., Horowitz, A.R., Prabhaker, N., 2001. Insecticidal control and resistance management for *Bemisia tabaci*. *Crop Protect.* 20, 739–765.
- Peleg, B.A., 1988. Effect of a new phenoxy juvenile hormone analog on California red scale (Homoptera: Diaspididae), Florida wax scale (Homoptera: Coccidae) and the ectoparasite *Aphytis holoxanthus* DeBache (Hymenoptera: Aphelinidae). *J. Econ. Entomol.* 81, 88–92.
- Perera, S.C., Ladd, T.R., Dhadialla, T.S., Krell, P.J., Sohi, S.S., et al., 1999. Studies on two ecdysone receptor isoforms of the spruce budworm, *Choristoneura fumiferana*. *Mol. Cell. Endocrinol.* 152, 73–84.
- Perez-Farinos, G., Smagghe, G., Marco, V., Tirry, L., Castañera, P., 1998. Effects of topical application of hexaflumuron on adult sugar beet weevil *Aubeononymus mariae-franciscas* on embryonic development: pharmacokinetics in adults and embryos. *Pestic. Biochem. Physiol.* 61, 169–182.
- Peterson, J.K., Kashian, D.R., Dodson, S.I., 2000. Methoprene and 20-OH-ecdysone affect male production in *Daphnia pulex*. *Environ. Toxicol. Chem.* 20(3), 582–588.
- Pimprikar, G.D., Georghiou, G.P., 1979. Mechanisms of resistance to diflubenzuron in the house fly, *Musca domestica* L. *Pestic. Biochem. Physiol.* 12, 10–22.
- Pons, S., Riedl, H., Avilla, J., 1999. Toxicity of the ecdysone agonist tebufenozide to codling moth (Lepidoptera: Tortricidae). *J. Econ. Entomol.* 92, 1344–1351.
- Pradeep, A.R., Nair, V.S.K., 1989. Morphogenetic effects of precocene II in the brown planthopper *Nilaparvata lugens* Stal. (Homoptera: Delphacidae). *Acta Entomol. Biochem.* 86(3), 172–178.
- Rasa, Cordellia G., Meola, Roger W., Shenker, R., 2000. Effects of a new insect growth regulator, CGA-255'728, on the different stages of the cat flea (Siphonaptera: Pulicidae). *Entomol. Soc. Am.* 37(1), 141–145.
- Reiji, I., Shinya, N., Takashi, O., Yasushi, T., Keiji, T., et al., 2000. Ecdysone mimic insecticide, chromafenozide: field efficacy against the apple tortrix. *Annu. Rep. Sankyo Res. Lab.* 52, 59–62.
- Retnakaran, A., 1970. Blocking of embryonic development in the spruce budworm, *Choristoneura fumiferana* (Lepidoptera: Tortricidae), by some compounds with juvenile hormone activity. *Can. Entomol.* 102, 1592–1596.
- Retnakaran, A., 1973a. Hormonal induction of supernumerary instars in the spruce budworm, *Choristoneura fumiferana* (Lepidoptera: Tortricidae). *Can. Entomol.* 105, 459–461.
- Retnakaran, A., 1973b. Ovicidal effect in the white pine weevil, *Pissodes strobi* (Coleoptera: Curculionidae), of a synthetic analogue of juvenile hormone. *Can. Entomol.* 105, 591–594.
- Retnakaran, A., 1974. Induction of sexual maturity in the white pine weevil, *Pissodes strobi* (Coleoptera: Curculionidae), by some analogue of juvenile hormone. *Can. Entomol.* 106, 831–834.
- Retnakaran, A., Gelbic, I., Sundaram, M., Tomkins, W., Ladd, T., et al., 2001. Mode of action of the ecdysone agonist tebufenozide (RH-5992), and an exclusion mechanism to explain resistance to it. *Pest Mgt. Sci.* 57, 951–957.
- Retnakaran, A., Granett, J., Ennis, T., 1985. Insect growth regulators. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 12. Pergamon, Oxford, pp. 529–601.
- Retnakaran, A., Hiruma, K., Palli, S.R., Riddiford, L.M., 1995. Molecular analysis of the mode of action of RH-5992, a lepidopteran-specific, non-steroidal ecdysteroid agonist. *Insect Biochem. Mol. Biol.* 25, 109–117.
- Retnakaran, A., Macdonald, A., Tomkins, W.L., Davis, C.N., Brownright, A.J., et al., 1997. Ultrastructural effects of a non-steroidal agonist, RH-5992, on the sixth instar larvae of spruce budworm, *Choristoneura fumiferana*. *J. Insect Physiol.* 43, 55–68.
- Retnakaran, A., Oberlander, H., 1993. Control of chitin synthesis in insects. In: Muzzarelli, R.A.A. (Ed.), *Chitin Enzymology*. European Chitin Society, pp. 89–99.
