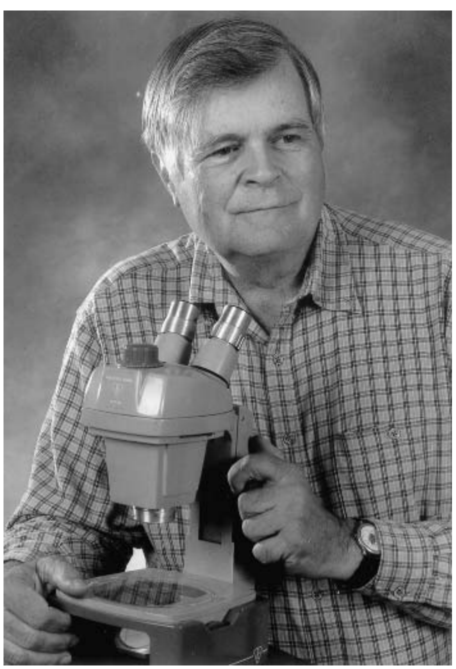
ENTOMOPATHOGENIC NEMATOLOGY

Edited by
Randy Gaugler

Entomopathogenic Nematology

DEDICATION



This volume is dedicated to George O. Poinar, Jr, in recognition of a life devoted to nematological research. His early work elucidating the complex relationship between *Steinernema* and *Heterorhabditis* and their bacterial symbionts formed a crucial bridge between the pioneering studies of Rudolf Glazer and all those who followed. Dr Poinar was a pivotal figure in the establishment of Biosys, a key milestone in the development of entomopathogenic nematodes. He has authored or co-authored five books and more than 400 publications on nematode systematics, structure, bionomics, paleontology, natural enemies, epizootiology, and host–parasite relationships. Recent interests reflect a passion for the evolutionary history of nematodes as revealed by fossils in amber. To have published across such a breath of topics is exceptional, but to have made significant contributions to each is extraordinary.

Entomopathogenic Nematology

Edited by

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Preface

The field of entomopathogenic nematology has experienced exponential growth over the past decade. A hundred different laboratories explore these nematodes and their bacterial symbionts in more than 60 countries from every inhabited continent. Despite research breadth that extends from molecular biology to field ecology, the discipline is unified by a common interest in biological control. Thirty years ago, the idea of using nematodes to control pest populations was a vague promise held by the handful of researchers working with these obscure insect parasites. Today, they are no longer a laboratory curiosity but have begun to gain acceptance as environmentally benign alternatives to chemical insecticides. Twenty-five thousand hectares of Florida citrus are treated with nematodes annually. Nematodes are applied against insect pests of cranberries (black vine weevil, cranberry girdler), turfgrass (mole cricket, cutworms, armyworms, fleas, billbugs), artichokes (plume moth), mushrooms (sciariid flies), apples and peaches (*Carposina*), ornamentals (black vine weevil, fungus gnats), and many other pests of horticulture, agriculture, home and garden.

Realization of entomopathogenic nematode practical use spurred developments across a far broader scientific front. Recent years have seen an intensive worldwide search for fresh genetic material resulting in thousands of new isolates. This, in turn, drove a need for improved taxonomic tools and soon doubled the number of described species. Molecular insights generated with the closely related (same superfamily) and exhaustively studied nematode *Caenorhabditis elegans* are now being applied to entomopathogenic species – no other biological control agent can tap into such a huge and relevant knowledge base to exploit cutting-edge methodologies. Advances or breakthroughs have also been made in elucidating foraging strategies, room temperature formulations, ecological roles, population dynamics, and innumerable other facets of entomopathogenic nematology.

This volume captures the full breath of basic and applied information on entomopathogenic nematodes. Experts from nine countries contribute authoritative chapters offering a comprehensive account of up-to-date findings, including the latest achievements in genetic engineering, biodiversity, fermentation, toxins, soil ecology,

host–parasite interactions, symbiosis, safety considerations and management. The book begins by reviewing fundamental biology and setting a taxonomic and phylogenetic foundation. The extensive and far-flung survey record for these ubiquitous nematode species is analysed within the context of a comprehensive database that summarizes their distribution. Emphasis then shifts to functional processes involved in parasitism and nematode ecology. This broad-based effort attempts to integrate work on entomopathogenic nematodes with better-studied nematode species. Adaptations in evolving from a primitive free-living existence to parasitism receive special emphasis. Subsequent chapters illustrate technological advances relevant to nematodes as biological insecticides. Current control methodologies in nematode commercial evolution are stressed with analysis of critical issues identified by earlier chapters that impact on nematodes application, production, quality and commercialization strategies. Research gaps, of which there are many, are identified and promising approaches suggested. The focus is on setting a research agenda that will permit entomopathogenic nematodes to reach their full potential.

Entomopathogenic nematodes are a nematode–bacterial complex; a symbiotic relationship based on mutualism. Unlike the earlier volume (*Entomopathogenic Nematodes in Biological Control*, 1990, CRC Press), the bacterial symbiont now moves from background to sharing centre stage. Emphasis is placed on the bacterial partner's role in maintaining the symbiotic relationship and in killing the host, as well as the extraordinary promise that a bewilderingly diverse array of different metabolites the bacteria produce appear to offer biotechnology. Thus, whereas the focus for commercial development had been exclusively nematode oriented, the bacteria seem poised to become the new focal point.

This volume is dedicated to George O. Poinar Jr, as an acknowledgement of his deep influence on my own research efforts on entomopathogenic nematodes, particularly during my graduate and postdoctoral training. I also wish to thank the many contributors who made the book possible. Finally, I express my gratitude to Cheryl, who assisted in this endeavour in innumerable ways.

Randy Gaugler
April 2001

1 Taxonomy and Systematics

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1.1. Introduction

The discovery of entomopathogenic nematode species and the rate at which they have been described is correlated with the historical need for biological alternatives to manage insect pests. After the initial discovery and subsequent development of *Steinernema glaseri* as a biological control agent in the early 20th century, research on entomopathogenic nematodes remained somewhat dormant as chemical-based pest control measures remained cheap, effective and relatively unregulated. Upon recognition of their negative environmental effects in the 1960s, pesticides gradually became more

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restricted, less effective, and much more costly. Consequently, the search for biological alternatives to chemical-based pest management programmes received renewed attention from scientists. By the 1980s, fuelled by an enormous infusion of resources from government and industry, research on entomopathogenic nematodes rapidly expanded. The search for new species of nematodes that could provide effective control of a persistent pest – and be marketed as such – was on.

1.2. General Biology

The invasive stage (= infective juveniles) of *Steinernema* carries cells of a symbiotic bacterium in the anterior part of the intestine (genus *Xenorhabdus*). When infective juveniles enter the haemocoel of a host, they release the bacterium. The bacterium multiplies rapidly in the insect blood and kills the insect by septicaemia, usually within 24–48 h. The nematodes then change to feeding third-stage juveniles, feed on the bacteria, and moult in succession to the fourth stage where they become adult females and males of the first generation. After mating, the females lay eggs that hatch as first-stage juveniles and moult successively to the second, third and fourth stages. The fourth-stage juveniles develop into adult females and males of the second generation. The adults mate and the eggs produced by the second-generation females hatch as first-stage juveniles that moult to the second stage. Nematode reproduction continues until resources in the cadaver are depleted, usually allowing for two to three generations (long cycle, Fig. 1.1). If the food supply is limited, the eggs produced by the first-generation females develop directly into infective juveniles (short cycle, Fig. 1.1). As resources in the cadaver are extinguished, late second-stage juveniles cease to feed and incorporate a pellet of bacteria in the bacterial chamber, or vesicle. They then moult to pre-infective and infective stages, retaining the cuticle of the second stage as a sheath. The infective juveniles then typically leave the cadaver in search of a new host. Infective juveniles do not feed and can survive in the soil for several months.

The life cycle of *Neosteinernema* is similar to that of *Steinernema*, except that *Neosteinernema* has only one generation, females move out into the environment and the offspring become infective juveniles in the female body before emerging from it. A bacterial endosymbiont of *Neosteinernema* has been observed, but remains uncharacterized. Little is known of this nematode beyond its taxonomic description.

The life cycle of *Heterorhabditis* is also similar to that of *Steinernema*, but the hermaphroditic female in *Heterorhabditis* replaces the first generation in *Steinernema*, and the symbiotic bacterium is *Photorhabdus* instead of *Xenorhabdus*.

1.3. Origins

Poinar (Poinar, 1983, 1993) proposed a mid-Palaeozoic origin (375 million years ago) for *Steinernema* and *Heterorhabditis*. He also postulated that independent, convergent evolution produced their similar morphology and life history traits. Based on similarities of the buccal capsule and male tail morphology, Poinar suggested that *Heterorhabditis* evolved from a '*Pellioditis*-like' ancestor in a sandy, marine environment, while *Steinernema* arose from terrestrial 'proto-Rhabditonema' ancestors (Poinar, 1993).

A preliminary evolutionary tree of the Nematoda based on 18S ribosomal DNA (Blaxter *et al.*, 1998) bolsters Poinar's ideas concerning the origins of *Heterorhabditis*

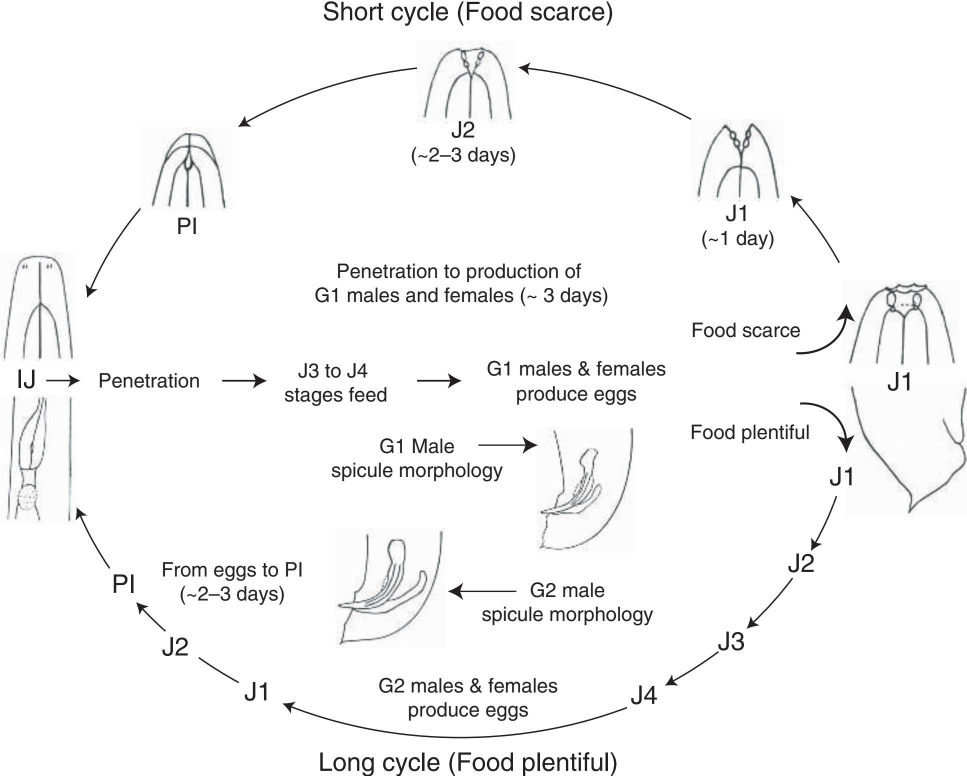


Fig. 1.1. Diagram of the life cycle of *Steinernema* with illustrations of different stages that are important for identification (based on *S. scapterisci*). G1 = first generation, G2 = second generation, J1 = first-stage juvenile, J2 = second-stage juvenile, J3 = third-stage non-infective juvenile, PI = pre-infective stage juvenile, IJ = third-stage infective juvenile, J4 = fourth-stage juvenile. Morphological differences between long and short cycles of *Heterorhabditis* have also been observed (Wouts, 1979).

and *Steinernema*. The tree depicts *Heterorhabditis* as being most closely related to an order of vertebrate parasites, the Strongylida. *Heterorhabditis* and Strongylida share a most recent common ancestor with *Pellioditis* (Rhabditoidea, a superfamily of order Rhabditida). The same tree depicts *Steinernema* as being most closely related to the Panagrolaimoidea and Strongyloidae (both superfamilies of Rhabditida), and as a member of a larger clade that includes plant parasitic, fungivorous, and bactivorous species of the orders Aphelenchida, Tylenchida and the Cephalobidae (Order of the Rhabditida). Thus, preliminary molecular data suggest that *Heterorhabditis* arose from a free-living bactivorous ancestor, while reconstruction of the trophic habits of Steinernematid ancestors remains ambiguous.

Poinar's hypothesis that *Heterorhabditis* and *Steinernema* do not share an exclusive common ancestry is upheld by Blaxter *et al.* (1998) and is consistent with other analyses (Sudhaus, 1993; Liu *et al.*, 1997; Adams *et al.*, 1998). All suggest that *Heterorhabditis* and *Steinernema* independently evolved relationships with bacteria and insects from disparate, unrelated ancestors. However, Burnell and Stock (2000) hint that these

findings may be premature, and offer an astonishing scenario – that the genus *Steinernema* is paraphyletic, with *Heterorhabditis* arising from within the Steinernematidae.

1.4. Molecular Systematics

Hampering efforts to describe new species of entomopathogenic nematodes is a rapidly diminishing pool of taxonomists. To a non-specialist, different species of *Steinernema*, and especially *Heterorhabditis*, look similar. Although excellent morphology-based taxonomic keys exist (Hominick *et al.*, 1997), the task of sorting through numerous field-collected nematode samples and making species level taxonomic identifications can be frustrating. Recent advances in molecular biology and evolutionary theory, while no substitute for the expertise of a taxonomist, can provide nematologists with additional tools for identifying, delimiting and representing species of entomopathogenic nematodes (Liu *et al.*, 2000). Several recent species descriptions integrate morphological and molecular genetic data (Gardner *et al.*, 1994; Liu and Berry, 1996a; Stock *et al.*, 1996; Stock, 1997; Stock *et al.*, 1998; Van Luc *et al.*, 2000).

Molecular characters can be used to identify, diagnose and delimit species in the same way as morphological characters. The allure of molecular data is their clear genetic basis, a compelling advantage given the significant amount of environmental and host-induced morphological variation displayed by entomopathogenic nematodes. Potentially sensitive, objective and powerful, molecular characters can also be dangerous when wielded carelessly. Below we discuss the utility of some common molecular markers used to identify and diagnose species of entomopathogenic nematodes.

1.4.1. Species identification

In theory, a nematode's entire genome can be drawn upon for molecular genetic markers to discriminate a particular taxonomic rank. Nevertheless, for ease of comparison, markers that have the greatest representation in genetic databases are the most convenient starting points for thoroughly investigating the taxonomic status of an unidentified entomopathogenic nematode. Consequently, markers that are not easily databased or cannot produce consistently replicable results among comparable taxa and investigators are falling out of favour. Often overlooked are those that give an indication of population structure (subspecific variation), vital information for determining the extent of species boundaries.

Based on representation in genetic databases, the most popular marker for identifying unknown nematodes is the DNA sequence of the 18S or small ribosomal subunit (SSU) RNA gene (Blaxter *et al.*, 1998; Dorris *et al.*, 1999). For non-nematologists, or those unfamiliar with entomopathogenic nematodes, this marker can distinguish an unknown sample as being a nematode and point to whether it is a heterorhabditid or steinernematid. A table of nematode 18S rDNA PCR primers is posted at: www./nema.cap.ed.ac.uk/biodiversity/sourhope/nemoprimers.html

For assigning material to species groups the molecular marker of choice for *Heterorhabditis* and *Steinernema* is DNA sequence of the internal transcribed spacer region of the ribosomal gene array (ITS rDNA). This marker exhibits substantial variation among species of *Heterorhabditis* and *Steinernema* (Adams *et al.*, 1998; Szalanski

et al., 2000; Nguyen *et al.*, 2001) and is well-represented in public DNA sequence databases. Evidence of variation within populations exists for some entomopathogenic nematodes (B. Adams and T. Powers, unpublished data), and such variation needs to be assessed carefully, especially if the marker is used for phylogenetic studies. Other loci that have proved useful for discrimination at the species level are the cytochrome oxidase (COII)-16S (Szalanski *et al.*, 2000) and ND4 (Liu *et al.*, 1999) mitochondrial genes, and the regions of the 28S ribosomal subunit (Stock *et al.*, 2001).

Satellite DNA probes have been proposed as diagnostic tools for identifying *Heterorhabditis* and *Steinernema* (Grenier *et al.*, 1995, 1996, 1997; Abadon *et al.*, 1998). These probes can rapidly determine whether the nematode in question belongs to one of the few taxa thus far characterized, but, as with all markers, is of limited diagnostic utility until probes have been constructed for all taxa. The probes investigated thus far appear to be species specific, but less sensitive than rDNA ITS or IGS (intergenic spacer) data (Stack *et al.*, 2000). One drawback to satellite DNA probes is that negative results can only reveal those taxa to which a sample does not belong (essentially, all other species). In addition, satellite DNA probes do not produce data that can be used to recover phylogenetic relationships.

Randomly amplified polymorphic DNA (RAPD) has been used to explore genetic diversity among species and strains of entomopathogenic nematodes (Hashmi and Gaugler, 1998), determine phylogenetic relationships (Liu and Berry, 1996b), and complement descriptions of new species (Gardner *et al.*, 1994; Liu and Berry, 1996a; Stock *et al.*, 1996). RAPD analyses are extremely sensitive to slight changes in the amount and quality of the DNA template and PCR cycling parameters, and the failure to replicate the results of other researchers makes it a poor choice for use in systematics. However, with careful laboratory procedures and explicit standardization, RAPD analyses can be a powerful tool for exploring subspecific variation. For example, it has been successfully used to examine inbreeding among different populations of *Heterorhabditis* (Shapiro *et al.*, 1997).

Similar to the information content of RAPD, amplified fragment length polymorphism (AFLP) can also be a powerful molecular systematics tool. As an alternative to RAPD, AFLP has more stringent amplification conditions that improve the replicability of results. AFLP markers have yet to be applied to entomopathogenic nematodes, but proved fruitful for investigating subspecific relationships and genetic variation among closely related species of *Meloidogyne* (Semblatt, 1998). The biggest disadvantage of AFLP methods for systematic studies is that they tend to generate dominant rather than codominant alleles (Mueller and Wolfenbarger, 1999) which can produce difficulties in identifying the homologous markers needed for character-based, as opposed to distance-based (phenetic) phylogenetic analyses. Applied to entomopathogenic nematodes, AFLP genotyping and fingerprinting is an appropriate tool for exploring population structure, but will probably see more use in mapping interesting trait loci (Qin *et al.*, 2000; van der Voort *et al.*, 2000).

Few DNA sequencing projects have sampled multiple populations of a single entomopathogenic nematode species. Such projects are essential to defining species based on fixed characters. Among populations of *Heterorhabditis* small amounts of subspecific variation have been observed at the rDNA ITS (Adams *et al.*, 1998) and mtDNA ND4 loci (Blouin *et al.*, 1999; Liu *et al.*, 1999). Such variation probably exists at comparable levels within and among populations of steinernematids (but see

Szalanski *et al.*, 2000). While sequences of microsatellite loci have become a popular marker for population-level studies in other organisms (Jarne and Lagoda, 1996; Goldstein and Pollock, 1997), they have yet to be employed successfully for *Heterorhabditis* and *Steinernema*. Efforts to find microsatellite markers with subspecific variation in *Heterorhabditis* and *Steinernema* using traditional genomic library screening have failed, and enrichment techniques (Casey and Burnell, 2001) have only produced small repeat motifs ($n < 5$) from the rDNA regions (D. Casey, Maynooth, 2001, personal communication).