- Retnakaran, A., Tomkins, W.L., Primavera, M.J., Palli, S.R., 1999. Feeding behavior of the first-instar *Choristoneura fumiferana* and *Choristoneura pinus pinus* (Lepidoptera: Tortricidae). *Can. Entomol.* 131, 79–84.
- Retnakaran, A., Wright, J.E., 1987. Control of insect pests with benzoylphenylureas. In: Wright, J.E., Retnakaran, A. (Eds.), *Chitin and Benzoylphenyl Ureas*. Dr. W. Junk Publishers, Dordrecht, Netherlands, pp. 205–282.
- Reynolds, S.E., Blakey, J.K., 1989. Cyromazine causes decreased cuticle extensibility in larvae of the tobacco hornworm, *Manduca sexta*. *Pestic. Biochem. Physiol.* 35, 251–258.
- Riddiford, L.M., 1971. Juvenile hormone and insect embryogenesis. *Mitt. Schweiz. Ent. Ges.* 44, 177–186.

- Riddiford, L.M., 1994. Cellular and molecular actions of juvenile hormone. I. General considerations and premetamorphic actions. *Adv. Insect Physiol.* 24, 213–274.
- Riddiford, L.M., 1996. Juvenile hormone: the status of its “status quo” action. *Arch. Insect Biochem. Physiol.* 32, 271–286.
- Riddiford, L.M., Cherbas, P., Truman, J.W., 2001. Ecdysone receptors and their biological functions. *Vit. Horm.* 60, 1–73.
- Riddiford, L.M., Hiruma, K., Zhou, X., Nelson, C.A., 2003. Insights into the molecular basis of the hormonal control of molting and metamorphosis from *Manduca sexta* and *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* 33, 1327–1338.
- Riddiford, L.M., Williams, C.M., 1967. The effects of juvenile hormone analogues on the embryonic development of silkworms. *Proc. Natl Acad. Sci. USA* 57, 595–601.
- Riedl, H., Blomefield, T.L., Gilomee, J.H., 1999. A century of codling moth control in South Africa. II. Current and future status of codling moth management. *J. S. Afr. Soc. Hort. Sci.* 8, 32–54.
- Riedl, H., Brunner, J.F., 1996. Insect growth regulators provide new pest control tools for Pacific Northwest orchards. In: Proc. 92nd Annu. Mtg. Washington State Hort. Assoc., pp. 189–200.
- RohMid, L.L.C., 1996. RH-0345, Turf and Ornamental Insecticide. *Technical Infor. Bull.*, 9.
- Roller, H., Dahm, K.H., Sweely, C.C., Trost, B.M., Ange, W., 1967. The structure of the juvenile hormone. *Chem. Int. Ed. Engl.* 6, 179.
- Romanuk, M., 1981. Structure-activity relationships in selected groups of juvenoids. In: Sehna, F., Zabra, A., Menn, J.J., Cymborowsti, B. (Eds.), Regulation of Insect Development and Behaviour, Part 1. Wroclaw Technical Univeristy Press, Wroclaw, Poland, pp. 247–260.
- Rup, P.J., Baniwal, A., 1985. Responses of *Zaprionus paravittiger* (Drosophilidae: Diptera) to anti-juvenile hormone, precocene. *Insect Sci. Appl.* 6(6), 671–675.
- Saleh, D.S., Zhang, J., Wyatt, G.R., Walker, V.K., 1998. Cloning and characterization of an ecdysone receptor cDNA from *Locusta migratoria*. *Mol. Cell. Endocrinol.* 143, 91–99.
- Sariaslani, F.S., McGee, L.R., Ovenall, D.W., 1987. Microbial transformation of Precocene II: Oxidative reactions by *Streptomyces griseus*. *Appl. Environ. Microbiol.* 53(8), 1780–1784.
- Saul, S.H., Mau Ronald, F.L., Oi, D., 1995. Laboratory trials of methoprene-impregnated waxes for disinfecting papayas and peaches of the Mediterranean fruit fly (Diptera: Tephritidae). *J. Econ. Entomol.* 78, 652–655.
- Saul, S.H., Mau Ronald, F.L., Kobayashi, R.M., Tsuda, D.M., et al., 1987. Laboratory trials of methoprene-impregnated waxes for preventing survival of adult oriental fruit flies (Diptera: Tephritidae) from infested papayas. *J. Econ. Entomol.* 80, 494–496.
- Sauphanor, B., Bouvier, J.C., 1995. Cross-resistance between benzoylureas and benzoylhydrazines in the codling moth, *Cydia pomonella* L. *Pestic. Sci.* 45, 369–375.
- Sauphanor, B., Brosse, V., Monier, C., Bouvier, J.C., 1998a. Differential ovicidal and larvicidal resistance to benzoylureas in the codling moth, *Cydia pomonella*. *Entomol. Exp. Appl.* 88, 247–253.
- Sauphanor, B., Bouvier, J.C., Brosse, V., 1998b. Spectrum of insecticide resistance in *Cydia pomonella* (Lepidoptera: Tortricidae) in southeastern France. *J. Econ. Entomol.* 91, 1225–1231.
- Saxena, R.C., Dixit, O.P., Sukumaran, P., 1992. Laboratory assessment of indigenous plant extracts for anti-juvenile hormone activity in *Culex quinquefasciatus*. *Indian J. Med. Res.* 95, 204–206.
- Sbragia, R., Bisabri-Ershadi, B., Rigterink, R.H., 1983. XRD-473, a new acylurea insecticide effective against *Heliothis. Brighton Crop Prot. Conf.* 1, 417–424.
- Schooley, D.A., Baker, F.C., 1985. Juvenile hormone biosynthesis. In: Kerkut, G.A., Gilbert, K.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 7. Pergamon, Oxford, pp. 363–389.
- Sempere, L.F., Sokol, N.S., Dubrovsky, E.B., Berger, E.M., Ambros, V., 2003. Temporal regulation of microRNA expression in *Drosophila melanogaster* mediated by hormonal signals and Broad-Complex gene activity. *Devel. Biol.* 259, 9–18.
- Sen, S.E., Garvin, G.M., 1995. Substrate requirements for lepidopteran farnesol dehydrogenase. *J. Agric. Food Chem.* 43, 820–825.
- Sevala, V.L., Davey, K.G., 1989. Action of juvenile hormone on the follicle cells of *Rhodnius prolixus*: Evidence for a novel regulatory mechanism involving protein kinase c. *Experientia.* 45, 355–356.
- Shaw, P.W., Walker, J.T.S., 1996. Biological control of woolly apple aphid by *Aphelinus mali* in an integrated fruit production programme in Nelson. In: Proc. 49th N.Z. Plant Prot. Conf., pp. 59–63.
- Sheets, J., Karr, L.L., Dripps, J.E., 2000. Kinetics and uptake, clearance, transfer, and metabolism of hexaflumuron by eastern subterranean termites (Isoptera: Rhinotermitidae). *J. Econ. Entomol.* 93, 871–877.
- Shemshedini, L., Wilson, T.G., 1990. Resistance of juvenile hormone and an insect growth regulator in *Drosophila* is associated with an altered cytosolic juvenile hormone-binding protein. *Proc. Natl Acad. Sci. USA* 87(6), 2072–2076.
- Shimuzu, B., Nakagawa, Y., Hattori, K., Nishimura, K., Kurihara, N., et al., 1997. Molting hormone and larvicidal activities of aliphatic acyl analogs of dibenzoylhydrazine insecticides. *Steroids* 62, 638–642.
- Singh, G., Sidhu, H.S., 1992. Effect of juvenile hormone analogues on the morphogenesis of apteriform mustard sphid, *Lipaphis Erysimi* (Kalt.). *J. Insect Sci.* 5(1), 73–74.
- Slama, K., 1995. Hormonal status of RH-5849 and RH-5992 synthetic ecdysone agonists (ecdysoids) examined

- on several standard bioassays for ecdysteroids. *Eur. J. Entomol.* 92, 317–323.
- Slama, K., Romanuk, M., Sorm, F., 1974. *Insect Hormones and Bioanalogs*. Springer, New York, NY, 477 pp.
- Slama, K., Williams, C.M., 1996. The juvenile hormone. V. The sensitivity of the bug, *Pyrrhocoris apterus*, to a hormonally active factor in american paper-pulp. *Biol. Bull.* 130, 235–246.
- Smagghe, G., Braeckman, B.P., Huys, N., Raes, H., 2003a. Cultured mosquito cells *Aedes albopictus* C6/36 (Dip., Culicidae) responsive to 20-hydroxyecdysone and non-steroidal ecdysone agonist. *J. Appl. Entomol.* 127, 167–173.
- Smagghe, G., Carton, B., Decombel, L., Tirry, L., 2001. Significance of absorption, oxidation, and binding to toxicity of four ecdysone agonists in multi-resistant cotton leafworm. *Arch. Insect Biochem. Physiol.* 46, 127–139.
- Smagghe, G., Carton, B., Heirman, A., Tirry, L., 2000a. Toxicity of four dibenzoylhydrazine correlates with evagination-induction in the cotton leafworm. *Pest. Biochem. Physiol.* 68, 49–58.
- Smagghe, G., Carton, B., Wesemael, W., Ishaaya, I., Tirry, L., 1999a. Ecdysone agonists – mechanism of action and application on *Spodoptera* species. *Pestic. Sci.* 55, 343–389.