Although less sensitive and more difficult to database than DNA sequences, digesting PCR amplified genes with restriction enzymes (PCR RFLP) is a quick alternative to DNA sequencing. Restriction fragment profiles of the ITS (internally transcribed spacer) region exist for several species of *Heterorhabditis* and *Steinernema* (Joyce *et al.*, 1994; Nasmith *et al.*, 1996; Reid *et al.*, 1997; Pamjav *et al.*, 1999; Stack *et al.*, 2000), and can also be easily generated from the ITS sequences deposited in public databases, such as GenBank. Hominick *et al.* (1997) provide clear, user-friendly PCR RFLP protocols for the identification of *Steinernema* and *Heterorhabditis*. Using their approach new species candidates can be rapidly mined from survey samples by non-specialists and forwarded to taxonomic experts for further scrutiny. For example, sampled nematodes can be PCR amplified, digested by a few diagnostic enzymes, and have their resulting banding patterns compared with those predicted from previously studied species. Where banding patterns are identical it can be assumed that they belong to the same species (until further analysis indicates otherwise). Those that exhibit novel patterns are more immediate candidates for closer genetic and morphological examination.

1.4.2. Phylogenetic analysis

For phylogenetic analyses at the species level, most of the aforementioned markers have been explored, yet an ideal marker for resolving relationships among species of *Heterorhabditis* and *Steinernema* has yet to be found (Adams *et al.*, 1998; Liu *et al.*, 1999; Nguyen *et al.*, 2001). With the possible exception of morphological characters, DNA sequence data is the molecular marker most amenable for phylogenetic reconstruction. RFLP data, when scored correctly (i.e. as mapped cleavage sites) could only produce robust phylogenetic trees if we were to assume convergent cleavage sites are rare. Similarly, the assumption that comigrating RAPD amplicons are homologous is difficult to justify, and it has been suggested that this is a suboptimal marker for estimating relationships among entomopathogenic nematodes (Liu and Berry, 1996b).

Among the DNA sequences analysed thus far, 18S markers tend to be too conservative to confidently resolve relationships among all *Heterorhabditis* or *Steinernema* species (Liu *et al.*, 1997). On the other hand, substitution rates within ITS, COII-16S, and ND4 makers are too high, resulting in excessive homoplasy and poor homology statements (multiple sequence alignments) (Adams *et al.*, 1998; Liu *et al.*, 1999; Nguyen *et al.*, 2001; but see Szalanski *et al.*, 2000). Another caveat of all of the aforementioned studies utilizing these markers is inadequate taxon sampling (missing species). Often taxa are excluded from analyses due to the unfortunate practice of researchers withholding proprietary material. When only the disclosure of live material is an issue, preserved specimens or DNA can be forwarded to systematists who need the

information to produce up-to-date research. Such cooperation poses little threat where concerns about patenting biocontrol applications are involved, while additional material can substantially enhance phylogenetic reconstruction (Graybeal, 1998; Hillis, 1998; Poe, 1998).

Note on the names of species: In 1997 Stock *et al.* and Hominick *et al.* suggested changing the specific epithet of several entomopathogenic nematodes because the International Code of Zoological Nomenclature requires the ending of the species-group name to agree in gender with the generic name (4th edition, article 34.2). Before 1982, most species of *Steinernema* were in the genus *Neoaplectana*, which is feminine in gender. After 1982 (Wouts *et al.*, 1982) the genus *Neoaplectana* became the junior synonym of *Steinernema. Steinernema* is neutral in gender, so the trivial names of *Steinernema* must be corrected to accommodate this change.

In 1998, Liu *et al.* objected to the name changes claiming that in several instances it was unclear whether the authors of the original species descriptions regarded the names as nouns or adjectives. If so, and the evidence of usage is not decisive, then the names are to be treated as nouns and should not be changed (4th edition, article 34.2.1). Liu *et al.* argued that the specific names of *S. affinis, S. anomali, S. neocurtillis* and *S. riobravis* could be either nouns or adjectives. However, this is not entirely true. *Anomali* and *neocurtillis* may be nouns in genitive, *affinis* is an adjective, and subsequent name changes made by the authors of the original description leave little doubt as to their intent. In this chapter we follow Hominick *et al.* (1997) and the rules established by the ICZN (International Com Zoc: Nom):

S. affinis should be *S. affine* because *affinis* is an adjective.
S. intermedia should be *S. intermedium* because *intermedia* is an adjective.
S. cubana should be *S. cubanum* because the original author was involved in the name change, inferring intent (Hominick *et al.*, 1997).
S. neocurtillis should be *S. neocurtillae*, because the original author was involved in the name change, inferring intent (Hominick *et al.*, 1997).
S. oregonensis should be *S. oregonense*, because *oregonensis* is an adjective.
S. puertoricensis should be *S. puertoricense* because *puertoricensis* is an adjective.
S. rara should be *S. rarum* because *rara* is an adjective. It has been altered consistently elsewhere (i.e. *Caenorhabditis rara, Stegellesta rara, Ditylenchus rarus*.)
S. riobravis should be changed to *S. riobrave* because *riobravis* constitutes a malformation, regardless of its intent as noun or adjective.
H. indicus should be *H. indica* because *indicus* is an adjective (*indica, indicus*, and *indicum* are often used as adjectives to agree in gender with a generic name).
For all other species the original names should be used.

1.5. Taxonomy and Systematics of Steinernematidae (Chitwood and Chitwood, 1937)

1.5.1. Morphological review (based on SEM observations)

Males: Male morphological characters are the most important in the taxonomy of Steinernematidae. **Head:** Swollen or not (Fig. 1.2A and B), four cephalic papillae often larger than six labial papillae. Anterior end with or without perioral disc. **Posterior region:** The number of genital papillae is important, for example (Fig. 1.2C) this male

has ten pairs of genital papillae anterior to the cloaca (the normal number is six or seven). In the tail region, usually there are one single papilla, preanal, ventral, two pairs adanal (Fig. 1.2D*a,b*), two pairs subterminal (left arrows), and one pair subdorsal (right arrow). **Tail tip:** Either with mucron or without mucron. **Spicule:** The spicule is large

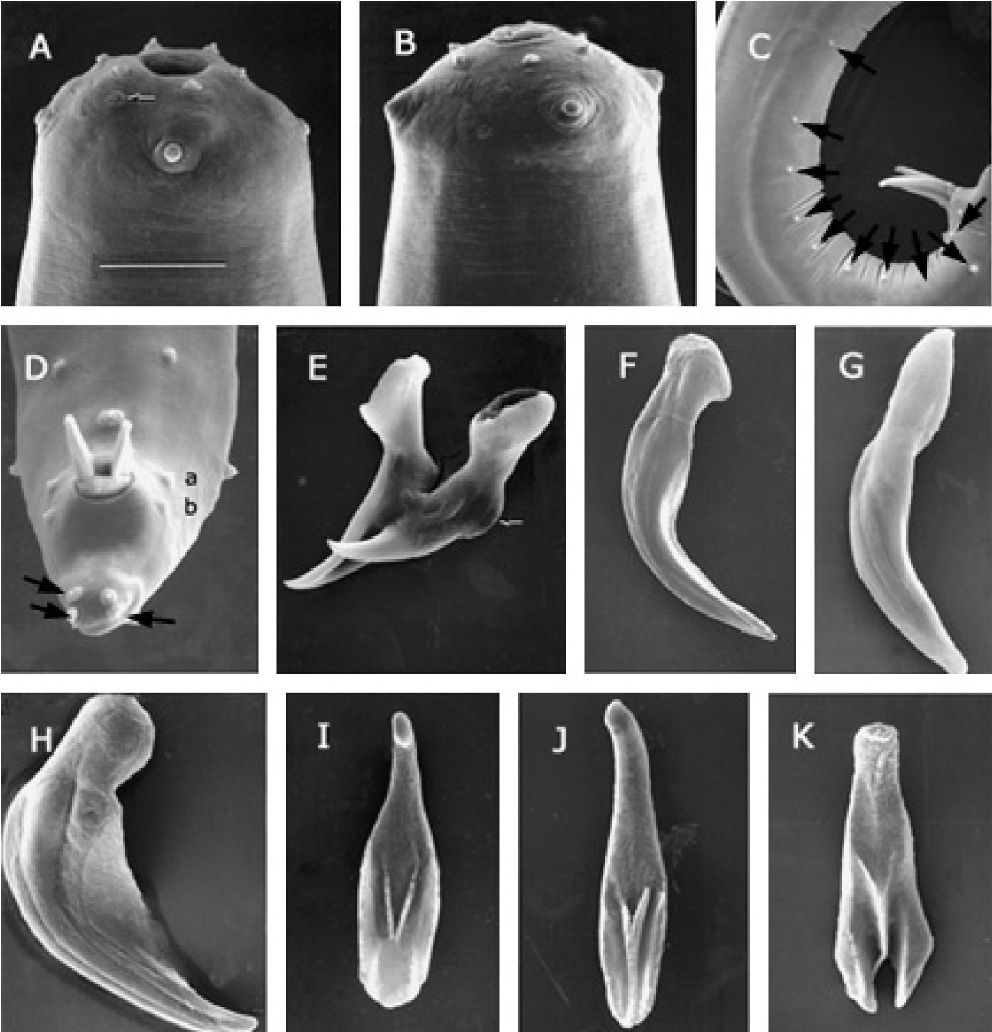


Fig. 1.2. SEM micrographs of male morphological structures of the family Steinernematidae. A, B, Heads showing stomal opening, labial and cephalic papillae; C, posterior part of a male showing spicules and pre-anal genital papillae (arrows); D, ventral view of posterior region of a male showing a single pre-anal papilla, a pair of subventral papillae, a pair of lateral papillae; two pairs of adanal papillae (*a* and *b*), two pairs of caudal, subterminal papillae (two left arrows) and one pair of caudal, subdorsal papillae; E, spicules of *Neosteinernema*; F–H, spicules of *Steinernema*: F, spicule head wider than long; G, spicule head longer than wide; H, spicule showing large velum and prominent rostrum; I–K; gubernacula of *Steinernema*: I, cuneus V-shaped; J, cuneus Y-shaped; K, cuneus arrowhead-shaped. Scale bar: A, B = 10 µm, C = 50 µm, D–K = 22 µm.

and foot-shaped (Fig. 1.2E) in *Neosteinernema*. Spicule head is either longer than wide or as long as wide, or wider than long (Fig. 1.2F–H); blade almost straight or curved; velum and rostrum present or absent (Fig. 1.2G and H). **Gubernaculum:** In ventral view it tapers anteriorly abruptly or gradually (Fig. 1.2I–K); the cuneus is V-shaped, Y-shaped or arrowhead-shaped.

Females: Some female characters are important in the taxonomy of Steinernematidae. Anterior end (Fig. 1.3A) is similar for all species. The appearance, and the presence or absent of epiptygma (Fig. 1.3C and D) is important. Tail tip round, with mucron, bluntly pointed, can be used as a diagnostic character (Fig. 1.3B).

Infective juveniles: Head with four cephalic papillae and six labial papillae or with only four cephalic papillae without labial papillae. The lateral field pattern (Fig. 1.4) and the arrangement of ridges in the lateral field from head to tail is an important

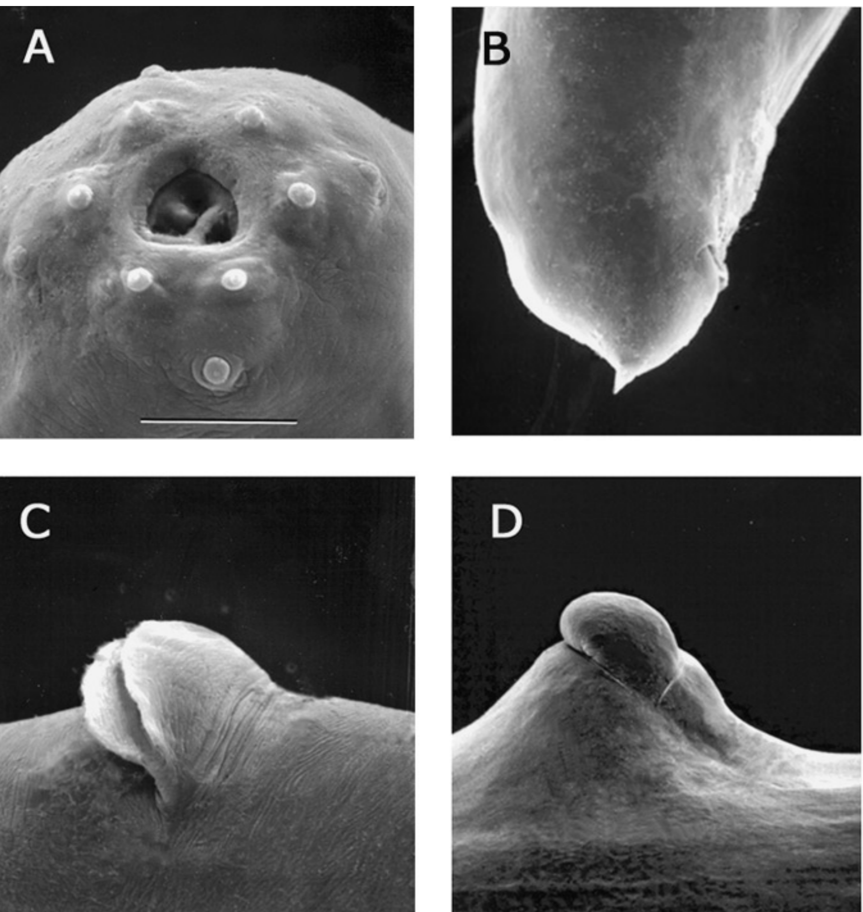


Fig. 1.3. SEM micrographs of morphological structures of females of *Steinernema*. A, Face view showing labial and cephalic papillae; B, tail with a mucron; C, double-flapped epiptygma; D, vulva with a thick flap. Scale bar: A = 10 µm, B = 52 µm, C, D = 20 µm.

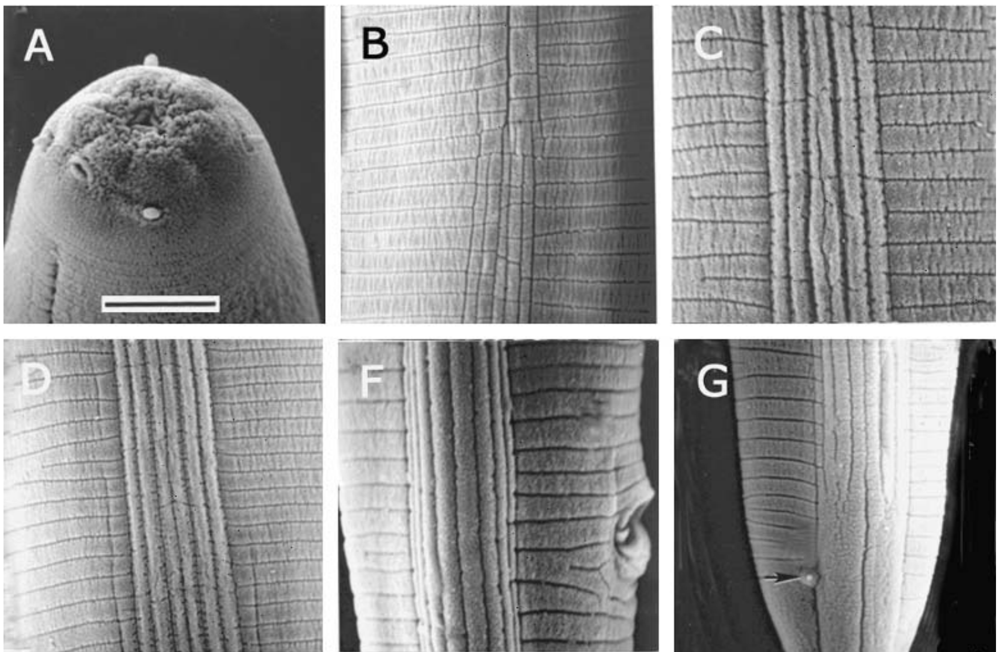


Fig. 1.4. Lateral field pattern of infective juvenile *Steinernema glaseri*. The formula of lateral field pattern is 2, 5, 6, 8, 2 ridges. Scale bar: A, E = 6 μm, B, D, F = 7.5 μm, C = 5 μm.

taxonomic character. The formula for the pattern in Fig. 1.4 is 2, 5, 6, 8, 2 ridges. Phasmids small, large or absent.

Table 1.1 lists the species of *Steinernema*, their taxonomic authority, host isolation, collection location, and publicly databased DNA sequences of diagnostic and systematic utility.

1.5.2. Taxonomy

Taxonomic status: Steiner (1923) described the first entomopathogenic nematode, *Aplectana kraussei*. Travassos (1927) changed the generic name to *Steinernema*. Two years later Steiner described another genus and species *Neoaplectana glaseri*, which resembled *Steinernema kraussei*. Filipjev (1934) placed *Steinernema* and *Neoaplectana* in the new subfamily Steinernematinae, and stated that *Neoaplectana* was probably congeneric with *Steinernema*. Chitwood and Chitwood (1937) raised the subfamily Steinernematinae to the family Steinernematidae. Wouts *et al.* (1982) re-examined the species *S. kraussei* and *N. glaseri* and concluded the two genera were identical and that *Neoaplectana* was a junior synonym of *Steinernema*. In 1994 Nguyen and Smart described a new genus, *Neosteinernema*, added it to the family and amended the family description to accommodate the new genus. Currently, the family contains only the two genera. *Steinernema* has 25 species, *Neosteinernema* has one.

Diagnosis: (After Nguyen and Smart, (1994) following the description of the new genus *Neosteinernema*): Cephalobina, Rhabditida. **Males:** (Fig. 1.2) Smaller than female.

Anterior end usually with six labial papillae, four large cephalic papillae and usually with perioral disc. Oesophagus rhabditoid with metacorpus slightly swollen, narrow isthmus surrounded by nerve ring, and large basal bulb with reduced valve. Testis single, reflexed. Spicules paired. Gubernaculum long, sometimes as long as spicule. Bursa absent. Tail tip rounded, digitate or mucronate. One single and 10–14 pairs of genital papillae present with 7–10 pairs precloacal.

Females: (Fig. 1.3) Large, size variable. Cuticle may be smooth or annulated. Lateral fields absent. Excretory pore distinct, mostly anterior to nerve ring. Head rounded or truncate, rarely offset. Six lips present, partly or completely fused, each lip with one labial papilla (Fig. 1.3A), sometimes additional papillae-like structures present near labial papillae. There are four cephalic papillae. Amphids present, small. Stoma collapsed; cheilorhabdions pronounced, forming a ring resembling two large sclerotized dots in lateral view. Other parts of stoma form an asymmetrical funnel with thick anterior end. Oesophagus rhabditoid with metacorpus slightly swollen, narrow isthmus surrounded by nerve ring, and large basal bulb with reduced valve. Oesophago-intestinal valve usually pronounced. Reproductive system didelphic, amphidelphic, reflexed. Vulva at mid-body, sometimes on a protuberance (Fig. 1.3D), with (Fig. 1.3C) or without epiptygma. Females oviparous or ovoviviparous with juveniles developing up to the infective stage (infective juvenile) before emerging from the body of the female. Tail longer or shorter than anal body width, with or without prominent phasmids.

Infective juveniles: Third-stage infective juvenile (Fig. 1.4) with collapsed stoma. Body slender, with or without a sheath (cuticle of second-stage juvenile). Cuticle annulated. Lateral fields present with 1–9 incisures and 3–8 smooth ridges forming a specific pattern. (The formula for the pattern in Fig. 1.4 is 2, 5, 6, 8, 2 ridges). Oesophagus and intestine appear reduced. Excretory pore distinct, anterior to nerve ring. Tail conoid or filiform. Phasmids, located about mid-tail, prominent, inconspicuous, or not observed. Type genus *Steinernema* (Travassors, 1927).

1.5.2.1. Diagnosis and identification of steinernematid species

The diagnostic characters and identification of species of *Steinernema* are presented in a polytomous key (Table 1.2). For ease of use, the nematodes are arranged in the order of body length of infective juveniles, and important characters are bold-faced. An on-line dichotomous key, which is updated as new research is published is also available (www.ifas.ufl.edu/~kbn/steinkey.htm).

How to use the key: To identify a nematode, the following steps should be considered: (i) Entomopathogenic nematodes exhibit host-induced morphological and morphometric variation. For uniformity, the nematode should be reared in *Galleria mellonella* when possible (Nguyen and Smart, 1996; Hominick *et al.*, 1997); (ii) Variation exists among individuals of the same species. To account for this we recommend measuring at least ten infective juveniles and males of the target nematode; (iii) Match the infective juvenile body length of the nematode with those that have similar body lengths in the key; (iv) Compare the collected data with the measurements in **bold face** letters and **specific characters** of selected nematodes; (v) Determine which nematodes in the key resemble the ones under investigation; (vi) The original descriptions include further detail and should be consulted to confirm your findings.

Table 1.1. Taxonomic authority, host isolation, collection location and GenBank accession numbers of valid *Steinernema* species.

Species	Authority	Isolated from	Locality	DNA Genbank accession #
S. abbasi	Elawad, Ahmad and Reid, 1997	Soil	Sultanate, Oman	
S. affine	(Bovien, 1937) Wouts, Mracek, Gerdin and Bedding, 1982	Bibionidae	Skive, Denmark	
S. arenarium syn.	(Artyukhovsky, 1967) Wouts, Mracek, Gerdin and Bedding, 1982	Soil	Central Russia	ITS1: AF192985
N. arenaria	Artyukhovsky, 1967			
N. anomali	Kozodoi, 1984	*Anomala dubia*		COII-16S: AF192994
N. anomalae	(Kozodoi, 1984) Curran, 1989			
S. bicornutum	Tallosi, Peters and Ehlers, 1995	Soil	Vojvodina, Yugoslavia	ITS1,2: AF121048
S. carpocapsae syn.	(Weiser, 1955) Wouts, Mracek, Gerdin and Bedding, 1982	*Cydia pomonella*	Czechoslovakia	ITS1, 2: AF121049 COII-16S: AF192995
N. carpocapsae	Weiser, 1955			ITS1: AF192987
N. feltiae	sensu Stanuszek, 1974, nec Filipjev, 1934			18S: AF036604 18S:U70633
S. caudatum	Xu, Wang and Li, 1991	Soil	Guangdong, China	
S. ceratophorum	Jian, Reid and Hunt, 1997	Soil	Liaoning, China	
S. cubanum	Mracek, Hernandez and Boemare, 1994	Soil	Pinar del Rio, Cuba	
S. feltiae (= *bibionis*) syn.	(Filipjev, 1934) Wouts, Mracek, Gerdin and Bedding, 1982	Bibionidae	Skive, Denmark	ITS1, 2: AF121050 COII-16S: AF192991
N. feltiae	Filipjev, 1934	*Heliothis armigera*	Australia	18S: U70634
N. bibionis	Bovien, 1937			
S. bibionis	(Bovien, 1937) Wouts, Mracek, Gerdin and Bedding, 1982			
S. glaseri syn. *N. glaseri*	(Steiner, 1929) Wouts, Mracek, Gerdin and Bedding, 1982 Steiner, 1929	*Popillia japonica*	New Jersey, USA	ITS1,2: AF122015 COII-16S: AF192994 18S: U70640
S. intermedium syn.	(Poinar, 1985) Mamiya, 1988	Soil	South Carolina, USA	ITS1, 2: AF122016
N. intermedia	Poinar, 1985			18S: U70636
S. karii	Waturu, Hunt and Reid, 1997	Soil	Central Province, Kenya	
S. kushidai	Mamiya, 1988	*Anomala cuprea*	Hamakita, Japan	ITS1: AF192984
S. kraussei syn.	(Steiner, 1923) Travassos, 1927	*Cephaleia abietis*	Germany	
Aplectana kraussei	Steiner, 1923			
S. longicaudum	Shen and Wang, 1991	Soil	Shandong, China	
S. monticolum	Stock, Choo and Kaya 1997	Soil	Gyeongnam, Korea	ITS1, 2: AF122017
S. neocurtillae syn.	Nguyen and Smart, 1992	*Neocurtilla hexadactilla*	Florida, USA	ITS1, 2: AF122018
S. neocurtillis	Nguyen and Smart, 1992			
S. oregonense syn.	Liu and Berry, 1996a	Soil	Oregon, USA	ITS1, 2: AF122019
S. oregonensis	Liu and Berry, 1996a			
S. puertoricense syn.	Roman and Figueroa, 1994	Soil	Loiza, Puerto Rico	
S. puertoricensis	Roman and Figueroa, 1994			
S. rarum syn.	(Doucet, 1986) Mamiya, 1988	Soil	Cordoba, Argentina	
S. rara	Doucet, 1986			
S. riobravis	Cabanillas, Poinar and Raulston, 1994	*Helicoverpa zea*	Texas, USA	ITS1: AF192994

Table 1.1. *Continued.*

Species	Authority	Isolated from	Locality	DNA Genbank accession #
S. ritteri	Doucet and Doucet, 1990	Soil	Cordoba, Argentina	
S. scapterisci syn. *N. carpocapsae*	Nguyen and Smart, 1990 Nguyen and Smart, 1988	*Scapteriscus vicinus*	Rivera, Uruguay	ITS1,2: AF122020
S. siamkayai	Stock, Somsook and Reid, 1998	Soil	Petchabun, Thailand	
S. tami	Luc, Nguyen, Reid and Spiridonov, 2000	Soil	Cat Tien, Vietnam	

1.5.2.2. Genus Neosteinernema (Nguyen and Smart, 1994) (Fig. 1.5)

Diagnosis: Males smaller than female, posterior part with one ventral and 13–14 pairs of genital papillae, eight of the pairs preanal; phasmids prominent (Fig. 1.5D), tail tip digitate; spicule foot-shaped (Fig. 1.5C) with a hump on dorsal side. Gubernaculum almost as long as spicule. **Females** with face view as in *Steinernema* (Fig. 1.5A); phasmids prominent (Fig. 1.5B), on a protuberance, located in posterior half of tail; tail longer than anal body width (T/ABW = 1.10–1.68); ovoviviparous, juveniles moulting and becoming infective juveniles before exiting the female body. **Infective juveniles** with slightly swollen head (Fig. 1.5E); phasmid large, tail elongate or filiform, as long as oesophagus, usually curved at end (Fig. 1.5F), ratio c about 5.5.

Type and only species: *Neosteinernema longicurvicauda* (Nguyen and Smart, 1994). **Diagnosis:** As in the genus.

1.5.3. Phylogenetic relationships

Early studies of phylogenetic relationships among *Steinernema* spp. included PCR RFLP analyses of the of the ITS rDNA region (Reid *et al.*, 1997), morphology and random amplified polymorphic DNA (RAPD; Liu and Berry, 1996b), and 18S ribosomal RNA gene sequences (Liu *et al.*, 1997). More recently, DNA sequences of the COII–16S, ITS and 28S rDNA regions have been employed (Szalanski *et al.*, 2000; Nguyen *et al.*, 2001; Stock *et al.*, 2001). Due primarily to difficulties in acquiring material representing all species of *Steinernema*, no phylogenetic analysis to date can be considered complete. A composite cladogram of three separate analyses is presented in Fig. 1.7A.

In general, patterns of phylogenetic relationships concur with overall morphological similarity. For example, *S. oregonense* is morphologically similar to *S. feltiae* except the E% (distance from anterior end/tail length × 100) is larger and it is missing the mucron on its tail terminus. These two taxa share 91.8% ITS DNA sequence identity, and appear as sister taxa in the best estimate of phylogenetic relationships for the genus (Fig. 1.7A). *S. glaseri* and *S. intermedium*, two species that would appear to be distantly related based on morphological characters (Nguyen and Smart, 1996) have an ITS DNA similarity ratio of only 58.1%, and are phylogenetically quite divergent (Nguyen *et al.*, 2001).

Table 1.2. Polytomous key for *Steinernema*. Ratios and abbreviations used in the keys: L = body length, W = greatest body width, EP = distance from anterior end to excretory pore, NR = distance from anterior end to nerve ring, ES = oesophagus length, a = L/W, b = L/ES, c = L/T (T = tail length), D% = EP/ES × 100, E% = EP/T × 100, ABW = anal body width, SL = spicule length, GL = gubernaculum length, IJ = infective juvenile; ratio SW = spicule length/ABW; ratio GS = gubernaculum length/spicule, MUC = mucron, A = absent, P = present; na = not available. All morphometric data were collected from original authors.

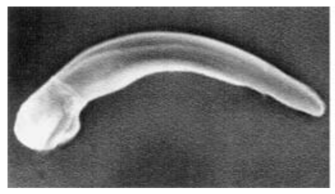
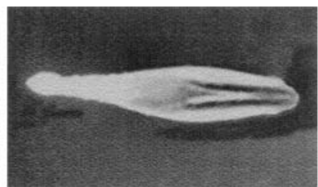
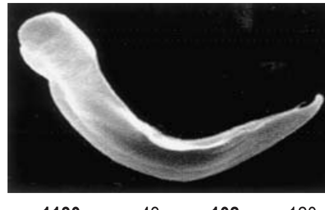
	Morphometric characters (range)										
Species	L	W	EP	NR	ES	T	a	b	c	D%	E%
S. cubanum	**1283**	37	**106**	116	148	**67**	35	8.6	19.2	70	**160**
Infective juvenile	1149–1508	33–46	101–114	106–130	135–159	61–77	na	na	na	na	na
Male	**SL = 58**(50–67); GL = 39(37–42); W = 97(77–117); **D% = 70**; SW = 1.41; GS = 0.7; **MUC = A**										
	Spicule and gubernaculum as in the micrographs:  										
S. puertoricense	**1171**	51	**95**	117	143	**94**	23	8.2	12.4	66	**101**
Infective juvenile	1057–1238	47–54	90–102	111–121	138–147	88–107	20–24	7.4–8.6	11.6–13.6	62–74	88–108
Male	**SL = 78**(71–88), GL = 40(36–45); W = 101(67–148); **D = 77**; SW = 1.52; GS = 0.5; **MUC = A**										
Female	Female with epiptygma.										
	Spicule and gubernaculum as in the micrographs: 										
S. glaseri	**1130**	43	**102**	120	162	**78**	29	7.3	14.7	65	**131**
Infective juvenile	864–1448	31–50	87–110	112–126	158–168	62–87	26–35	6.3–7.8	13.6–15.7	58–71	122–138
Male	**SL = 77** (64–90); GL = 55(44–59); W = 72 (54–92); **D% = 70**(60–78); **SW = 2.1** (1.64–2.43); GS = 0.7(0.6–0.85); **MUC = A**										
	Spicule and gubernaculum as in the micrographs:										
	Specific character: Spicule tip with a notch.										
S. caudatum	**1106**	36	**82**	109	156	**88**	31	7.1	12.6	52	**94**
Infective juvenile	933–1269	34–41	76–89	104–126	149–175	80–100	na	na	na	na	87–100
Male	**SL = 75**; GL = 52; W = 91; **D% = 71**; SW = 2.22; GS = 0.70; **MUC = A**										
	No illustrations available.										
	Specific characters: EP and T short, spicule tip not hook-like in shape.										

Table 1.2. *Continued*.

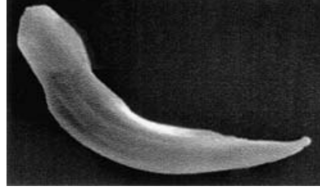
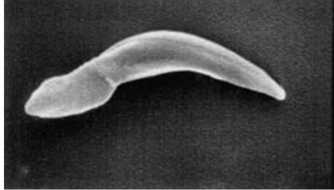
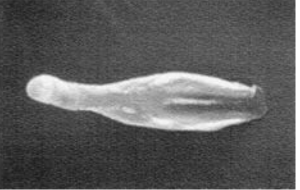
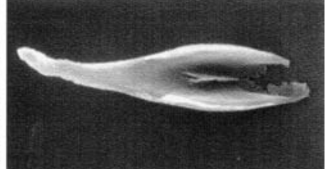
Species	Morphometric characters (range)									
	L	W	EP	NR	ES	T	a	b	c	D%	E%
S. longicaudum	**1063**	40	**81**	107	145	**95**	27	7.3	11.2	56	**85**
Infective juvenile	(No ranges available)										
Male	**SL = 77**; GL = 48; W = 155; **D% = 62**; SW = 1.60; GS = 0.62; **MUC = A** Spicule and gubernaculum as in the micrographs: **Specific characters:** IJ tail long; spicule reduced in width suddenly to form a tip with flattened terminus.										
S. arenarium	**1034**	46	**83**	109	138	**75**	26	7.6	13.8	55	**119**
Infective juvenile	724–1408	28–77	76–86	100–120	123–160	64–84	17–34	5.9–10.8	9.4–16.9	52–59	106–130
Male	**SL = 84**(81–91); GL = 55(49–60); W = 188(184–219); **D% = 93**(88–102); SW = 2.1; GS = 0.65(0.60–0.66); **MUC = A**. Spicule and gubernaculum as in the micrographs: **Specific character:** Spicule tip ball-shaped. 										
S. oregonense	**980**	34	**66**	na	132	**70**	30	7.6	14.0	50	**100**
Infective juvenile	820–1110	28–38	60–72	na	116–148	64–78	24–37	6.0–8.0	12.0–16.0	40–60	90–110
Male	**SL = 71**(65–73); GL = 56(52–59); W = 138(105–161); **D% = 73**(64–75); SW = 1.51; GS = 0.79; **MUC = A** Spicule and gubernaculum as in the micrographs: **Specific character:** This nematode is very similar to *Steinernema feltiae* except male without a mucron and IJ longer.  										
S. kraussei	**951**	33	**63**	105	134	**79**	29	7.1	12.1	47	**80**
Infective juvenile	797–1102	30–36	50–66	99–111	119–145	63–86	na	na	na	na	na
Male	**SL = 55**(52–57); GL = 33(23–38); W = 128(110–144); **D% = 53**; SW = 1.10; GS = 0.71; **MUC = P** Spicule and gubernaculum as in the micrographs: **Specific characters:** Spicule short, D% low. 										

Table 1.2. *Continued*.

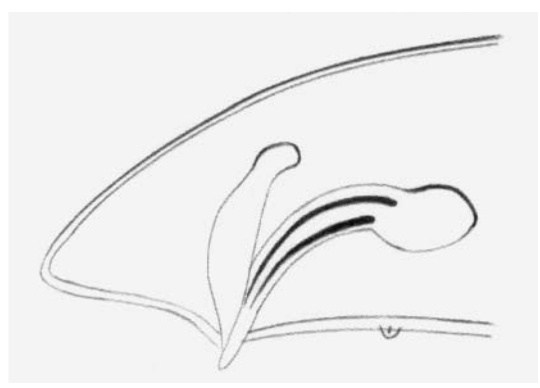
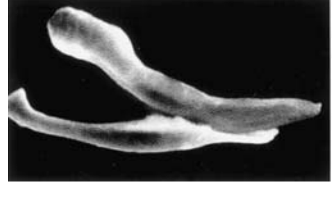
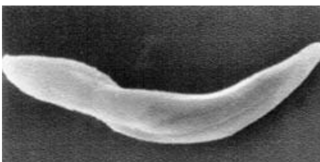
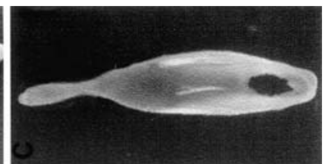
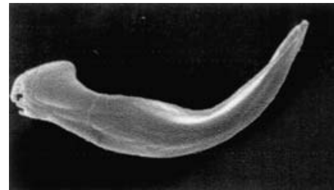
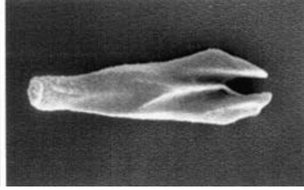
	Morphometric characters (range)										
Species	L	W	EP	NR	ES	T	a	b	c	D%	E%
S. karii	**932**	33	**74**	105	134	**74**	29	6.8	12.6	57	**96**
Infective juvenile	876–982	31–35	68–80	97–112	122–147	64–80	na	na	na	na	na
Male	**SL = 83**(73–91); GL = 57(42–64); W = 136(107–166); **D% = 66**(57–78); **MUC = A** Spicule and gubernaculum as in the drawing (redrawn after Waturu *et al.*, 1997):										
	Specific character: Spicule long.										
S. neocurtillae	**885**	34	**18**	107	144	**80**	26	6.1	11.2	12	**23**
Infective juvenile	741–988	28–42	14–22	100–119	130–159	64–97	22–29	5.4–6.7	9.1–14.2	10.0–15.0	18–30
Male	**SL = 58**(52–64); GL = 52(44–59); W = 111(77–144); **D% = 19**(13–26); SW = 1.43(1.18–1.64); **GS = 0.89**(0.82–0.93); **MUC = P**. Spicule and gubernaculum as in the micrographs:										
	Specific characters: EP extremely short; GS very high; cannot be produced in *Galleria*.										
S. feltiae	**849**	26	**62**	99	136	**81**	31	6.0	10.4	45	**78**
Infective juvenile	736–950	22–29	53–67	88–112	115–150	70–92	29–33	5.3–6.4	9.2–12.6	42–51	69–86
Male	**SL = 70**(65–77); GL = 41(34–47); W = 75(60–90); **D% = 60**; SW = 1.13(0.99–1.30); GS = 0.59(0.52–0.61); **MUC = P**. Spicule and gubernaculum as in the micrographs:										
	Specific characters: Spicule head very long; male with long mucron.	 									

Table 1.2. *Continued.*

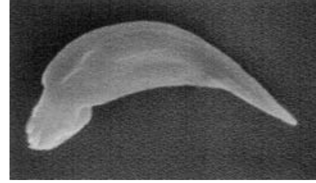
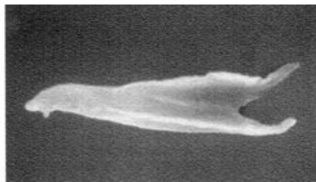
Species	Morphometric characters (range)										
	L	W	EP	NR	ES	T	a	b	c	D%	E%
S. bicornutum	**769**	29	**61**	92	124	**72**	27	6.2	10.7	50	**84**
Infective juvenile	648–873	25–33	53–65	88–100	113–135	63–78	23–29	5.6–6.9	9.7–12.0	40–60	80–100
Male	**SL = 65**(53–70); GL = 48(38–50); W = 109(80–127); **D% = 52**(50–60); **SW = 2.22**(2.18–2.26); GS = 0.72); **MUC = A**. Spicule and gubernaculum as in the micrographs:										

Note: 2 horn-like structures on lip region of IJ.

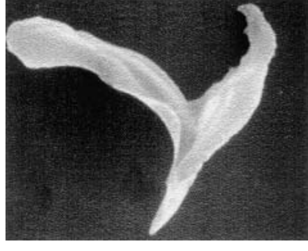
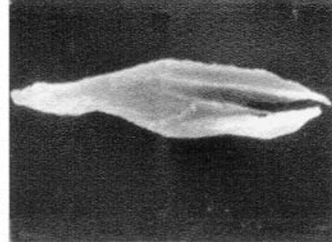
Species	L	W	EP	NR	ES	T	a	b	c	D%	E%
S. monticolum	**706**	37	**58**	88	124	**77**	19	5.7	9.3	47	**76**
Infective juvenile	612–821	32–46	54–62	81–93	120–131	71–95	14–22	5.0–6.4	7.6–11.1	44–50	63–86
Male	**SL = 70**(61–80); GL = 45(35–54); W = 160(117–206); **D% = 55**(49–61); SW = 1.40(1.20–1.50); GS = 0.6 (0.5–0.7); **MUC = P.** Spicule and gubernaculum as in the micrographs:										

Specific characters: IJ tail long; E% low

Species	L	W	EP	NR	ES	T	a	b	c	D%	E%
S. ceratophorum	**706**	27	**55**	92	123	**66**	26	na	10.6	45	**84**
Infective juvenile	591–800	23–34	47–70	79–103	108–144	56–74	24–28	na	8.8–12.9	40–56	74–96
Male	**SL = 71**(54–90); GL = 40(25–45); W = 146(104–185); **D% = 51**(33–65); **SW = 1.4**(1.0–2.0); GS = 0.6(0.4–0.8);**MUC = A**; Spicule and gubernaculum as in the micrographs:										

Note: 2 horn-like structures on lip region of IJ as in *S. bicornutum.*

Species	L	W	EP	NR	ES	T	a	b	c	D%	E%
S. affine	**693**	30	**62**	95	126	**66**	23	5.5	10.5	49	**94**
Infective juvenile	608–880	28–34	51–69	88–104	115–134	64–74	21–28	5.1–6.0	9.5–11.5	43–53	74–108
Male	**SL = 70**(67–86); GL = 46(37–56); W = 118(95–164); **D% = 61**; SW = 1.17; GS = 0.66; **MUC = P** Spicule and gubernaculum as in the micrographs:										

Specific character: Spine-like structure in IJ tail tip (sheath).