- Smagghe, G., Degheele, D., 1994. The significance of pharmacokinetics and metabolism to the biological activity of RH-5992 (Tebufenozide) in *Spodoptera exempta*, *Spodoptera exigua*, and *Leptinotarsa decemlineata*. *Pestic. Biochem. Physiol.* 49, 224–234.
- Smagghe, G., Degheele, D., 1997. Comparative toxicity and tolerance for the ecdysteroid mimic tebufenozide in a laboratory and field strain of cotton leafworm (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 90, 278–282.
- Smagghe, G., Dhadialla, T.S., Derycke, S., Tirry, L., Degheele, D., 1998. Action of the ecdysteroid agonist tebufenozide in susceptible and artificially selected beet armyworm. *Pestic. Sci.* 54, 27–34.
- Smagghe, G., Dhadialla, T.S., Lezzi, M., 2002. Comparative toxicity and ecdysone receptor affinity of non-steroidal ecdysone agonists and 20-hydroxyecdysone in *Chironomus tentans*. *Insect Biochem. Mol. Biol.* 32, 187–192.
- Smagghe, G., Eelen, H., Verschelde, E., Richter, K., Degheele, D., 1996a. Differential effects of nonsteroidal ecdysteroid agonists in Coleoptera and Lepidoptera: Analysis of evagination and receptor binding in imaginal discs. *Insect Biochem. Mol. Biol.* 26, 687–695.
- Smagghe, G., Gelman, D., Tirry, L., 1997. *In vivo* and *in vitro* effects of tebufenozide and 20-hydroxyecdysone on chitin synthesis. *Arch. Insect Biochem. Physiol.* 41, 33–41.
- Smagghe, G., Medina, P., Schuyesmans, S., Tirry, L., Viñuela, E., 2000b. Insecticide resistant monitoring of tebufenozide for managing *Spodoptera exigua* (Hübner [1808]). *Bol. San. Veg. Plagas* 26, 475–481.
- Smagghe, G., Nakagawa, Y., Carton, B., Mourad, A.K., Fujita, T., *et al.*, 1999b. Comparative ecdysteroid action of ring-substituted dibenzoylhydrazines in *Spodoptera exigua*. *Arch. Insect Biochem. Physiol.* 41, 42–53.
- Smagghe, G., Pineda, S., Carton, B., Del Estal, P., Budia, F., Viñuela, E., 2003b. Toxicity and kinetics for methoxyfenozide in greenhouse-selected *Spodoptera exigua* (Lepidoptera: Noctuidae). *Pest Mgt. Sci.* 59, 1203–1209.
- Smagghe, G., Salem, H., Tirry, L., Degheele, D., 1996b. Action of a novel insect growth regulator tebufenozide against different developmental stages of four stored product insects. *Parasitica* 52, 61–69.
- Smagghe, G., Viñuela, E., Budia, F., Degheele, D., 1996c. *In vivo* and *in vitro* effects of the nonsteroidal ecdysteroid agonist tebufenozide on cuticle formation in *Spodoptera exigua*: An ultrastructural approach. *Arch. Insect Biochem. Physiol.* 32, 121–134.
- Smagghe, G., Viñuela, E., Van Limbergen, H., Budia, F., Tirry, L., 1999c. Nonsteroidal moulting hormone agonists: effects on protein synthesis and cuticle formation in Colorado potato beetle. *Entomol. Exp. Appl.* 93, 1–8.
- Sohi, S.S., Palli, S.R., Retnakaran, A., 1995. Forest insect cell lines responsive to 20-hydroxyecdysone and two nonsteroidal ecdysone agonists, RH-5849 and RH-5992. *J. Insect Physiol.* 41, 457–464.
- Soltani, N., Aribi, N., Berghiche, H., Lakbar, S., Smagghe, G., 2002. Activity of RH-0345 on ecdysteroid production and cuticle secretion in *Tenebrio molitor* pupae *in vivo* and *in vitro*. *Pestic. Biochem. Physiol.* 72, 83–90.
- Soltani, N., Besson, M.T., Delachambre, J., 1984. Effect of diflubenzuron on the pupal-adult development of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae): growth and development, cuticle secretion, epidermal cell density and DNA synthesis. *Pestic. Biochem. Physiol.* 21, 256–264.
- Song, Q., Ma, M., Ding, T., Ballarino, J., Wu, S.-J., 1990. Effects of a benzodioxole, J2581 (5-ethoxy-6-(4-methoxyphenyl)methyl-1,3-benzodioxole), on vitellogenesis and ovarian development of *Drosophila melanogaster*. *Pestic. Biochem.* 37(1), 12–23.
- Sonoda, M., Kuwano, E., Taniguchi, E., 1995. Precocious metamorphosis induced by 1,5-disubstituted imidazoles is counteracted by tebufenozide (RH-5992), an ecdysteroid agonist. *Nippon Noyaku Gakkaishi* 20, 325–327.