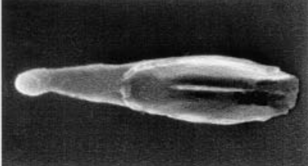


Table 1.2. *Continued*.

Species	Morphometric characters (range)										
	L	W	EP	NR	ES	T	a	b	c	D%	E%
S. intermedium	**671**	29	**65**	93	123	**66**	23	5.3	10.0	51	**96**
Infective juvenile	608–800	25–32	59–69	85–99	110–133	53–74	20–26	5.0–6.0	9.3–10.8	48–58	89–108
Male	**SL = 91**(84–100); GL = 64(56–75); W = 168(113–207); **D% = 67**(58–76); SW = 1.24(1.03–1.39); GS = 0.69(0.62–0.77). MUC = A. Spicule and gubernaculum as the micrographs: **Specific character:** Spicule is the largest in *Steinernema* spp.										
S. riobrave	**622**	28	**56**	87	114	**54**	23	5.4	11.6	49	**105**
Infective juvenile	561–701	26–30	51–64	84–89	109–116	46–59	20–24	4.9–6.0	10.1–12.4	45–55	93–111
Male	**SL = 67**(62.5–75.0); GL = 51(47.5–56.2); W = 133(116–159); **D% = 71**(60–80); SW = 1.14; GS = 0.76; **MUC = A**. Spicule and gubernaculum as in the micrographs: **Specific character**: Width of gubernaculum neck as wide as posterior corpus.										
S. kushidai	**589**	26	**46**	76	111	**50**	22.5	5.3	11.7	41	**92**
Infective juvenile	524–662	22–31	42–50	70–84	106–120	44–59	19–25	4.9–5.9	9.9–12.9	38–44	84–95
Male	**SL = 63**; GL = 44; W = 97; **D% = 51**; SW = 1.50; GS = 0.70; **MUC = A.** Spicule and gubernaculum as in the micrographs: **Specific character**: SL/spicule width about 3.9 **Note:** Cannot reproduce in *Galleria*.										
S. scapterisci	572	24	**39**	97	127	**54**	24	4.5	10.7	31	**73**
Infective juvenile	517–609	18–30	36–48	83–106	113–134	48–60	20–31	4.0–4.6	9.2–11.7	27–40	60–80
Male	**SL = 83(72–92); GL = 65(59–75); W = 156(97–213); D% = 38**(32–44); **SW = 2.52**(2.04–2.80); GS = 0.78; **MUC = P.** Spicule and gubernaculum as in the micrographs: **Specific characters:** Female: Epiptygma very large; Excretory duct with ellipse-shaped structure. Male: long spicule. **Note:** Develops poorly in *Galleria*.										

Table 1.2. *Continued*.

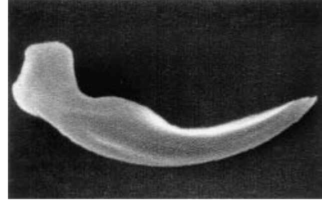
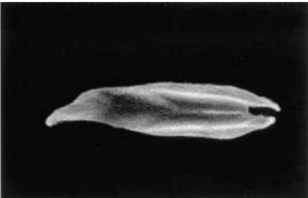
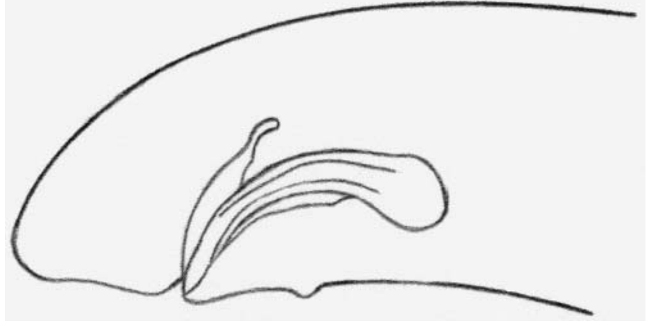
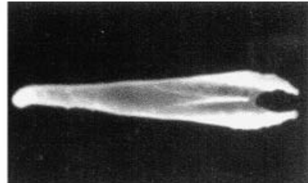
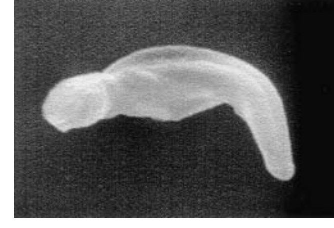
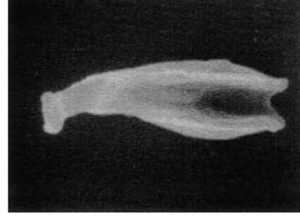
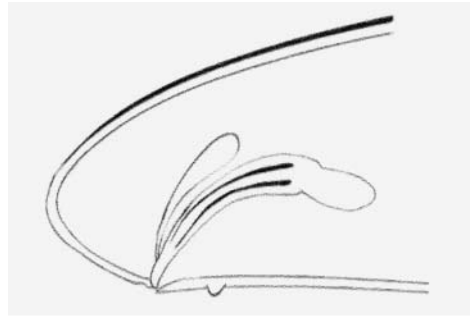
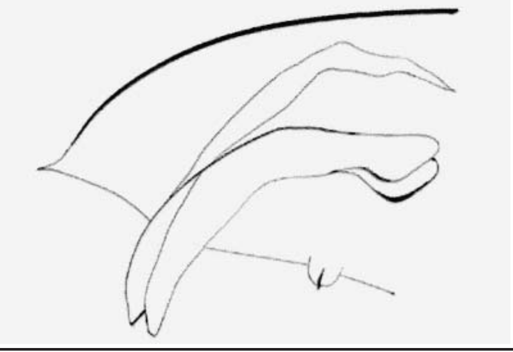
Species	Morphometric characters (range)										
	L	W	EP	NR	ES	T	a	b	c	D%	E%
S. carpocapsae	**558**	25	**38**	85	120	**53**	21	4.4	10.0	26	**60**
Infective juvenile	438–650	20–30	30–60	76–99	103–190	46–61	19–24	4.0–4.8	9.1–11.2	23–28	54–66
Male	**SL = 66**(58–77); GL = 47(39–55); W = 101(77–130); **D% = 41**(27–55); **SW = 1.72**(1.40–2.00); GS = 0.71(0.59–0.88). **MUC = P**. Spicule and gubernaculum as in the micrographs: **Specific characters:** Spicule head wider than long. Gubernaculum with short anterior part.  										
S. abbasi	**541**	29	**48**	68	89	**56**	18	6.0	9.8	53	**86**
Infective juvenile	496–579	27–30	46–51	64–72	85–92	52–61	17–20	5.5–6.6	8.1–10.8	51–58	79–94
Male	**SL = 65**(57–74); GL = 45(33–50); W = 87(82–98); **D% = 60**(51–68); SW = 1.56(1.07–1.87); GS = 0.7(0.58–0.85). **MUC = A**. Spicule and gubernaculum as in the drawing (redrawn from Elawad *et al.*, 1997): **Specific character:** Mucron in male absent. 										
S. tami	**530**	23	**36**	na	117	**50**	23	5.0	10.0	31	**73**
Infective juvenile	400–600	19–29	34–41	na	110–123	42–57	19–28	3.7–5.1	9.0–11.0	28–34	67–86
Male	**SL = 77**(71–84); G L = 48(38–55); W = 129(98–161); **D% = 44**(30–60); **SW = 2.0**(1.4–3.0); GS = 1.6; **MUC = P**. Spicule and gubernaculum as in the micrographs: **Specific characters:** Short nematode with long spicule, shaft prominent. SW greater than that of other short nematodes. 										

Table 1.2. *Continued*.

	Morphometric characters (range)										
Species	L	W	EP	NR	ES	T	a	b	c	D%	E%
S. rarum	**511**	23	**38**	70	102	**51**	23	**4.7**	9.8	35	**72**
Infective juvenile	443–573	18–26	32–40	60–88	89–120	44–56	20–26	4.1–5.6	8.7–11.0	30–39	63–80
Male	**SL = 47**(42–52); GL = 34(23–38); W = 123(100–142); **D% = 50**(44–51); SW = 0.94(0.91–1.05); GS = 0.71(0.55–0.73). **MUC = P**. Spicule and gubernaculum as in the micrographs. **Specific characters:** Short nematode. Spicule short, strongly curved posteriorly.  										
S. ritteri	**510**	22	**43**	73	92	**49**	24	5.5	10.6	46	**88**
Infective juvenile	470–590	19–24	40–46	68–85	85–95	44–54	19–31	4.9–6.3	9.2–13.1	44–50	79–97
Male	**SL = 69**(58–75); GL = 44(33–50); W = 130(110–176); **D% = 47**(44–50); SW = 1.56(1.44–1.57); GS = 0.64(0.57–0.67); **MUC = A.** Spicule and gubernaculum as in the drawings (redrawn from Doucet and Doucet, 1990): **Specific characters:** Short nematode. Mucron absent in first-generation male. 										
S. siamkayai	**446**	21	**35**	72	95	**36**	21	4.7	11.3	37	**96**
Infective juvenile	398–495	18–24	29–38	68–80	80–107	31–41	19–23	4.0–6.1	10.3–14.8	31–43	95–112
Male	**SL = 77.5**(75–80); GL = 54(47–65); W = 140(107–159); **D% = 42**(35–49); SW = 1.7(1.4–2.2); GS = 0.7(0.6–0.8); **MUC = P**. Spicule and gubernaculum as in drawing (redrawn from Stock *et al.*, 1999). **Specific characters:** Very short nematode. Spicule long for a short nematode. Epiptygma present in females. 										

1.6. Taxonomy and Systematics of Heterorhabditidae (Poinar, 1976)

1.6.1. Morphological review (based on SEM observations)

Males: Bursa with nine papillae (Fig. 1.6D). The bursa structure is similar in all species. Spicules (Fig. 1.6E) and gubernaculum in ventral view (Fig. 1.6F) can be used as diagnostic characters.

Females: Head region with six forward-directed papillae (Fig. 1.6A and C) and is similar for almost all species, except the labial papillae is curved outward in *Heterorhabditis hawaiiensis*. The pattern of the vulva (Fig. 1.6B) is a good diagnostic character.

Infective juveniles: Morphological characters of infective juveniles are in Fig. 1.6G–I. Morphological characters have been poorly investigated and are unreliable for species differentiation. Identification is based primarily on morphometrics and molecular data.

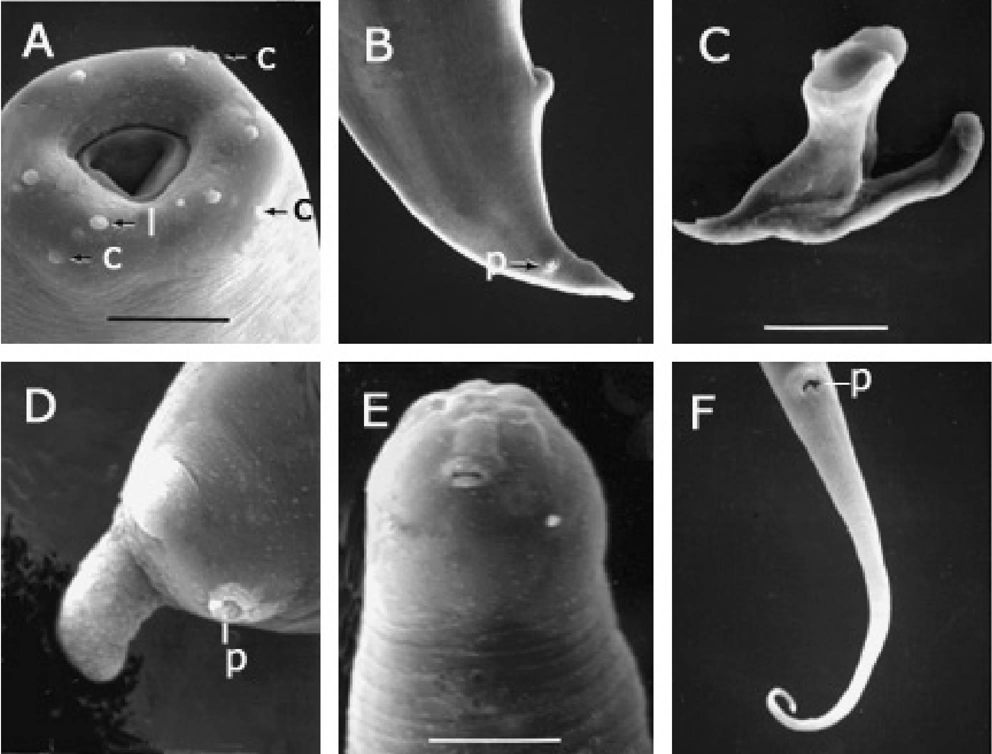


Fig. 1.5. SEM micrographs of *Neosteinernema longicurvicauda*. A, Face view showing cephalic papillae (c), labial papillae (l), and anterior end of stoma; B, female tail with prominent phasmid and pointed tail; C, foot-shaped spicules and gubernaculum; D, tail end with digitate tail tip and prominent phasmid (p); E, swollen head of infective juvenile showing amphid and cephalic papillae; F, curved tail of infective juvenile with large phasmid. Scale bar in A: A = 12 μm, B = 30 μm; scale bar in C: C = 23 μm, D = 5 μm; scale bar in E: E = 3 μm, F = 17 μm.

Table 1.3 summarizes all currently recognized species of *Heterorhabditis*, their taxonomic authority, host isolation, collection location, and publicly databased DNA sequences that are of diagnostic and systematic utility.

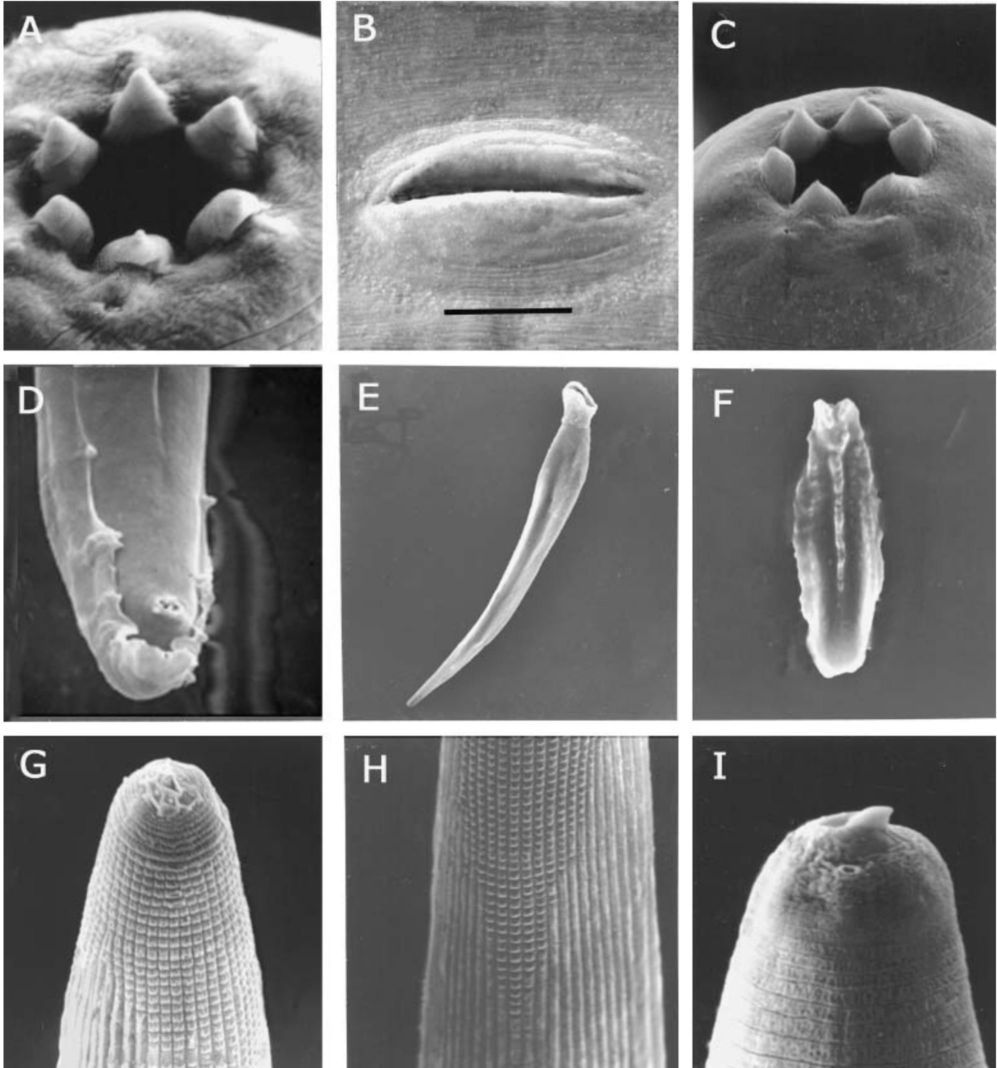


Fig. 1.6. SEM micrographs of *Heterorhabditis* spp. A, Face view of a first-generation female showing lips, labial papillae and amphids; B, vulval appearance; C, face view of a second-generation female; D, bursa; E, spicule; F, gubernaculum, ventral view; G, H, anterior regions of a third-stage infective juvenile with the cuticle of the second-stage juvenile showing tessellate structure and longitudinal ridges; I, anterior region of an infective juvenile showing dorsal tooth in anterior end and prominent amphid. Scale bar in B: A = 5 μm, B = 8.6 μm, C = 6 μm, D = 20 μm, E = 15 μm, F = 8.6 μm, G = 8.6 μm, H = 10 μm, I = 3.8 μm.

Table 1.3. Taxonomic authority, host isolation, collection location and GenBank accession numbers of valid *Heterorhabditis* species.

Species	Authority	Isolated from	Locality	DNA Genbank accession #
H. argentinensis[a]	Stock, 1993	*Graphognathus* sp.	Santa Fe, Argentina	ITS1: AF029706
H. bacteriophora syn.	Poinar, 1975	*Heliothis punctigera*	Brecon, Australia	18S: AF036593 ND4: AF066888
H. heliothidis	(Khan, Brook and Hirschmann, 1976) Poinar, Thomas and Hess, 1977	*Heliothis zea*	USA	ITS1: AF066890 18S: U70628
Chromonema heliothidis	Khan, Brook and Hirschmann, 1976			
H. brevicaudis	Liu, 1994	Soil	Fugian, China	
H. hawaiiensis[a]	Gardner, Stock and Kaya, 1994	Soil	Hawaii, USA	ITS1: AF029707
H. indica	Poinar, Karunakar and David, 1992	*Scirpophaga excerptalis*	Coimbatore, India	ITS1: AF029710 18S: U70625 ND4: AF066878
H. marelatus syn.	Liu and Berry, 1996a	Soil	Oregon, USA	ITS1: AF029713 18S: U70630 ND4: AF066881
H. hepialius	Stock, 1997			
H. megidis	Poinar, Jackson and Klein, 1987	*Popillia japonica*	Ohio, USA	ITS1: AF029711 18S: U70631 ND4: AF066885
H. zealandica	Poinar, 1990	*Heteronychus arator*	Auckland, New Zealand	ITS1: AF029705
Species inguirenda:				
H. poinari	Kakulia and Mikaia, 1997	Soil	Georgia	

[a] These species are probable synonyms of *H. bacteriophora* and *H. indica* respectively (Adams *et al.*, 1998).

1.6.2. Taxonomy

Poinar erected the family Heterorhabditidae in 1976 when he described the genus and species *Heterorhabditis bacteriophora*. The family contains only one genus, *Heterorhabditis*. The genus currently has eight species; a new species from Ireland is in the process of being described (A.M. Burnell, Maynooth, 2000, personal communication). A ninth species, *H. poinari*, has been published (Kakulia and Mikaia, 1997) but several inadequacies (morphometric values, number of specimens measured, lack of estimates of variance, and availability of type material) suggest validation by additional study.

Diagnosis (amended based on additional information revealed by SEM): Obligate insect parasites. Infective juveniles carry symbiotic bacteria. Both hermaphroditic and amphimictic females present.

Hermaphroditic females: Head truncate to slightly rounded, six conical lips well developed, separate, each with a terminal papilla (Fig. 1.6A); one or two small raised structures sometimes visible at the base of each lip; amphidial opening small. Stoma wide but shallow; cheilorhabdions present, forming a ring, which, in lateral view

resembles two refractile elongate structures. Other parts of the stoma fused to form a collapsed posterior portion. Posterior part of stoma surrounded by oesophagus. Oesophagus without metacorpus; isthmus slender; basal bulb swollen; valve in basal bulb reduced. Nerve ring at middle of isthmus. Excretory pore usually posterior to end of oesophagus. Vulva median, slit-like, surrounded by elliptical rings; ovotestis amphidelphic, reflexed. Oviparous, later becoming ovoviviparous. Tail pointed, longer than anal body width, postanal swelling usually present.

Amphimictic females: Similar to, but usually smaller than, hermaphroditic female; labial papillae prominent (Fig. 1.6C). Reproductive system amphidelphic, reflexed. Vulva not functional for egg deposition (eggs hatch in female body), but functional for mating.

Males: Testis one, reflexed. Spicules paired, separate, and slightly curved ventrally (Fig. 1.6E). Spicule head short, offset from lamina by a constriction. Gubernaculum usually about half as long as spicule length. Bursa peloderan with nine pairs of genital papillae (Fig. 1.6D).

Infective juveniles: Third-stage infective juvenile usually with sheath (cuticle of second-stage juvenile). Sheath with anterior tessellate pattern and longitudinal ridges throughout the body (Fig. 1.6G and H); cuticle of infective juvenile striated with one smooth band marginated by two ridges in lateral fields. Head with a prominent dorsal tooth anteriorly (Fig. 1.6I). Mouth and anus closed. Stoma appearing as a closed chamber with parallel walls. Oesophagus and intestine reduced. Excretory pore posterior to nerve ring. Symbiotic bacterial cells found in intestine. Tail pointed. Type and only genus *Heterorhabditis* (Poinar, 1976).

1.6.2.1. Diagnosis and identification of *Heterorhabditis species*

The diagnostic characters and identification of species of *Heterorhabditis* are presented in a polytomous key (Fig. 1.4). For ease of use, the nematodes are arranged in the order of body length of infective juveniles. Important characters are bold-faced. An on-line dichotomous key, which is updated as new research is published, is also available (ifas.ufl.edu/~kbn/hetekey.htm).

How to use the key: As in Steinernematidae.

1.6.3. Phylogenetic relationships

An estimate of phylogenetic relationships based on the analysis of Adams *et al.* (1998) is presented in Fig. 1.7B. Their study relied on ITS-1 rDNA sequences, and is compatible with one based on mitochondrial ND4 sequences (Liu *et al.*, 1999) except for the position of *H. marelatus*, which is depicted in the best estimate of Liu *et al.* as branching basally from the remaining taxa. Relationships among closely-related sister taxa are well established (*H. indica* + *H. hawaiiensis, H. bacteriophora* + *H. argentinensis, H. megidis* + 'Irish type') while support for deeper nodes is dubious. For example, Adams *et al.* (1998) expressed doubts as to the position of *H. zealandica* relative to *H. marelatus* and the *H. megidis* + 'Irish type' clade. Their concern was based on lower bootstrap and decay index support, but also the stability of *H. zealandica* under different alignment and tree-building parameters. The study of Liu *et al.* (1999) did not include *H. zealandica* or the 'Irish type' species, but still points to conflicting support for nodes involving *H. marelatus* and *H. megidis*. The discordance between the two

studies obviates the conclusion that relationships among *Heterorhabditis* are firmly established, especially among the more distantly related species.

1.7. Conclusions and Future Prospects

The taxonomic history of most species of entomopathogenic nematodes may be brief, but in the last 10 years they have undergone a rigorous scrutiny that rivals nematodes of even more anthropocentric concern. The impetus for such a burst of effort stems primarily from their potential as biological control agents. The result has not merely been the formation of a robust taxonomic foundation and the production of

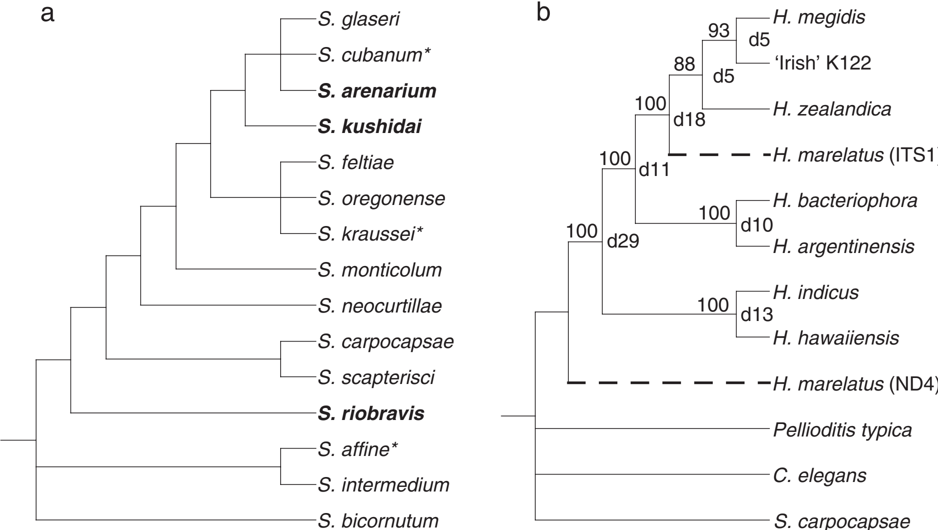


Fig. 1.7. Phylogenetic relationships among *Steinernema* and *Heterorhabditis*. (a) Best estimate of phylogenetic relationships among species of *Steinernema*. Tree is a composite of three separate analyses (Reid *et al*., 1997; Szalanski *et al*., 2000 Nguyen *et al*., 2001) and is rooted based on an analysis of 5.8S rDNA using *Heterodera avenae, Heterodera glycines, Nacobbus aberrans, Meloidogyne arenaria, Aphelenchus avenae, Ascaris suum, Toxocara canis, Heterakis gallinarum* and *Caenorhabditis elegans* (Nguyen *et al*., 2001). Taxa represented by partial ITS and COII-16S sequences (Szalanski *et al*., 2000) appear in bold type. Taxa represented by ITS PCR RFLP (Reid *et al*., 1997) are indicated with an asterisk. Where taxa were redundant among the three analyses, only the phylogenetic position of *S. bicornutum* was discordant, with Reid *et al*. (1997) placing it as sister taxon to *S. scapterisci* and *S. carpocapsae*. The tree is congruent with the study of Liu *et al*. (Liu and Berry, 1996b) based on RAPD and morphological characters, but differs significantly from a phylogeny based on partial 18S rDNA sequences (Liu *et al*., 1997). (The tree resulting from the 18S analysis is so great that attempts to integrate it into a consensus of the other analyses collapses most of the resolution.); (b) Phylogenetic relationships among species of *Heterorhabditis*. Tree is from Adams *et al*. (1998) and is compatible with that of Liu *et al*. (1999) except for the position of *H. marelatus*. Based on ND4 mtDNA sequences, the best estimate of Liu *et al*. depicts *H. marelatus* as branching basally from the remaining taxa. Bootstrap frequencies (100 replicates) and decay indices (preceded by the letter 'd') are from Adams *et al*. (1998).

Table 1.4. Polytomous key to *Heterorhabditis*. Ratios and abbreviations used in the keys: L = body length, W = greatest body width, EP = distance from anterior end to excretory pore, NR = distance from anterior end to nerve ring, ES = oesophagus length, a = L/W, b = L/ES, c = L/T (T = tail length), D% = EP/ES × 100, E% = EP/T × 100, ABW = anal body width, SL = spicule length, GL = gubernaculum length, IJ = infective juvenile; ratio SW = spicule length/ABW; ratio GS = gubernaculum length/spicule. MUC = mucron, A = absent, P = present; TR = testis reflexion; na = not available. All morphometric data were collected from original authors.

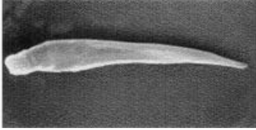
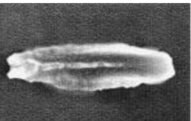
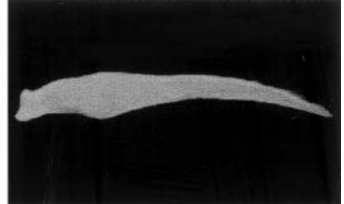
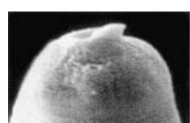
	Morphometric characters (range)										
Species	L	W	EP	NR	ES	T	a	b	c	D%	E%
H. megidis	**768**	29	**131**	109	155	**119**	26	5.0	6.5	85	**110**
Infective juvenile	736–800	27–32	123–142	104–115	147–160	112–128	23–28	4.6–5.9	6.1–6.9	81–91	103–120
Male	**SL = 49**(46–54); GL = 21(17–24); W = 47(44–50); **D% = 122**; **TR = 128**; **SW = 1.35**(1.30–1.40); GS = 0.43										
	Identifying structures as in the micrographs: Dorsal tooth with membranous ring, spicule and gubernaculum.										
	Specific character: Long IJ with membranous ring around oral aperture.										
H. zealandica	**685**	27	**112**	100	140	**102**	25	4.9	**6.6**	80	**108**
Infective juvenile	570–740	22–30	94–123	90–107	135–147	87–119	24–26	4.2–5.0	6.2–6.7	70–84	103–109
Male	**SL = 51**(48–55); GL = 22(19–25); W = 41(36–45); **D% = 118**; **TR = 132**(88–173); SW = 1.81(1.60–2.09); GS = 0.43										
	Identifying structures as in the micrographs: Vulva pattern, and spicule.										
	Specific character: Spicule with prominent rostrum. **Note:** Infected *Galleria* usually turns green.										
H. marelatus	**654**	28	**102**	99	133	**107**	24	4.9	**6.1**	77	**96**
Infective juvenile	588–700	24–32	81–113	83–113	121–139	99–117	21–29	4.7–5.4	5.5–6.6	60–86	89–110
Male	**SL = 45**(42–50); GL = 19(18–22); W = 51(48–56); D% = 113; **TR = 91**(67–136); SW = 1.75(1.73–1.78); GS = 0.41										
	Note: No difference in morphological characters were found. Diagnosis based on morphometrics										
H. bacteriophora	**588**	23	**103**	85	125	**98**	25	4.5	**6.2**	84	**112**
Infective juvenile	512–671	18–31	87–110	72–93	100–139	83–112	17–30	4.0–5.1	5.5–7.0	76–92	103–130
Male	**SL = 40**(36–44); GL = 20(18–25); W = 43(38–46); **D% = 117**; **TR = 76**(61–89); **SW = 1.28**(1.20–1.34); GS = 0.50										
	Identifying structures are in the micrographs: Infective juvenile head, vulva tatern, spicule.										
											

Table 1.4. *Continued.*

	Morphometric characters (range)										
Species	L	W	EP	NR	ES	T	a	b	c	D%	E%
H. hawaiiensis	**575**	25	**114**	92	133	**90**	23	4.3	**6.4**	86	**127**
Infective juvenile	506–631	21–28	95–132	79–103	115–181	82–108	na	na	na	na	na
Male	**SL = 47**(40–51); GL = 22(18–26); W = 63(49–84); **D% = 110**; **TR = 174**(114–198); SW = 1.73(1.44–1.95); GS = 0.47										
	Identifying structures are in the micrographs: spicule and gubernaculum. **Note:** This nematode may be junior synonym of *H. indica*.										
H. brevicaudis	**572**	22	**111**	101	124	**76**	26	4.6	**7.6**	90	**147**
Infective juvenile	528–632	20–24	104–116	96–104	120–136	68–80	na	na	6.6–8.6	na	na
Male	**SL = 47**(44–48); GL = 22(20–24), W = 43(40–48); **D% = 88**; **TR = 194**(162–240); SW = na; GS = 0.47										
	Note: This nematode has very short tail, more studies are needed to clarify this character. Type material is currently unavailable.										
H. indica	**528**	20	**98**	82	117	**101**	26	4.5	**5.3**	84	**94**
Infective juvenile	479–573	19–22	88–107	72–85	109–123	93–109	25–27	4.3–4.8	4.5–5.6	79–90	83–103
Male	**SL = 43**(35–48); GL = 21(18–23); W = 42(35–46); **D% = 122**; **TR = 106**(78–132); SW = 1.90(1.80–2.00); GS = 0.49										
	Note: Morphological characters of this species are very similar to that of *H. hawaiiensis*.										

phylogenetic hypotheses. Studies in taxonomy and systematics that were initially fuelled to facilitate applied research in pest management programmes have concomitantly laid the groundwork for the use of entomopathogenic nematodes to investigate biological questions that are basic in nature. *Heterorhabditis* has a small genome, is easily cultured, and belongs to the same taxonomic family as *C. elegans*, endowing it as a prime candidate for comparative proteomics and molecular developmental studies. The distribution of host specificity and different host-finding behaviours among species of *Steinernema* make it an excellent candidate for studying historical behavioural ecology. The presently established systematic framework enables studies of historical ecology and evolution (Brooks and McLennan, 1991; Harvey and Pagel, 1991), including the history, molecular mechanics, and genetic maintenance of parasitism and symbiosis. Entomopathogenic nematodes are also set to play an important role in teasing out the evolutionary genetics of symbiotic interactions, and in creating and testing theoretical models of pathogenicity, host specificity and speciation.

The current systematic knowledge base will be reinforced and improved by the training of systematists in traditional and molecular methods for recovering and

representing entomopathogenic nematode diversity. Greater phylogenetic resolution and explanatory power will come from integrated studies that combine morphological and molecular character sets. Developments in molecular biology and comparative genomics will significantly expand our repertoire of molecular markers and analytical tools. In the future the processing of samples collected by non-specialists can become streamlined, increasing the speed and efficiency of biodiversity surveys. Collaborative efforts of basic and applied scientists, leveraged with the historical framework provided by nematode systematists, will eventuate more effective biocontrol programmes and elevate the status of entomopathogenic nematodes and their symbiotic bacteria to that of model biological organisms.

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2 Biology, Taxonomy and Systematics of *Photorhabdus* and *Xenorhabdus*

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2.1. Introduction

2.1.1. Habitat and position in the Proteobacteria

Xenorhabdus and *Photorhabdus* are two bacterial genera mutualistically associated with infective juveniles of entomopathogenic nematodes from the genera *Steinernema* and *Heterorhabditis*, respectively. Most *Xenorhabdus* and all *Photorhabdus* are pathogenic for insects when injected into the haemocoel. In addition, non-symbiotic strains

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of *Photorhabdus asymbiotica* have been identified as opportunistic pathogens for humans (Farmer *et al.*, 1989) and another group of *Photorhabdus*, not yet identified at the level of a species, causes infections needing antibiotherapy (Peel *et al.*, 1999).

All isolations made during the last 20 years, from wild nematodes (*c.* 1000), established the presence of *Xenorhabdus* in *Steinernema* and *Photorhabdus* in *Heterorhabditis* juvenile intestines (Forst *et al.*, 1997). When sometimes other bacterial strains were found associated with *Steinernema* (Lysenko and Weiser, 1974; Aguillera *et al.*, 1993; Elawad *et al.*, 1999), it was shown that they were contaminants of the cuticle (Bonifassi *et al.*, 1999) and in all cases the symbiont was found in the infective juvenile gut. In *Heterorhabditis, Ochrobactrum* spp. (Babic *et al.*, 2000) and *Providencia rettgeri* (Jackson *et al.*, 1995) have sometimes been isolated and were probably also located between the cuticle and the juvenile sheath.

Xenorhabdus and *Photorhabdus*, as with other insect and vertebrate symbionts, are members of the γ-subclass of Proteobacteria. Both are negative for nitrate-reductase, and *Xenorhabdus* is negative for catalase, two major positive characters of the Enterobacteriaceae. Nevertheless, they have the enterobacterial common antigen (Ramia *et al.*, 1982). Moreover, if we look at the tree of the ribosomal data-set project (Larsen *et al.*, 1997), both genera branch deeply in the family of the Enterobacteriaceae without any common ancestor. Their nearest neighbour is the genus *Proteus*.

2.1.2. Fundamental and applied interests

The study of symbiont properties, such as cellular exportation, exoenzymatic activities, special metabolites, pathogenic processes, capabilities to differentiate into multicellular populations for the colonization of different habitats, are bringing new insights for microbiology. This is particularly true for the study on pathogenic mechanisms and specific metabolic pathways. Presumably researchers will soon also be able to propose an evolutionary history for these mechanisms with the comparative data given by these bacterium–invertebrate interactions.

The foremost applied interest in studying these bacteria concerns nutritional requirements for improving mass production of the nematodes for biological control of insects. We know that the quality of the symbiont inoculum, in terms of viability and preservation from microbial contamination, must be maintained for effective commercial production (Ehlers *et al.*, 1990, 1998). The second interest of these bacteria is to use their secondary metabolites which have a commercial potential in the pharmaceutical and agroforestry industries (Webster *et al.*, 1998). The third interest is the occurrence of protein toxins identified in *Photorhabdus* (Bowen *et al.*, 1998) that enhanced the use of the bacteria alone for biological control. Programmes to insert toxin genes into plant genomes for crop protection have been proposed, as for *Bacillus thuringiensis* ('Bt'). In addition, to counter resistance development in target insects consuming these transgenic 'Bt' plants, the insertion of both toxin genes (from *B. thuringiensis* and *Photorhabdus*) into the same plant has been suggested (ffrench-Constant and Bowen, 1999).

2.2. Biology of *Xenorhabdus* and *Photorhabdus* Bacteria

2.2.1. Life cycle – a vegetative multiplication in insects and a quiescence in nematodes

Parasitism starts when nematodes enter by natural openings (mouth, anus, spiracles) or directly through the integument (mainly *Heterorhabditis* nematodes) of the insects. *Steinernema* spp. induce a toxicogenesis (Boemare *et al.*, 1982, 1983b) and produce an immune depressive factor active against antimicrobial peptides from the insects (Götz *et al.*, 1981); nothing is known for *Heterorhabditis* in this respect. Infective juveniles of both genera release their bacterial symbionts in the host body cavity and develop into fourth-stage juveniles and adults. The insects die mainly due to a septicaemia. Sometimes a bacterial toxaemia precedes the resulting septicaemia (Forst *et al.*, 1997). Nematodes reproduce in the insect cadaver and feed on the symbiont biomass and insect tissues metabolized by the bacteria.