- Sotomatsu, T., Nakagawa, Y., Fujita, T., 1987. Quantitative structure-activity studies of benzoylphenylurea larvicides. IV. Benzoyl ortho substituent effects and molecular conformation. *Pestic. Biochem. Physiol.* 27, 156–164.
- Spindler, K.D., Spindler-Barth, M., Londershausen, M., 1990. Chitin metabolism: a target for drugs against parasites. *Parasitol. Res.* 76, 283–288.
- Spindler-Barth, M., Spindler, K.-D., 1998. Ecdysteroid resistant subclones of the epithelial cell line from *Chironomus tentans* (Insecta, Diptera). I. Selection and

- characterization of resistant clones. *In Vitro Cell. Devel. Biol. Animal* 34, 116–122.
- Spindler-Barth, M., Turberg, A., Spindler, K.-D., 1991. On the action of RH 5849, a nonsteroidal ecdysteroid agonist, on a cell line from *Chironomus tentans*. *Arch. Insect Biochem. Physiol.* 16, 11–18.
- Srivastava, U.S., Jaiswal, A.K., 1989. Precocene II Induced effects in the aphid *Aphid craccivora* Koch. *Insect Sci. Appl.* 10(4), 471–475.
- Sterk, G., Hassan, S.A., Baillo, M., Bakker, F., Bigler, F., *et al.*, 1999. Results of the seventh joint pesticide testing programme carried out by the IOBC/WPRS-Working Group Pesticides and Beneficial Organisms. *BioControl* 44, 99–117.
- Staal, G.B., 1975. Insect growth regulators with juvenile hormone activity. *Annu. Rev. Entomol.* 20, 417–460.
- Staal, G.B., 1982. Insect control with growth regulators interfering with the endocrine system. *Entomol. Exp. Appl.* 31, 15–23.
- Stevens, P.S., Stevens, D., 1994. An insect growth regulator for controlling leafrollers in kiwifruit. In: Proc. 47th N.Z. Plant Prot. Conf., pp. 310–313.
- Strand, M.R., Goodman, W.G., Baehrecke, E.H., 1991. The juvenile hormone titer of *Trichoplusia ni* and its potential role in embryogenesis of the polyembryonic wasp *Copidosoma floridanum*. *Insect Biochem.* 21(2), 205–214.
- Suh, C.P.-C., Orr, D.B., Van Duyn, J.W., 2000. Effect of insecticides on *Trichogramma exiguum* (Trichogrammatidae: Hymenoptera) preimaginal development and adult survival. *J. Econ. Entomol.* 93(3), 577–583.
- Suhr, S.T., Gil, E.B., Senut, M.-C., Gage, F.H., 1998. High level transactivation by a modified *Bombyx* ecdysone receptor in mammalian cells without exogenous retinoid X receptor. *Proc. Natl Acad. Sci. USA* 95, 7999–8004.
- Sun, X., Barrett, B.A., 1999. Fecundity and fertility changes in adult codling moth (Lepidoptera: Tortricidae) exposed to surfaces treated with tebufenozide and methoxyfenozide. *J. Econ. Entomol.* 92, 1039–1044.
- Sun, X., Barrett, B.A., Biddinger, D.J., 2000. Fecundity and fertility changes in adult redbanded leafroller and obliquebanded leafroller (Lepidoptera: Tortricidae) exposed to surfaces treated with tebufenozide and methoxyfenozide. *Entomol. Exp. Applic.* 94, 75–83.
- Sundaram, K.M.S., Sundaram, A., Sloane, L., 1996. Foliar persistence and residual activity of tebufenozide against spruce budworm larvae. *Pestic. Sci.* 47, 31–40.
- Sundaram, M., Palli, S.R., Ishaaya, I., Krell, P.J., Retnakaran, A., 1998a. Toxicity of ecdysone agonists correlates with the induction of CHR3 mRNA in the spruce budworm. *Pestic. Biochem. Physiol.* 62, 201–208.
- Sundaram, M., Palli, S.R., Krell, P.J., Sohi, S.S., Dhadialla, T.S., *et al.*, 1998b. Basis for selective action of a synthetic molting hormone agonist, RH-5992 on lepidopteran insects. *Insect Biochem. Mol. Biol.* 28, 693–704.
- Sundaram, M., Palli, S.R., Smagghe, G., Ishaaya, I., Feng, Q.L., *et al.*, 2002. Effect of RH-5992 on adult development in the spruce budworm, *Choristoneura fumiferana*. *Insect Biochem. Mol. Biol.* 32, 225–231.
- Swevers, L., Drevet, J.R., Lunke, M.D., Iatrou, K., 1995. The silkworm homolog of the *Drosophila* ecdysone receptor (B1 Isoform): cloning and analysis of expression during follicular cell differentiation. *Insect Biochem. Mol. Biol.* 25, 857–866.