There are two different physiological states in the life cycle of the symbionts corresponding to two different ecological niches. The first is a phoretic state in the resting stage of the nematode host: *Xenorhabdus* occurs naturally in a special intestinal vesicle of *Steinernema* infective juveniles (Bird and Akhurst, 1983), while *Photorhabdus* are mainly located in the anterior part of *Heterorhabditis* infective juvenile guts (Boemare *et al.*, 1996). The second state is a vegetative state when the bacteria multiply inside the insects after inoculation in the haemolymph. Consequently, the symbionts alternate between a poor and a rich nutrient existence. If we compare with several other bacteria that are symbiotic with animals (see Forst and Clarke, Chapter 3 this volume), the life cycle of these bacteria is unique: they are symbiotic with nematodes and pathogenic with insects.

2.2.2. Virulence of bacteria and contribution of nematodes

In general terms, except for the rare strains which are pathogenic by ingestion, the bacterial infection starts after the penetration of the nematode into the body cavity of insects. No development in the insect gut has been reported. Depending on the three partners of this interaction (insect, nematode, bacteria), the pathogenic process may be the result of some insect resistance according to insect species, the action of one of the partners (bacteria or nematode), or the action of both partners (bacteria and nematodes).

According to Bucher (1960), when the LD_{50} is $<$ 10,000 cells the bacterium may be considered as entomopathogenic. Most *Xenorhabdus* strains are highly pathogenic for larvae of the greater wax moth, *Galleria mellonella*, with LD_{50}s $<$ 100 cells (Akhurst and Boemare, 1990). However, *X. poinarii* has little pathogenicity for *G. mellonella* when injected alone (LD_{50} $>$ 5000 cells), although it is highly pathogenic when co-injected with axenic (germ-free) *Steinernema glaseri*, its natural host (Akhurst, 1986b). Axenic *S. scapterisci* and its *Xenorhabdus* symbiont alone are also not pathogenic to *G. mellonella*. The combination of both partners re-establishes the pathogenicity of the complex towards *G. mellonella* (Bonifassi *et al.*, 1999).

All *Photorhabdus* strains examined to date have been reported to be entomopathogenic, the LD_{50} usually being $<$ 100 cells when injected into *G. mellonella*

(Fischer-Le Saux *et al.*, 1999b). Consequently *Photorhabdus* have to be considered as highly entomopathogenic. In addition some of them are entomopathogenic by ingestion (ffrench-Constant and Bowen, 1999). The toxin produced is active on the insect digestive epithelium, not only from the gut lumen, but also from the body cavity (Blackburn *et al.*, 1998).

2.2.3. Antimicrobial barriers preserve the symbiosis

There is a large production of anti-microbial compounds at the end of *Xenorhabdus* spp. *in vitro* multiplication (Paul *et al.*, 1981; Akhurst, 1982; McInerney *et al.*, 1991a,b; Sundar and Chang, 1993; Li *et al.*, 1996, 1997). It was demonstrated that they were also produced in insects (Maxwell *et al.*, 1994). Similarly there is production of antimicrobial compounds from *Photorhabdus* spp. *in vitro* (Paul *et al.*, 1981; Richardson *et al.*, 1988; Li *et al.*, 1995; Hu *et al.*, 1997) and *in vivo* (Hu *et al.*, 1998). All these molecules show a large scale of antibiotic activity (see Webster *et al.*, Chapter 5, this volume). Conversely, bacteriocins produced by both genera are active against closely related bacteria such as other species of *Xenorhabdus*, *Photorhabdus* and the nearest genus *Proteus* (Boemare *et al.*, 1992; Thaler *et al.*, 1995). So antimicrobial barriers play an important role to protect the specificity of the symbiosis by eliminating microbial competitors (Boemare *et al.*, 1993b; Thaler *et al.*, 1997).

2.2.4. Gnotobiology

Nematodes reproduce in a state of natural monoxenic microcosm (containing only one bacterial species) occurring during the insect parasitism and offer a sort of monoxenic sanctuary inside their gut during the resting stage. These two features are exceptional in terms of microbial ecology and are at the origin of the definition of the concept of natural monoxeny occurring with the entomopathogenic nematodes (Bonifassi *et al.*, 1999). After the death of an organism, the putrefaction normally proceeds from the intestinal microflora of the dead organism. This is not true with parasitism by entomopathogenic nematodes: the insect cadaver is essentially a mummified bag containing only *Xenorhabdus* or *Photorhabdus* cells that work with nematodes on insect tissues and provide their own biomass for nutrition of the nematodes. When juvenile larvae are escaping the insect cadaver, they recruit some symbiont cells in their intestine for the next infection assuring continuation of the mutualistic association. During long-term starvation in the resting infective juveniles, about 100 cells of *Xenorhabdus* or *Photorhabdus* are maintained. Two possibilities have been hypothesized: either bacteria are digested by starving juveniles and there is a threshold of about 100 bacterial cells that are maintained (Selvan *et al.*, 1993), or bacteria are not digested. The latter, where bacteria are not digested and are well preserved, supposes a special behaviour of the infective juveniles, and a special quiescent physiology of the bacteria.

Another interesting feature is that the reproduction of nematodes on artificial diet is possible. This point was at the origin of the mass production of the nematodes for biological control. As we are able to axenize nematodes and to cultivate the bacteria on nutrient agars, combinations have been tested to establish the reliability of the symbiosis. Such gnotobiological experiments show that some combinations with non-symbiotic bacteria, as *E. coli*, allow development of the nematodes

(Ehlers *et al.*, 1990). Steinernematidae and Heterorhabditidae belong to Rhabditida, a nematode group adapted to feed on various microorganisms. They retained the ancestral microbivorous behaviour allowing them to feed and reproduce on microorganisms other than their own symbiont. However, these non-symbiotic bacteria do not support long-term experimental associations (Akhurst and Boemare, 1990; Ehlers *et al.*, 1990; Han and Ehlers, 1998). Similarly, *Photorhabdus* isolates do not support *in vitro* culture of any *Steinernema* species or *Xenorhabdus* spp. of any *Heterorhabditis* (Akhurst, 1983; Akhurst and Boemare, 1990; Han *et al.*, 1990; Gerritsen and Smits, 1993). Consequently some non-symbiotic bacteria are able to create provisional associations with these nematodes, but the naturally occurring symbiotic bacteria provide the most efficient development. This paradox underlines our inadequate understanding about the needs provided by most of the bacteria for the nutritional requirements of the microbivorous nematodes, and for the special nutritional or hormonal substances provided by the symbionts for entomopathogenic nematodes.

2.2.5. Intraspecific biodiversity

Phase variation can be easily detected by dye adsorption and antibiotic production (Akhurst, 1980) for any symbiotic strain. Only phase I of the symbionts have been isolated from wild nematodes in natural conditions. After *in vitro* subculture a variable proportion of clones is affected in colony and cell morphologies (Boemare and Akhurst, 1988), motility (Givaudan *et al.*, 1995), production of endo- and exo-enzymes, respiratory enzymatic activities (Smigielski *et al.*, 1994), and secondary metabolites (Akhurst, 1980, 1982). Revertants have been obtained with *X. nematophila* (Akhurst and Boemare, 1990; Givaudan *et al.*, 1995) indicating that variation in *Xenorhabdus* fits the classical definition of phase variation. In bacteriology, phase variation is commonly defined as a reversible genetic event usually mediated by DNA instability such as DNA inversion occurring at a significant frequency. In the case of *Photorhabdus* no reversion has been confirmed. In fact, the terminology of phenotypic conversion should be more appropriate. For *Xenorhabdus* and *Photorhabdus*, 'phase variation' is commonly used to refer to an alternative balance in expression/non-expression of physiological traits from the same genome. For every character which can be quantified (e.g. luminescence, antibiotic production) the difference between phases is quantitative, and it is highly probable that this is also true for other phase-related characters emphasizing the occurrence of a genetic regulation (see Forst and Clarke, Chapter 3 this volume).

2.2.5.1. Physiological significance

Phase I variants provide essential nutrients for the nematodes by killing and metabolizing the insect host and producing a range of antimicrobial agents. Although phase II variants may also kill the insect host, they are less effective in providing growth conditions for the nematodes (Akhurst, 1980, 1982; Akhurst and Boemare, 1990). In addition, some *Photorhabdus* phase II variants may be deleterious for their nematode partner (Ehlers *et al.*, 1990; Gerritsen and Smits, 1993). There is no satisfactory explanation for the ecological role of phase II: is it a survival form, ancestral bacterial form of the symbiont, or a shift in relationship with the life cycle of the nematode?

2.2.5.2. Consequences for taxonomic studies

This phenotypic conversion has been the source of dissimilarities between results published by different laboratories. It occurs at an intra-specific level for all *Xenorhabdus* and *Photorhabdus* but does not affect the phenotypic characters that discriminate the species (Akhurst and Boemare, 1988).

When testing a bacteriological property, even if only one variant is positive among other variants of the same strain that are negative, the response has to be compiled as positive for the given strain. In fact for taxonomic purposes we have to be consistent with the definition of all the potential expression of the studied strain.

2.3. Taxonomy and Systematics

Initially only one genus, *Xenorhabdus*, encompassed all the symbionts of the entomopathogenic nematodes (Thomas and Poinar, 1979). The species previously named '*Xenorhabdus luminescens*' clearly formed a DNA relatedness group distinct from all other *Xenorhabdus* strains (Boemare *et al.*, 1993a; Akhurst *et al.*, 1996) and had also significant differences in phenotypic characters (Fischer-Le Saux *et al.*, 1999b). This resulted in the transfer of *X. luminescens* into a new genus, *Photorhabdus*, as *Photorhabdus luminescens* comb. nov. (Boemare *et al.*, 1993a). *Xenorhabdus* strains are clearly distinguished from strains of *Photorhabdus* by the 16S rDNA signature sequences. *Xenorhabdus* have a TTCG sequence at positions 208–211 (*E. coli* numbering) of the 16S rDNA, while *Photorhabdus* have a TGAAAG sequence at positions 208–213 (Rainey *et al.*, 1995; Szállás *et al.*, 1997).

A fast method using the polymorphism of the 16S rRNA genes (Brunel *et al.*, 1997; Fischer-Le Saux *et al.*, 1998) gives an excellent estimation of the diversity of the genera by using efficient restriction endonucleases providing the most discriminative restriction patterns (Fig. 2.1). Both genera have a particular cell morphology showing, mainly in phase I variants, protein inclusions inside the protoplasm that are highly characteristic (Fig. 2.2).

The names of the bacteria are modified from the previous descriptions to comply with bacteriological nomenclature. Thus, according to rule 65 (2) of the Code of Nomenclature of Bacteria, *Photorhabdus* and *Xenorhabdus*, genera ending by *rhabdus* (from *rhabdos*, rod in Greek as a feminine word), becomes in modern Latin a feminine word (Euzéby and Boemare, 2000). Consequently, '*X. nematophilus*' and '*X. japonicus*' become *X. nematophila* and *X. japonica*, and the new species of *Photorhabdus, P. temperata* and *P. asymbiotica*. Details related to the description of the genera and the species are described in Boemare and Akhurst (2000, 2001) and Akhurst and Boemare (2001).

2.3.1. Genus *Xenorhabdus*

A comprehensive phenotypic study led to the elevation of the subspecies previously described (Akhurst, 1983) to species status, as *X. nematophila* (*X. nematophilus*), *X. bovienii, X. poinarii* and *X. beddingii* (Akhurst and Boemare, 1988). *Xenorhabdus japonica* (*X. japonicus*), symbiotically associated with *Steinernema kushidai*, was described later (Nishimura *et al.*, 1994), meaning that today five species are recognized. DNA/DNA hybridization (Suzuki *et al.*, 1990; Boemare *et al.*, 1993a; Akhurst *et al.*, 1996) and 16S rDNA analyses (Suzuki *et al.*, 1996; Brunel *et al.*, 1997; Szállas *et al.*, 1997; Fischer-Le Saux *et al.*, 1998) validated the inclusion of five species in the genus *Xenorhabdus*.

However, DNA/DNA hybridization analyses and multivariate analyses of phenotypic data confirmed that some strains should be assigned to several new species (e.g. the symbionts of *S. arenarium, S. puertoricense, S. riobrave, S. scapterisci* and *S. serratum*), but too few to warrant a decision on their taxonomic status (Bonifassi *et al.*, 1999; Fischer-Le Saux *et al.*, 1998).

2.3.1.1. Main characters of the genus

Xenorhabdus cells are asporogenous, rod-shaped cells (0.3–2 × 2–10 μm), and Gram negative. They are facultatively anaerobic, with both respiratory and fermentative types

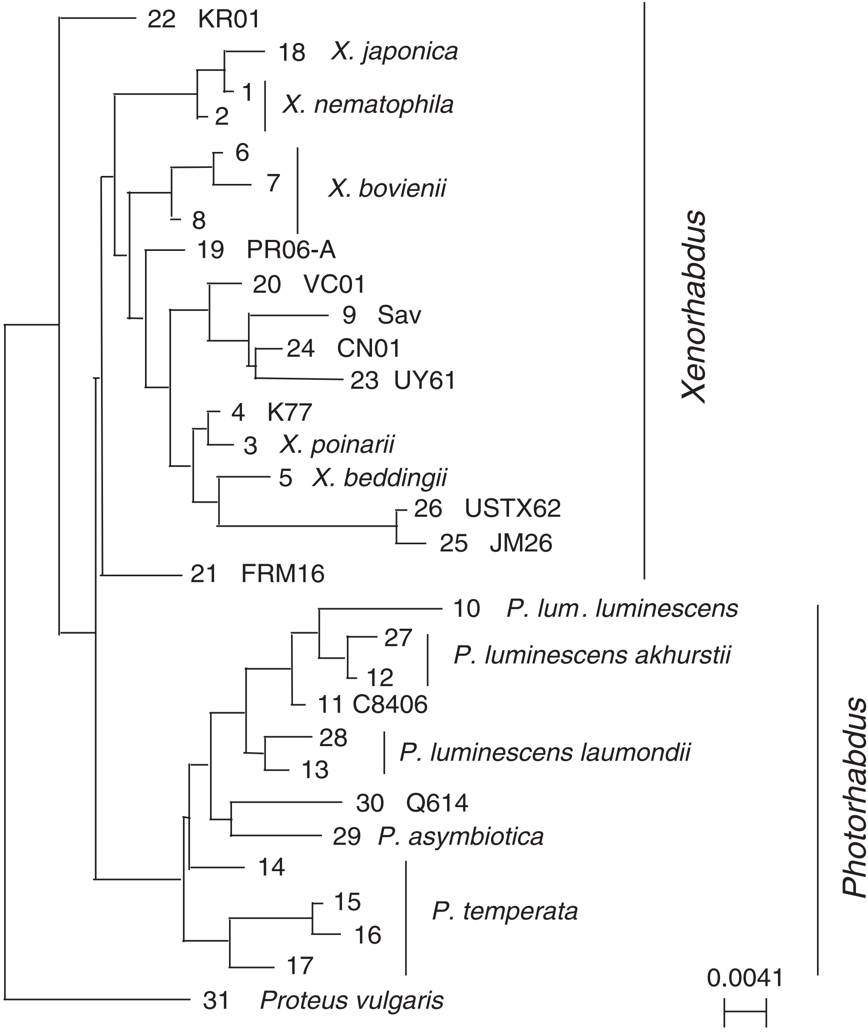

Fig. 2.1. The neighbour-joining method was applied to 30 defined genotypes from a total of 117 strains. The number of the genotypes according to Fischer-Le Saux *et al.* (1998) is followed by the name of the strain or the species (from Boemare and Akhurst, 2000). Scale bar: number of substitutions per site.

of metabolism. The optimum temperature is usually 28°C or less; a few strains grow at 40°C. Acid (no gas) is produced from glucose, and some other carbohydrates are poorly fermented. Catalase is negative and nitrate is not reduced to nitrite. The genus is negative for most tests used to differentiate Enterobacteriaceae. Phase shift occurs to varying degrees in growth stationary cultures (Boemare and Akhurst, 1988). Phase I variants produce protoplasmic crystalline inclusions in stationary period cultures (Boemare *et al.*, 1983a), are motile by means of peritrichous flagella and may swarm on 0.6–1.2% agar (Givaudan *et al.*, 1995), and are lecithinase positive on egg yolk agar. Phase II variants lack dye adsorption (Akhurst, 1980), antibiotic production (Akhurst, 1982), protein inclusions and some other characteristics of phase I variants. Lipase is detected with Tween–20® (Sigma, St Louis, USA) and most strains hydrolyse Tweens–40, 60, 80 and 85; lipolytic responses are more positive with phase II than phase I variants. Some phase II strains are motile, but this is generally a property of phase I variants. Most strains are positive for deoxyribonuclease and protease. Biochemical identification of *Xenorhabdus* within the family Enterobacteriaceae and the main characters of the species are summarized in Tables 2.1 and 2.2. *Xenorhabdus* are only known from the intestinal lumen of entomopathogenic nematodes of the Family Steinernematidae and insects infected by these nematodes.

2.3.1.2. *Xenorhabdus beddingii*

The upper threshold for growth is 39°C. Both variants are motile (Givaudan *et al.*, 1995). Phase I is highly unstable, producing a very stable phase II. The strains hydrolyse aesculin and the pigmentation is lightly brown. They are associated with two undescribed species of *Steinernema* from Australia (Akhurst, 1986a), one of which may be *S. longicaudum* which was described from China.

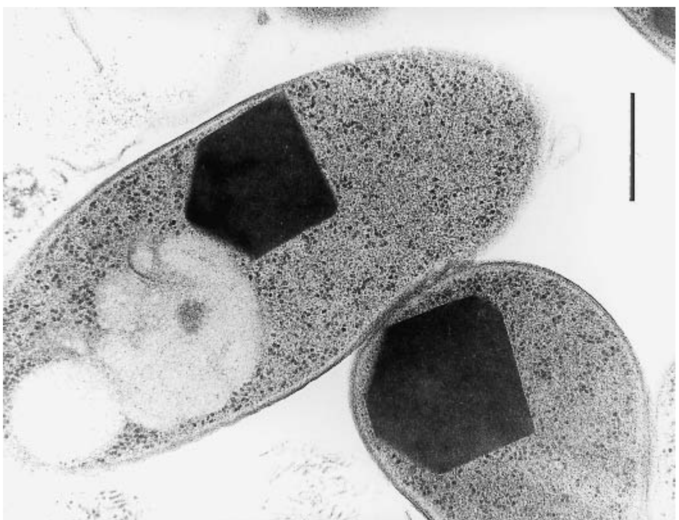

Fig. 2.2. Protoplasmic inclusions in *Photorhabdus temperata temperata*. Transmission electron microscopy after fast freeze fixation and cryo-substitution. After uranyl acetate and lead citrate dying, crystalline inclusions are highly contrasted (black areas) and the 'myelinic membranes' (clear areas) are poorly contrasted. The functional significance of these structures is unclear. Scale bar = 0.5 μm.

2.3.1.3. *Xenorhabdus bovienii*

This species grows until 32°C; some strains will grow at 5°C. Some phase II variants may be motile (Givaudan *et al.*, 1995). It is associated with several species of steinernematid nematodes (*S. feltiae, S. intermedium, S. kraussei, S. affine*) from temperate regions (Boemare and Akhurst, 1988; Fischer-Le Saux *et al.*, 1998).

2.3.1.4. *Xenorhabdus japonica*

Strains of the species grow until 35°C. Pigmentation is yellowish brown. It is only associated with *S. kushidai* in Japan. Properties of this species are similar to *X. nematophila,* and *X. japonica* may be a subspecies of *X. nematophila*.

2.3.1.5. *Xenorhabdus nematophila*

The upper threshold for growth in nutrient broth is 35°C. Neither phase is pigmented, but the phase I strongly adsorbs dyes (Akhurst, 1980). Surface appendages and proteins have been described with phase I variants in ultrastructural and molecular terms: occurrence of glycocalyx and fimbriae (Brehélin *et al.*, 1993; Moureaux *et al.*, 1995), a specific outer membrane protein OpnP (Forst *et al.*, 1995), and flagella with the regulation of their synthesis (Givaudan *et al.*, 1995, 1996; Givaudan and Lanois, 2000). Several strains are lysogenic, producing phages and bacteriocins after thermal or mitomycin C induction (Boemare *et al.*, 1992, 1993b; Baghdiguian *et al.*, 1993). This species is only found associated with *S. carpocapsae*.

2.3.1.6. *Xenorhabdus poinarii*

This is the most heat tolerant *Xenorhabdus* species, the upper threshold for growth for several strains being at 40°C. The intensity of pigmentation in phase I varies from light to reddish brown. May be motile phase II variants. In some strains, phase I does not produce antimicrobials; in other strains both phases do. Associated with *S. glaseri* (Akhurst, 1986b) and *S. cubanum* (Fischer-Le Saux *et al.*, 1999a). It is not pathogenic for greater wax moth (*Galleria mellonella)* larvae unless associated with its nematode partner.

Table 2.1. Characteristics differentiating *Xenorhabdus* and *Photorhabdus*, from the nearest genus, *Proteus*, within the Enterobacteriaceae.[a]

	Xenorhabdus	*Photorhabdus*	*Proteus*
Bioluminescence	–	+	–
Catalase	–	+	+
Annular haemolysis	–	d	–
Urea hydrolysis	–	d	+
Indole	–	d	d
H_2S production	–	–	[+]
Nitrate reduction	–	–	+
Acid from mannose [*Xenorhabdus*]	+	+	–

[a] + = 90–100% of strains are positive; [+] = 76–89% are positive; d = 26–75% are positive; [–] = 11–25% are positive; – = 0–10% are positive.

Table 2.2. Characters discriminating *Xenorhabdus* spp.[a]

	X. nematophila	*X. bovienii*	*X. poinarii*	*X. beddingii*	*X. japonica*
Isolated from:					
S. carpocapsae	+	–	–	–	–
S. feltiae, S. intermedium, S. affine, S. kraussei	–	+	–	–	–
S. glaseri, S. cubanum	–	–	+	–	–
Unidentified, *Steinernema* spp.	–	–	–	+	–
S. kushidai	–	–	–	–	+
Upper threshold T°C for growth	35	32	40	39	35
Pathogenicity for lepidopteran insects	+	+	–	+	–
Motility	d	d	d	+	d
Pigmentation	ow	y	br	lb	yb
Simmon's citrate	+	+	+	+	–
Aesculin hydrolysis	–	–	d	+	–
Phenylalanine deaminase	d	[–]	[–]	–	d^w
Tryptophan deaminase	–	+	–	$+^w$	–
Acid production from:					
myo-Inositol	$+^w$	d^w	–	–	–
Ribose	–	+	–	+	–
Salicin	–	–	–	+	–
Utilization of:					
Diaminobutane	–	[+]	–	–	–
D,L-Glycerate	+	+	[–]	+	–
L(–)Histidine	–	+	[+]	+	–
myo-Inositol	$+^w$	d^w	–	–	–
Ribose	–	[+]	–	+	–
L-Tyrosine	–	[+]	–	+	–
Lecithinase (egg yolk agar)	d	d	–	d	d
Lipase (Tween-80)	d	+	$+^w$	$+^w$	–

[a] All tests were done at 28°C; + = 90–100% of strains are positive; [+] = 76–89% are positive; d = 26–75% are positive; [–] = 11–25% are positive; – = 0–10% are positive. The superscript w (e.g. $[+]^w$) indicates a weak reaction; F = fermentative. Pigmentation: ow = off-white; y = yellow; br = brown; lb = light brown; yb = yellowish brown.

2.3.2. Genus *Photorhabdus*

Poinar *et al.* (1977) isolated a bacterium from *Heterorhabditis bacteriophora* (Poinar, 1976), and proposed inclusion of this luminous species in the genus *Xenorhabdus, as 'X. luminescens'* (Thomas and Poinar, 1979). It was later transferred to *Photorhabdus luminescens* comb. nov. (Boemare *et al.*, 1993a).

By using the hydroxyapatite method (Akhurst *et al.*, 1996) and the S1 nuclease method (Fischer-Le Saux *et al.*, 1999b) to analyse DNA–DNA *Photorhabdus*

heteroduplexes, two DNA relatedness groups associated with *Heterorhabditis* nematodes and another which contains human clinical specimens (Farmer *et al.*, 1989), were recognized.

Species and subspecies among *Photorhabdus* were delineated by applying a polyphasic approach, combining 16S rDNA, DNA–DNA hybridization and phenotypic data (Fischer-Le Saux *et al.*, 1999b), as described below. On the basis of 16S rDNA sequencing, the new Australian clinical strains (Peel *et al.*, 1999) will probably constitute a new species (R.J. Akhurst, Australia, 2000, personal communication). The one non-luminous strain (Akhurst and Boemare, 1986) could be yet another species (Akhurst *et al.*, 1996).

2.3.2.1. Main characters of the genus

Photorhabdus cells are asporogenous, rod-shaped (0.5–2 × 1–10 μm), Gram negative, and motile by means of peritrichous flagella. They are facultatively anaerobic, having both respiratory and a fermentative metabolism. The optimum growth temperature is usually 28°C; some strains grow at 37–38°C. All strains are catalase positive, but do not reduce nitrate. They are negative for many characters of Enterobacteriaceae. They hydrolyse gelatin, and most strains are haemolytic for sheep and horse blood, some producing an unusual annular haemolysis on sheep blood at 25°C. All are lipolytic on Tween-20 and many on Tweens-40, 60, 80 and 85. Glucose is acidified without gas. Fructose, D-mannose, maltose, ribose, and *N*-acetylglucosamine are also acidified. Fermentation from glycerol is weak. Fumarate, glucosamine, L-glutamate, L-malate, L-proline, succinate and L-tyrosine are utilized as sole carbon and energy sources. Spontaneous phase shift occurs in subcultures, inducing the appearance of phase II clones. Phase I variants, which are the wild clones freshly isolated from the natural environment, produce protoplasmic inclusions synthesized inside a high proportion of cells (50–80%) during the stationary period (Fig. 2.2), and are luminous, emitting generally several wavelengths as white light, usually detectable by the dark-adapted eye. The intensity varies within and between strains and may only be detectable by photometer or scintillation counter in some isolates. Only one non-luminous isolate (Akhurst and Boemare, 1986) is known among the approximately 150 reported strains. Phase I variants produce light more than 100-fold greater than phase II variants. Phase I variants produce pink, red, orange, yellow or green pigmented colonies on nutrient agar, and especially on rich media (tryptic soy agar, egg yolk agar). Phase II variants are less pigmented or produce a different pigment. Phase II variants are mainly characterized by the loss of neutral red adsorption on MacConkey agar, and production of antibiotics.

Unfortunately, it appears that the type strain of the genus from *P. luminescens* (strain Hb) is not a good representative of the *Photorhabdus* isolates, because as numerous new isolates are typed, none have been found to belong to this taxon. So far, strain Hm remains the only one closely related to type strain Hb. Differential characters within the Enterobacteriaceae and species characteristics are listed in Tables 2.1 and 2.3.

2.3.2.2. *Photorhabdus luminescens*

The upper threshold for growth in nutrient broth occurs at 35–39°C. Strains are indole positive. Most strains produce weak acid from fructose, *N*-acetylglucosamine,

Table 2.3. Characters discriminating *Photorhabdus* spp.[a]

	Photorhabdus luminescens isolation from: *Heterorhabditis* spp.	*P. luminescens luminescens* isolation from: *H. bacteriophora* group Brecon	*P. luminescens laumondii* isolation from: *H. bacteriophora* group HP88	*P. luminescens akhurstii* isolation from: *H. indica*	*Photorhabdus temperata* isolation from: *Heterorhabditis* spp.	*P. temperata temperata* isolation from: *H. megidis* Palaeartic group	*Photorhabdus asymbiotica* isolation from: human blood and wounds
Upper threshold T °C for growth	35–39	38–39	35–36	38–39	33–35	34	37–38
Protoplasmic inclusions	+	+	+	+	+	+	–
Dye adsorption	+	+	+	+	+	+	–
Antimicrobial production	+	+	+	+	+	+	–
Indole production	+	+	+	d	[–]	–	–
Simmon's Citrate	d	+	d	d	d	d	$+^{w}$
Aesculin hydrolysis	+	+	+	+	[+]	d	+
Urease, Christensen's	D	–	[+]	D	D	[–]	+
Phenylalanine deaminase	[–]	–	D	–	[+]	D	–
Tryptophan deaminase	[–]	–	D	–	$[–]^{w}$	–	–
Acid production from:							
myo-Inositol	d	+	[+]	[+]	d^{w}	[–]	d^{w}
D-Mannitol	d	d^{w}	–	+	[–]	–	–
Trehalose	$[+]^{w}$	$+^{w}$	$[+]^{w}$	$[+]^{w}$	[+]	+	[+]
Utilization of:							
L-Fucose	d	d	–	[+]	d	+	–
D,L-Glycerate	[–]	d	–	–	+	+	d
L(–)Histidine	d	+	$[+]^{w}$	d	[+]	+	d
myo-Inositol	+	+	+	+	[+]	d	d
D,L-Lactate	[–]	–	–	d^{w}	–	–	–
D-Mannitol	d	+	–	+	[–]	–	–
Lecithinase (egg yolk agar)	+	+	+	+	+	+	–
DNase	[–]	–	+	–	+	+	–
Annular haemolysis (sheep blood agar)	D	+	[–]	+	+	$+^{w}$	+
Annular haemolysis (horse blood agar)	d	+	–	d	+	+	+

[a] All tests were done at 28°C; + = 90–100% of strains are positive; [+] = 76–89% are positive; d = 26–75% are positive; [–] = 11–25% are positive; – = 0–10% are positive. The superscript w (e.g. $[+]^{w}$) indicates a weak reaction; F = fermentative.

glucose, glycerol, maltose, mannose, ribose, and trehalose. Some strains acidify mannitol. The natural habitat is in the intestinal lumen of entomopathogenic nematodes of *H. bacteriophora* (Brecon and HP88 subgroups) and *H. indica*. This species is divided in three subspecies.

2.3.2.3. *Photorhabdus luminescens* subsp. *luminescens*

The upper threshold for growth in nutrient broth occurs at 38–39°C. Aesculin is hydrolysed and indole is weakly positive. DNase, tryptophan deaminase and urease are negative. Annular haemolysis of sheep and horse blood agars is characteristic of the group. This subspecies does not use D,L-lactate as the sole source of carbon. Mannitol is used as the sole source of carbon and energy. It is symbiotically associated with nematodes from the Brecon subgroup of *H. bacteriophora,* the type species of the genus *Heterorhabditis* (Poinar, 1976).

2.3.2.4. *Photorhabdus luminescens* subsp. *laumondii*

This subspecies has an upper threshold for growth in nutrient broth that is lower than the previous subspecies (35–36°C). Aesculin is hydrolysed, indole and DNase are positive. The tryptophan deaminase is variable and urease is mostly positive. Haemolysis on sheep and horse blood agars is total and the *Photorhabdus* annular reaction is rare. It does not use L-fucose, D,L-glycerate, D,L-lactate, or mannitol. It is symbiotically associated with nematodes of the HP88 subgroup of *H. bacteriophora* isolated in South and North America, southern Europe, and Australia, corresponding to the satellite DNA probe of the nematode strain HP88 (Grenier *et al.*, 1996a,b).

2.3.2.5. *Photorhabdus luminescens* subsp. *akhurstii*

This subspecies, the first one described, has an upper threshold for growth in nutrient broth at 38–39°C. Aesculin is hydrolysed, but tryptophan deaminase and DNase are negative. Urease and indole are variable. Annular haemolysis is observed on sheep blood agar. The use of D,L-lactate as the sole source of carbon is variable and weak when positive. Mannitol is used and acidified, but the D,L-glycerate is negative. It is symbiotically associated with the nematode *H. indica* isolated in tropical and sub-tropical regions.

2.3.2.6. *Photorhabdus temperata*

Colonies are highly luminous. The upper threshold for growth in nutrient broth occurs at 33–35°C. The DNase is positive and most of the strains are indole negative. Aesculin hydrolysis and tryptophan deaminase are mostly positive. Urease is variable. Acid is produced from fructose, *N*-acetylglucosamine, glucose, mannose, ribose, and weakly from glycerol and maltose. Annular haemolysis often occurs on sheep and horse blood agars. This species uses D,L-glycerate, but does not use D,L-lactate as the sole source of carbon. Mannitol is not used by most strains. The natural habitat is in the intestinal lumen of entomopathogenic nematodes of *H. megidis*, of NC subgroup of *H. bacteriophora*, and of *H. zealandica*.

2.3.2.7. *Photorhabdus temperata* subsp. *temperata*

This subspecies often produces several intermediate forms between the two extreme phase I and II variants (Gerritsen *et al.*, 1992). Strains cannot grow above 34°C. Indole

is negative and DNase is positive. Hydrolyses aesculin but tryptophan deaminase is variable. Urease is mostly negative. Annular haemolysis on sheep and horse blood agars is observed in most isolates. The subspecies uses D,L-glycerate and L-fucose, but does not use D,L-lactate and mannitol, as the sole source of carbon. The natural habitat is in the intestinal lumen of entomopathogenic nematodes of the Palaearctic subgroup of *H. megidis*.

2.3.2.8. *Photorhabdus asymbiotica*

Although this species is not symbiotic with nematodes, it must be mentioned due to its similarities with the symbiotic *Photorhabdus*. The upper threshold for growth in nutrient broth occurs at 37–38°C. Produce a yellow or brown pigment. No phase I isolates have been detected and isolates do not absorb dyes, sometimes weakly produce antibiotics, and are negative for lecithinase on egg yolk agar. They are positive for urease, aesculin hydrolysis, and for Christensen's citrate, but weakly positive on Simmons' citrate. Tryptophan deaminase, indole, and DNase are negative. Acid is produced from fructose, *N*-acetylglucosamine, glucose, maltose, mannose, ribose, and weakly produced from glycerol. Protoplasmic inclusions are poorly produced. Tween-40 esterase is variable. The typical annular haemolysis of *Photorhabdus* of sheep and horse blood agars (Akhurst *et al.*, 1996) was first shown with this species (Farmer *et al.*, 1989). Does not use L-fucose, D,L-lactate, or mannitol. The natural habitat is uncertain. All isolates have been obtained from human clinical specimens.

2.4. Co-speciation Between the Bacterial Symbionts and the Nematode Hosts

When we compare the taxonomic data of *Xenorhabdus* with that of their host nematodes, the close relatedness of the two taxonomic structures reveals co-speciation between bacterium and nematode (Table 2.4). There are *X. nematophila* and *S. carpocapsae, X. japonica* and *S. kushidai*, three or four *Xenorhabdus*-*Steinernema* associations in the course of characterization, *P. luminescens akhurstii* and *H. indica, P. temperata temperata* and the palaearctic group of *H. megidis*.

2.4.1. Taxonomic uncertainties do not invalidate the concept of co-speciation

However, sometimes one species of *Xenorhabdus* can share several species of *Steinernema*. As mentioned previously *X. bovienii* is symbiotic for *S. affine, S. feltiae, S. kraussei* and *S. intermedium; X. beddingii* is harboured by two undefined species of nematodes from which one would be possibly *S. longicaudum* (R. Akhurst, Australia, 2000, personal communication); *X. poinarii* is symbiotic for *S. cubanum* and *S. glaseri*. Conversely, two *Photorhabdus* subspecies are harboured by one species of *Heterorhabditis* (*P. luminescens luminescens* and *P. luminescens laumondii* by *H. bacteriophora*). These reports do not modify the concept of co-speciation. They are essentially the result of taxonomic uncertainties corresponding to the definition of species in bacteriology and nematology. Bacteriologists cannot use the cross-breeding method used by other biologists to delineate species. A species in bacteriology is an artificial concept. It is defined as a group of strains sharing approximately 70% or greater DNA/DNA relatedness with 5°C or less of temperature variation of DNA denaturation (ΔT_m) (Wayne *et al.*, 1987). When other criteria will be more accurate, subdivisions would be possible.

Table 2.4. Species associations.

Steinernema and *Xenorhabdus*	
S. kraussei	*Xenorhabdus bovienii*
S. abbasi	*Xenorhabdus* sp.
S. arenarium (synonym: *S. anomalae*)	*Xenorhabdus* sp.
S. affine	*Xenorhabdus bovienii*
S. bicornutum	*Xenorhabdus* sp.
S. carpocapsae	*Xenorhabdus nematophila*
S. cubanum	*Xenorhabdus poinarii*
S. feltiae	*Xenorhabdus bovienii*
S. glaseri	*Xenorhabdus poinarii*
S. intermedium	*Xenorhabdus bovienii*
S. kushidai	*Xenorhabdus japonica*
S. longicaudum(?)	*Xenorhabdus beddingii*
S. monticulum	*Xenorhabdus* sp.
S. puertoricense	*Xenorhabdus* sp.
S. rarum	*Xenorhabdus* sp.
S. riobrave	*Xenorhabdus* sp.
S. scapterisci	*Xenorhabdus* sp.
S. serratum	*Xenorhabdus* sp.
Heterorhabditis and *Photorhabdus*	
H. bacteriophora subgroup Brecon	*Photorhabdus luminescens luminescens*
H. bacteriophora subgroup HP88	*Photorhabdus luminescens laumondii*
H. indica	*Photorhabdus luminescens akhurstii*
H. zealandica	*Photorhabdus temperata*
H. bacteriophora subgroup NC	*Photorhabdus temperata*
H. megidis Nearctic group (Ohio, Wisconsin)	*Photorhabdus temperata*
H. megidis Palaearctic group	*Photorhabdus temperata temperata*
Clinical opportunistic strains	*Photorhabdus asymbiotica*

For instance, different genotypes of PCR–RFLP of 16S rDNA have been recognized for *X. bovienii* (Brunel *et al*., 1997; Fischer-Le Saux *et al*., 1998), underlying the possibility that bacterial subspecies could be defined shortly based on 16S rDNA sequence, and surprisingly each one would correspond to *S. feltiae, S. affine, S. intermedium,* and *S. kraussei* (Boemare, unpublished data).

Similarly difficulties may also occur with the definition of nematode species as in the case of *S. cubanum* and *S. glaseri*. When *S. cubanum* was described, it was considered to be a related species of *S. glaseri* (Mrácek *et al*., 1994). Morphological characters and the restriction analysis of the intergenic transcribed spacer (ITS) of the ribosomal genes reveal high similarities between the two species (Hominick *et al*., 1997).

The only remaining enigma is the NC strain of *H. bacteriophora,* which harbours *P. temperata* and not a subspecies of *P. luminescens* as other symbionts of *H. bacteriophora*. The re-isolation of this group in nature is required to control for possible confusion in previous sampling.

2.4.2. Correlation between symbiont and host heat tolerances

Within both bacterial genera, the upper threshold for growth temperature appears to be a relevant character for distinguishing species (Tables 2.2 and 2.3). If we examine the ecology of the corresponding strains, it is noticeable that this character and the host species origin are correlated. For example, *X. bovienii* and *P. temperata* strains are 'cold' adapted symbionts of nematodes (*S. feltiae* and *H. megidis*) living in temperate cilmates, while *X. poinarii* and *P. luminescens akhurstii* are 'warm' adapted symbionts of nematodes (*S. glaseri, S. cubanum* and *H. indica*) from tropical and sub-tropical areas (Fischer-Le Saux *et al.*, 1999a,b). Thus, temperature tolerance appears to be an important property reflecting long-term adaptation to different climatic conditions in bacterium–helminthic complexes.

2.4.3. Mechanisms of co-evolution

The phenomenon of co-speciation results from co-evolution between both partners of the symbiosis. How is the specific association between bacteria and nematode maintained? It is likely that signal compounds are involved in the recognition for both partners. In the case of *Xenorhabdus*, preservation of the bacterial cells from digestion by the infective juveniles during the free life cycle of the host seems to be ensured by an anatomical sanctuary: the special vesicle in the intestine of the infective juveniles (Bird and Akhurst, 1983). This is a clear co-evolutionary trait, not appearing in other species of free-living soil-dwelling nematodes in the Order Rhabditida. Specific attachment by the symbiotic bacteria to this organ ensures the maintenance of the symbiosis. However, the molecular basis of such associations is unknown, even if bacterial fimbriae and glycocalyx are supposed to be the structures that support the specific attachment during starvation in the host (Brehélin *et al.*, 1993; Moureaux *et al.*, 1995).