- Takagi, M., Tsuda, Y., Wada, Y., 1995. Laboratory evaluation of a juvenile hormone mimic, pyriproxyfen, against *Chironomus fusciceps* (Diptera: Chironomidae). *J. Am. Mosquito Control Assoc.* 11(4), 474–475.
- Tatar, M., Yin, C.-M., 2001. Slow aging during insect reproductive diapause: Why butterflies, grasshoppers and flies are like worms. *Exp. Gerontol.* 36, 723–738.
- Taylor, M.A., 2001. Recent developments in ectoparasiticide. *Vet. J.* 161, 253–268.
- Thomas, H.E., Stunnenberg, H.G., Stewart, A.F., 1993. Heterodimerization of the *Drosophila* ecdysone receptor with retinoid X receptor and ultraspiracle. *Nature* 362, 471–475.
- Tobe, S.S., Ruegg, R.P., Stay, B.A., Baker, F.A., Miller, C.A., *et al.*, 1985. Juvenile hormone titer and regulation in the cockroach *Diploptera punctata*. *Experientia* 41, 1028–1034.
- Tobe, S.S., Stay, B., 1985. Structure and regulation of the corpus allatum. *Adv. Insect Physiol.* 18, 305–432.
- Tomlin, C.D.S. (Ed.) 2000. The Pesticide Manual, 12th edn. British Crop Protection Council Publications.
- Toya, T., Fukasawa, H., Masui, A., Endo, Y., 2002. Potent and selective partial ecdysone agonist activity of chromafenozide in sfg cells. *Biochem. Biophys. Res. Comm.* 292, 1087–1091.
- Tran, H.T., Askari, H.B., Shaaban, S., Price, L., Palli, S.R., *et al.*, 2000. Reconstruction of ligand-dependent transactivation of *Choristoneura fumiferana* ecdysone receptor in yeast. *J. Mol. Endocrinol.* 15, 1140–1153.
- Tran, H.T., Shaaban, S., Askari, H.B., Walfish, P.G., Raikhel, A.S., *et al.*, 2001. Requirement of co-factors for the ligand-mediated activity of the insect ecdysteroid receptor in yeast. *J. Mol. Endocrinol.* 27, 191–209.
- Trisyono, A., Chippendale, M., 1997. Effect of the nonsteroidal ecdysone agonists, methoxyfenozide and tebufenozide, on the European corn borer (Lepidoptera: Pyralidae). *J. Econ. Entomol.* 90(6), 1486–1492.
- Trisyono, A., Chippendale, M., 1998. Effect of the ecdysone agonists, RH-2485 and tebufenozide, on the Southwestern corn borer, *Diatraea grandiosella*. *Pestic. Sci.* 53, 177–185.
- Trisyono, A., Goodman, C.L., Grasela, J.J., McIntosh, A.H., Chippendale, G.M., 2000. Establishment and characterization of an *Ostrinia nubilalis* cell line, and its response to ecdysone agonists. *In Vitro Cell. Devel. Biol. Animal* 36, 400–404.
- Truman, J.W., Rountree, D.B., Reiss, S.E., Schwartz, L.M., 1983. Ecdysteroids regulate the release and action of eclosion hormone in the tobacco hornworm, *Manduca sexta* (L.). *J. Insect Physiol.* 29, 895–900.

- Tsunoda, K., Doki, H., Nishimoto, K., 1986. Effect of developmental stages of workers and nymphs of *Reti-culitermes speratus* (Kolbe) (Isoptera: Rhinotermitidae) on caste differentiation induced by JHA treatment. *Mat. Organismen Berlin* 21(1), 47–62.
- Tyagi, B.K., Kalyanasundaram, M., Das, P.K., Somachary, N., 1985. Evaluation of a new compound (VCRC/INS/A-23) with juvenile hormone activity against mosquito vectors. *Ind. J. Med. Res.* 82, 9–13.
- Uchida, M., Asai, T., Sugimoto, T., 1985. Inhibition of cuticle deposition and chitin biosynthesis by a new insect growth regulator buprofezin in *Nilaparvata lugens* Stål. *Agric. Biol. Chem.* 49, 1233–1234.
- Ujváry, I., Matolcsy, G., Riddiford, L.M., Hiruma, K., Horwath, K.L., 1989. Inhibition of spiracle and crochet formation and juvenile hormone activity of isothiocyanate derivatives in the tobacco hornworm, *Manduca sexta*. *Pestic. Biochem. Physiol.* 35, 259–274.
- Unger, E., Cigan, A.M., Trimnell, M., Xu, R.-J., Kendall, T., et al., 2002. A chimeric ecdysone receptor facilitates methoxyfenozide-dependent restoration of male fertility in ms45 maize. *Transgenic Res.* 11, 455–465.
- Unnithan, G.C., Andersen, J.F., Hisano, T., Kuwano, E., Feyereisen, R., 1995. Inhibition of juvenile hormone biosynthesis and methyl farnesoate epoxidase activity by 1,5-disubstituted imidazoles in the cockroach, *Diploptera punctata*. *Pestic. Sci.* 43, 13–19.
- Vail, K.M., Williams, D.F., 1995. Pharaoh ant (Hymenoptera: Formicidae) colony development after consumption of pyriproxyfen baits. *J. Econ. Entomol.* 88(6), 1695–1702.
- Van Daalen, J.J., Meltzer, J., Mulder, R., Wellinga, K., 1972. A selective insecticide with a novel mode of action. *Naturwissenschaften* 59, 312–313.
- Van de Veire, M., Smagghe, G., Degheele, D., 1996. Laboratory test method to evaluate the effect of 31 pesticides on the predatory bug, *Orius laevigatus* (Het.: Anthocoridae). *Entomophaga* 41, 235–243.
- Van Laecke, K., Degheele, D., 1991. Detoxification of diflubenzuron and teflubenzuron in the larvae of the beet armyworm (*Spodoptera exigua*) (Lepidoptera: Noctuidae). *Pestic. Biochem. Physiol.* 40, 181–190.
- Van Laecke, K., Smagghe, G., Degheele, D., 1995. Detoxifying enzymes in greenhouse and laboratory strain of beet armyworm (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 88, 777–781.
- Veech, J.A., 1978. The effect of diflubenzuron on the reproduction of free living nematodes. *Nematologica* 24, 312–320.
- Veras, M., Mavroidis, M., Kokolakis, G., Gourzi, P., Zacharopoulou, A., et al., 1999. Cloning and characterization of CcEcR: An ecdysone receptor homolog from the Mediterranean fruit fly *Ceratitidis capitata*. *Eur. J. Biochem.* 265, 798–808.
- Viñuela, E., Budia, F., 1994. Ultrastructure of *Ceratitidis capitata* Wiedemann larval integument and changes induced by cyromazine. *Pestic. Biochem. Physiol.* 48, 191–201.
- Vogt, H. (Ed.) 1994. Pesticides and Beneficial Organisms, IOBC/WPRS Bulletin. vol. 17. IOBC/WPRS Publications.
- Wakgari, W., Giliomee, J., 2001. Effects of some conventional insecticides and insect growth regulators on different phenological stages of the white – scale, *Ceroplastes destructor* Newstead (Hemiptera: Coccidae), and its primary parasitoid, *Aprostocetus ceroplastae* (Girault) (Hymenoptera: Eulophidae). *Intern. J. Pest Manag.* 47(3), 179–184.
- Walton, L., Long, J.W., Spivey, J.A., 1995. Use of Confirm insecticide for control of beet armyworm in cotton under Section 18 in MS and AL. *Proc. Beltwide Cotton Conf.* 2, 46–47.
- Watkinson, I.A., Clarke, B.S., 1973. The insect moulting hormone system as a possible target site for insecticidal action. *PANS* 14, 488–506.
- Wearing, C.H., 1998. Cross-resistance between azinphosmethyl and tebufenozide in the greenheaded leafroller, *Planotortrix octo*. *Pestic. Sci.* 54, 203–211.
- Wigglesworth, V.B., 1936. The function of the corpus allatum in the growth and reproduction of *Rhodnius prolixus* (Hemiptera). *Q. J. Microsc. Sci.* 79, 91–121.
- Wildboltz, T., 1988. Integrated pest management in Swiss apple orchards: stability and risks. *Entomol. Exp. Appl.* 49, 71–74.
- Williams, C.M., 1952. Physiology of insect diapause. IV. The brain and prothoracic glands as an endocrine system in the *Cecropia* silkworm. *Biol. Bull.* 103, 120–138.
- Williams, C.M., 1967. The juvenile hormone. II. Its role in the endocrine control of molting, pupation, and adult development in the *Cecropia* silkworm. *Biol. Bull.* 121, 572–585.
- Williams, D.R., Chen, J.-H., Fisher, M.J., Rees, H.H., 1997. Induction of enzymes involved in molting hormone (ecdysteroid) inactivation by ecdysteroids and an agonist, 1,2-dibenzoyl-1-tert-butylhydrazine (RH-5849). *J. Biol. Chem.* 272, 8427–8432.
- Williams, D.R., Fisher, M.J., Smagghe, G., Rees, H.H., 2002. Species specificity of changes in ecdysteroid metabolism in response to ecdysteroid agonists. *Pestic. Biochem. Physiol.* 72, 91–99.
- Williams, R.E., Berry, J.G., 1980. Evaluation of CGA 72662 as a topical spray and feed additive for controlling house fly breeding in chicken manure. *Poultry Sci.* 59, 2207–2212.
- Wilson, T.G., Ashok, M., 1998. Insecticide resistance resulting from an absence of target-site gene product. *Proc. Natl Acad. Sci. USA* 95(24), 14040–14044.
- Wilson, T.G., Fabian, J., 1986. A *Drosophila melanogaster* mutant resistant to a chemical analog of juvenile hormone. *Devel. Biol.* 118(1), 190–201.
- Wing, K.D., 1988. RH 5849, a non-steroidal ecdysone agonist: effects on a *Drosophila* cell line. *Science* 241, 467–469.
- Wing, K.D., Aller, H.E., 1990. Ecdysteroid agonists as novel insect growth regulators. In: Cassida, J.E. (Ed.), Pesticides and Alternatives: Innovative Chemical and

- Biological Approaches to Pest Control. Elsevier, pp. 251–257.
- Wing, K.D., Slaweck, R., Carlson, G.R., 1988. RH-5849, a nonsteroidal ecdysone agonist: effects on larval Lepidoptera. *Science* 241, 470–472.
- Wroblewski, V.J., Harshman, L.G., Hanzlik, T.N., Hammock, B.D., 1990. Regulation of juvenile hormone esterase gene expression in the tobacco budworm (*Heliothis virescens*). *Arch. Biochem. Biophys.* 278, 461–466.
- Wurtz, *et al.*, 2000. A new model for 20-hydroxyecdysone and dibenzoylhydrazine binding: A homology modeling and docking approach. *Protein Sci.* 9, 1073–1084.
- Wyatt, G.R., Davey, K.G., 1996. Cellular and molecular actions of juvenile hormone. II. Roles of juvenile hormone in adult insects. *Adv. Insect Physiol.* 26, 1–155.
- Xu, Y., Fang, F., Chu, Y.-X., Jones, D., Jones, G., 2002. Activation of transcription through the ligand-binding pocket of the orphan nuclear receptor spiracle. *Eur. J. Biochem.* 269, 6026–6036.
- Yanagi, M., Watanabe, T., Masui, A., Yokoi, S., Tsukamoto, Y., *et al.*, 2000. ANS-118: A novel insecticide. *Proc. Brighton Crop Prot. Conf.* 2, 27–32.
- Yanagi, M., 2000. Development of a novel lepidopteran insect control agent, chromofenozide (MATRIC), and prospect of success. *Agrochem. Jpn.* 76, 16–18.
- Yao, T.-P., Forman, B.M., Jiang, Z., Cherbas, L., Chen, J.-D., *et al.*, 1995. Functional ecdysone receptor is the product of *EcR* and *Ultraspiracle* genes. *Nature* 366, 476–479.
- Yao, T.-P., Segraves, W.A., Oro, A.E., McKeown, M., Evans, R.M., 1992. *Drosophila* ultraspiracle modulates ecdysone receptor function via heterodimer formation. *Cell* 71, 63–72.
- Yen, J.L., Batterham, P., Gelder, B., McKenzie, J.A., 1996. Predicting resistance and managing susceptibility to cyromazine in the Australian sheep blowfly *Lucilia cuprina*. *Aust. J. Exp. Agri.* 36, 413–420.
- Zhang, J., Cress, D.E., Palli, S.R., Dhadialla, T.S., 2003. cDNAs encoding ecdysteroid receptors of whitefly and their use in screening for pesticides. *PCT Int. Appl. WO 2003027266*. pp. 85.
- Zhang, J., Wyatt, G.R., 1996. Cloning and upstream sequence of a juvenile hormone-regulated gene from the migratory locust. *Gene* 175(1–2), 193–197.
- Zhou, X., Riddiford, L.M., 2002. Broad specifies pupal development and mediates the ‘status quo’ action of juvenile hormone on the pupal-adult transformation in *Drosophila* and *Manduca*. *Development* 129, 2259–2269.
- Zimowska, G., Mikólajczyk, P., Silhacek, D.L., Oberlander, H., 1994. Chitin synthesis in *Spodoptera frugiperda* wing discs. II. Selective action of chlorfluazuron on wheat germ agglutinin binding and cuticle ultrastructure. *Arch. Insect Biochem. Physiol.* 27, 89–108.
- Zitnanova, I., Adams, M.E., Zitnan, D., 2001. Dual ecdysteroid action on the epitracheal glands and central nervous system preceding ecdysis of *Manduca sexta*. *J. Exp. Biol.* 204, 3483–3495.
- Zoebelein, G., Hammann, I., Sirrenberg, W., 1980. BAY-SIR-8514, a new chitin synthesis inhibitor. *J. Appl. Entomol.* 89, 289–297.
- Zuo, J., Chua, N.-H., 2000. Chemical-inducible systems for regulated expression of plant genes. *Curr. Opin. Biotech.* 11, 146–151.

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