Poinar (1993) suggested that *Heterorhabditis* evolved from a marine ancestor. On the basis of biological, taxonomic and ecological arguments, the genus *Heterorhabditis* evolved from a *Pellioditis*-like ancestor in an arenicolous marine environment. As it seems likely that its symbiotic bacterium would have obtained the *lux* genes by horizontal genetic transfer from marine bacteria, the symbiosis may have originated at the seashore interface.

2.5. Conclusions and Future Prospects

Further microbial ecology studies are needed to explain the durability over many generations of these intestinal symbiotic associations. The concept of a natural monoxeny in these bacterium–helminthic symbioses must be accepted if we consider the following feature. In natural conditions, if any bacterium is carried in the infective juveniles, perhaps the resulting complex could kill insects, but the probability of the nematode reproducing is low.

Despite the high similarity of their life cycles, *Photorhabdus*–*Heterorhabditis* and *Xenorhabdus*–*Steinernema* symbioses are widely divergent. The similarities in the patterns of infectivity and mutualism with nematode should be considered to result from evolutionary convergence. Indeed the symbiotic, pathogenic, and phase variation properties, which are the conditions for such associations (Boemare *et al.*, 1997), do

not necessarily imply the same physiological mechanisms. Current genetic studies will probably formulate a clearer picture to explain the convergent evolution of *Photorhabdus-Heterorhabditis* and *Xenorhabdus-Steinernema* symbioses.

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3 Bacteria–Nematode Symbiosis

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3.1. Introduction

Xenorhabdus and *Photorhabdus* are motile Gram-negative bacteria that form a mutualistic symbiosis with entomopathogenic nematodes from the families Steinernematidae or Heterorhabditidae, respectively (Forst *et al.*, 1997a). While both bacteria belong to the family Enterobacteriaceae they do not reduce nitrate and ferment only a limited number of carbohydrates. The bacteria are carried in the gut of a specialized,

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free-living form of the nematode called the infective juvenile. In the soil, infective juveniles seek out insect larvae and gain entrance to the larval haemocoel. The symbiotic bacteria are released in the haemolymph where they proliferate and produce a wide range of toxins and hydrolytic exoenzymes that are responsible for the death and bioconversion of the insect larva into a nutrient soup that is ideal for nematode growth and reproduction. The nematodes reproduce until the nutrient supply becomes limiting at which time they develop into infective juveniles which are recolonized by the symbiotic bacterium. This remarkable co-dependent reproductive cycle is the result of a highly evolved interaction between the bacterium and the nematode.

An interesting aspect of both *Photorhabdus* and *Xenorhabdus* is the formation of phenotypic variant cells that arise at low frequency during prolonged incubation. The variant cells, referred to as phase II or secondary cells, are altered in numerous properties normally found in the so-called phase I or primary form of the bacteria isolated from the nematode.

The unique life cycle of *Photorhabdus* and *Xenorhabdus* involves the formation of a mutualistic symbiosis with one host, the nematode, and a vigorous pathogenic attack against a separate host, the insect. The bacteria benefit from this interaction by being protected from the competitive environment of the soil and by being transported to the nutrient-rich haemolymph of an insect. In turn, the nematode takes advantage of the pathogenic potential of the bacteria to help kill the insect host. The bacteria also supply the nutrient base for the growth and development of the nematode and suppress contamination of the insect cadaver by soil microorganisms. Recent studies in *Xenorhabdus* and *Photorhabdus* have allowed us to identify genetic systems that play important roles in the association between the nematode and the bacterium. Here we first describe the well-studied *Vibrio fisheri*–squid symbiotic model system. Subsequently we discuss in detail the various stages of the bacteria–nematode symbiosis and compare this symbiosis with the bacteria–squid system. The second part of the chapter focuses on our current understanding of the molecular mechanisms involved in this symbiosis and discusses the topic of variant cell formation. Noteable differences between the *Photorhabdus-Heterorhabditis* and *Xenorhabdus-Steinernema* systems will be presented throughout the chapter.

3.2. Symbiotic Relationships Between Bacteria and Animals

Many bacteria have long and mutually beneficial relationships with eukaryotic hosts. Several model systems that utilize naturally occurring associations between bacteria and animals have clearly established that symbiotic relationships involve complex molecular interactions that require coordinate gene expression within, and trans-species signalling between, the bacteria and the host. An example of this is the association between the bacterium, *Buchnera* spp. and its aphid host (Baumann *et al.*, 1995). *Buchnera* lives in specialized cells in the aphid called bacteriocytes and the bacterium is transmitted from generation to generation in the egg. These bacteriocytes provide the bacterium with most of the nutrients that are required for growth and, as a consequence, the genome of *Buchnera* spp. (0.64 Mb; Shigenobu *et al.*, 2000) has been greatly reduced compared with free-living bacteria such as *Escherichia coli* (4.6 Mb). Reduction of genome size is a common feature of bacteria that have an obligate interaction with eukaryotic cells. Despite its large-scale reduction in genome size, *Buchnera* spp. has

retained genes that are used to make essential amino acids and vitamins for the aphid host.

One of the best-studied bacteria–animal symbioses occurs between the Hawaiian bobtail squid, *Euprymna scolopes*, and the bioluminescent bacterium *Vibrio fischeri*. This relationship is not obligate and both partners can grow independently of each other. However, all *Euprymna* squid isolated from the wild are colonized by *V. fischeri* suggesting that there is a selective advantage favouring this association. *Vibrio fischeri* colonize a specialized organ in the squid, called the light organ where the bacteria grow to high cell density and emit light (Ruby, 1996). The squid provides the bacteria with nutrients, in the form of amino acids (Graf and Ruby, 1998). The squid use bacterial bioluminescence as a counter-illumination camouflage system to eliminate its own shadow that may be detected by predators looking up from below (McFall-Ngai, 1998).

Immediately after hatching the immature light organ of the juvenile squid lacks bacteria. The light organ is rapidly colonized by *V. fischeri* that are present in low concentrations (< 10 cells per ml of seawater) in the marine environment (Nyholm *et al.*, 2000). In the absence of *V. fischeri* in the marine environment, the light organ remains uncolonized. A negative selection mechanism against non-symbiotic microorganisms and positive selection for *V. fischeri* accounts for this selective colonization. The presence of *V. fischeri* induces the squid to produce mucus that enables it to selectively capture and enrich for the bacterium (Nyholm *et al.*, 2000). Moreover, *V. fischeri* induces developmental changes in the light organ that involves modulation of host gene expression systems. It is clear that reciprocal signalling between the host and the bacterium plays an integral role in the progression and maintenance of this symbiotic relationship (Lemus and McFall-Ngai, 2000; Nyholm *et al.*, 2000).

The bacteria modulate the immune response of the host in order to establish a suitable niche for the foundation of a stable relationship (McFall-Ngai, 1999; Lemus and McFall-Ngai, 2000). Bioluminescence appears to play an important role in this modulation (Ruby and McFall-Ngai, 1999). The enzyme luciferase, which is responsible for light production, has a high affinity for molecular oxygen. It has been hypothesized that this enzyme competes for oxygen with host enzymes that produce reactive oxygen species in response to a bacterial infection (Ruby and McFall-Ngai, 1999). Thus *V. fischeri* have a significant competitive advantage over dim mutant strains with respect to colonization of the light organ (Visick *et al.*, 2000). This modulation of host environment and/or signalling pathways might be a conserved principle in all bacteria–host relationships, both symbiotic and pathogenic.

3.3. The Bacteria–Nematode Symbiosis is a Cyclic Association

The symbiosis between the bacterium and nematode share similarities with the *Vibrio*–squid association. For example, the relationship is not obligate as both *Photorhabdus* and *Xenorhabdus* can be grown without the nematode under laboratory conditions. In addition there is a high degree of specificity imposed on the symbiotic relationship between the bacteria and the nematode. This specificity is generally more restrictive for the *Heterorhabditis-Photorhabdus* pair where one species of nematode retains one species of bacteria. In contrast, the *Steinernema-Xenorhabdus* symbiosis can be less restrictive whereby several different species of the nematode can grow on and retain the same *Xenorhabdus* species (Akhurst and Boemare, 1990).

While the life cycle of the respective bacteria–nematode complexes appear to be similar, upon closer examination differences between the *Heterorhabditis*-*Photorhabdus* and *Steinernema*-*Xenorhabdus* symbioses can be discerned. *P. luminescens* and *X. nematophila* are the most extensively studied species of *Photorhabdus* and *Xenorhabdus*, respectively. Comparisons of major phenotypic traits of these bacteria reveal important differences (Table 3.1). *P. luminescens* possesses catalase activity, is bioluminescent, and produces hydroxystilbene antibiotics and anthraquinone pigment compounds. The protoplasmic crystalline inclusions are composed of either 11.6 kDa (CipA) or 11.3 kDa (CipB) crystal proteins. Many, but not all, strains of *P. luminescens* possess urease activity, produce indole and are able to hydrolyse aesculin. In contrast, *X. nematophila* does not produce detectable levels of catalase, produces unique antimicrobial compounds (xenocoumacins, xenorhabdins, indole derivatives), does not possess pigments and produces protoplasmic crystalline inclusions composed of either 26 kDa or 22 kDa crystal proteins. *X. nematophila* does not possess urease activity, produce indole or hydrolyse aesculin. While comparison of *X. nematophila* and *P. luminescens* is useful for highlighting salient differences between these two genera, it is important to note that significant intrageneric variations in phenotypic traits and biological properties occur in both *Xenorhabdus* spp and *Photorhabdus* spp. (Akhurst, 1986; Boemare and Akhurst, 1988).

Several distinguishing differences also exist between the nematode families Heterorhabditidae and Steinernematidae (Table 3.2). The first generation adults of *Heterorhabditis* are hermaphroditic and the infective juveniles carry the symbiotic bacteria in the midgut of the intestine. First generation *Steinernema* are amphimictic males and females and the bacteria are carried in a specialized intestinal vesicle. While both nematodes belong to the Rhabditida family, *Heterorhabditis* branch in the clade containing *Caenorhabditis elegans* and are most closely related to the Strongylida group while the *Steinernema* branch in a separate clade and are more closely related to the Strongyloididae group (Blaxter *et al.*, 1998). Additional morphological features that distinguish these two groups of nematodes are listed in Table 3.2.

Significant differences also exist in the growth characteristics of the respective nematodes. Axenic (bacteria-free) *Steinernema* can grow on artificial medium while an

Table 3.1. Distinguishing traits of *Photorhabdus* and *Xenorhabdus*

Phenotypic trait	*P. luminescens*	*X. nematophila*
Catalase	+	–
Bioluminescence	+	–
Pigments	Anthraquinones	–
Antibiotics	Hydroxystilbenes	Xenocoumacins Xenorhabdins Indole derivatives
Crystal proteins	11.6 kDa 11.3 kDa	26 kDa 22 kDa
Urease activity	d	–
Indole production	d	–
Aesculin hydrolysis	d	–

d = depending on strain.

Table 3.2. Distinguishing features of *Heterorhabditis* and *Steinernema*.

Phenotypic trait	*Heterorhabditis*	*Steinernema*
First generation adults	Hermaphroditic	Males and females
Bacterial location	Last 2/3 of intestine	Within specialized intestinal vesicle
Phylogenetic relationships[a]	Rhabditida (Rhabditidae) and Stongylida	Rhabditida (Strongyloididae) and Rhabditida (Panagrolaimidae)
Retention of secondary form of bacteria	No	Yes
Infective juveniles	*With* cuticular tooth Excretory pore *below* nerve ring Lateral field with 2 lines	*Without* cuticular tooth Excretory pore *above* nerve ring Lateral field with 6–8 lines
First generation males	*With* bursa 9 pairs of bursal rays (genital papillae) or a reduction of this number	*Without* bursa 10 or 11 pairs + 1 single genital papillae

[a] After Blaxter *et al.* (1998).

artificial medium does not yet exist that supports axenic growth of *Heterorhabditis. Steinernema carpocapsae* can grow axenically and develop into infective juveniles when injected into *Galleria mellonella*, whereas axenic *Heterorhabditis* develop into first stage juvenile progeny but these juveniles die so infective juveniles are not formed (Han and Ehlers, 2000). Finally, axenic steinernematid nematodes grow on bacterial lawns of the secondary form of *Xenorhabdus*, while axenic heterorhabditid nematodes do not grow, or grow poorly, on the secondary form of *Photorhabdus*. Taken together, these observations support the idea that the congruence observed in the life cycles of these bacto-helminthic associations is the result of convergent evolution.

The bacteria–nematode symbiosis can be described as a cyclic association that starts and ends with the infective juveniles in the soil. For the purpose of this discussion, the life cycle has been divided into three stages, based on milestone events in the temporal development of the bacteria and nematode (Table 3.3).

3.3.1. Stage I

In this stage, the nematode exists as a non-feeding infective juvenile that is relatively resistant to environmental stresses, thereby allowing it to persist in the soil (Poinar, 1979). *Steinernema* infective juveniles carry the bacteria in a specialized intestinal vesicle. Recently, Ciche and Ensign (Madison, 2000, personal communication) found that *Photorhabdus* are carried in the midgut region of the intestine of *Heterorhabditis*. It is likely that interaction between specific receptors on the intestinal epithelium of the respective nematodes and cell surface molecules of the bacteria facilitate tissue-specific and species-specific colonization.

The infective juveniles locate the insect hosts and gain entry into the haemocoel. *Steinernema* infective juveniles enter the insect through respiratory spiracles, the mouth or anus and then migrate to the haemocoel. *Heterorhabditis* have a tooth-like appendage near the mouth that can be used to penetrate the cuticle allowing the nematode to enter the haemocoel directly (Table 3.2).

Table 3.3. Life cycle events in the bacteria–nematode symbiosis

Stage	Nematode life cycle	Bacteria life cycle
I	Infective juvenile in the soil Search for insect host Infective juvenile enters insect haemocoel	Bacteria retained in nematode gut
II (Early)	Recovery in the haemocoel	Bacteria released into haemolymph Production of virulence factors Death of insect
II (Late)	Nematode reproduction	Bacteria in stationary phase Production of antibiotics, exoenzymes, crystal protein Bioconversion of insect
III	Development of new infective juveniles	Colonization of the intestine of infective juveniles

3.3.2. Stage II (early)

This stage is associated with the recovery of the infective juvenile, the release of the bacteria into the haemolymph and the death of the insect.

3.3.2.1. Infective juvenile recovery

Once in the haemolymph, the nematode exits from the infective juvenile stage and reinitiates development. This step in nematode development is called recovery. *Heterorhabditis* infective juveniles recover and develop into self-fertilizing hermaphrodites while *Steinernema* infective juveniles develop into amphimictic male or female adults.

A hierarchy of food signals may control different stages of nematode development. *In vivo* experiments have shown that the infective juveniles of both *Steinernema* and *Heterorhabditis* can develop into adults even in the absence of bacteria (Han and Ehlers, 2000). Therefore, it is likely that the dominant recovery signal originates in the haemolymph of the insect. The bacteria also appear to produce signals that control nematode development. Grewal *et al.* (1997) have shown that the recovery of infective juveniles of *Steinernema scapterisci* is dependent on a specific signal that appears to originate from the bacterial symbiont. Strauch and Ehlers (1998) have reported that *Photorhabdus* secretes a food signal that stimulates nematode recovery on artificial media. This food signal was not produced until the bacteria reached the late exponential phase of growth. Thus, it is unlikely that the bacterial food signal is directly involved in infective juvenile recovery but it may be involved in controlling some later stage in nematode development.

3.3.2.2. Bacterial growth and pathogenicity

The insect haemolymph is protected from infection by a sophisticated immune system that combines both humoral and cellular responses (Kusch and Lemaitre, 2000). The cellular response is largely mediated by circulating haemocytes that can destroy

invading microorganisms by phagocytosis. If the invading organism is large (e.g. a nematode), the haemocytes have a coordinated response, called nodulation, whereby layers of haemocytes envelop the invading organism. The haemocytes activate an enzyme cascade called the phenoloxidase system that deposits a layer of melanin around the invading organism. Humoral responses include the production of lysozyme and anti-bacterial peptides that can be rapidly synthesized in response to an infection (Kusch and Lemaitre, 2000). *Steinernema carpocapsae* can secrete proteins that suppress the immune response of the insects, perhaps in preparation for the release of the bacteria (Wang and Gaugler, 1998; Simoes *et al.*, 2000). It is not yet known whether *Heterorhabditis* secretes similar proteins.

Most species of *Photorhabdus* and *Xenorhabdus* are highly virulent when injected directly into insect larvae. LD_{50} values as low as 1 bacterium per insect have been recorded (Clarke and Dowds, 1995). It is clear that these bacteria have developed highly successful mechanisms for evading the insect immune response. Some species of *Xenorhabdus*, such as *Xenorhabdus poinarii* and *Xenorhabdus japonica*, lack injectable toxicity towards certain insects but are virulent in conjunction with their respective nematode hosts (Akhurst, 1986; Yamanaka *et al.*, 1992). *Photorhabdus* may produce a *trans*-acting, heat-labile inhibitor of the insect phenoloxidase system (S. Boundy and S. Reynolds, Bath, 2000, personal communication). *Xenorhabdus* appear to avoid the immune response by either binding to or entering the insect haemocytes (Dunphy and Webster, 1991). As with their respective nematodes, it appears that *Photorhabdus* and *Xenorhabdus* may have developed different strategies for evading the host immune response.

Pathogenicity is a prerequisite step in the bacteria–nematode symbiosis. It is clear that the bacteria produce virulence factors that are responsible for the death of the insect larva. The genome of *Photorhabdus* is replete with genes encoding proteins that share sequence similarity with a wide range of virulence factors previously characterized in mammalian pathogens (ffrench-Constant *et al.*, 2000). The role of these putative virulence factors in insect pathogenicity has not yet been resolved.

3.3.3. Stage II (late)

This stage is characterized by the growth of the bacteria to high cell density and the bioconversion of the insect cadaver into a nutrient source suitable for nematode reproduction and development.

3.3.3.1. Bioconversion of the insect cadaver

The haemolymph is a rich source of nutrients and the bacteria can grow to a high cell density within the cadaver (Forst and Tabatabai, 1997b; Hu and Webster, 2000). At this stage, normally 24–48 h postinfection, the insect larva has succumbed to the infection. During this stationary stage the bacteria produce numerous molecules, including antimicrobial agents and exoenzymes that are important in the symbiotic process.

When cultured on artificial media *Photorhabdus* and *Xenorhabdus* produce different combinations of proteases, lipases, chitinases and lecithinases during the post-exponential phase of growth (Akhurst and Boemare, 1990; Thaler *et al.*, 1998). A metalloprotease from *P. luminescens* is produced during the post-exponential phase of

bacterial growth both *in vitro* and *in vivo* (Clarke and Dowds, 1995; Daborn *et al*., 2001). Therefore it is likely that *in vitro* studies of enzyme regulation will also be relevant to the *in vivo* situation.

3.3.3.2. Support of nematode growth and development

Nematode reproductive ability is dependent on the bacteria present in the insect cadaver. In the case of the *Photorhabdus*-*Heterorhabditis* association this dependence appears to be obligate and specific. *Heterorhabditis* will only grow when *Photorhabdus* are present in the insect cadaver (Han and Ehlers, 2000). However, this specificity is less stringent when *Heterorhabditis* are cultured on artificial media, indicating that the bacteria are important for the correct bioconversion of the insect host (R.-U. Ehlers, Kiel, 2000, personal communication). On the other hand, *Steinernema* nematodes can reproduce, to a limited degree, even if *Xenorhabdus* are not present in the insect (Han and Ehlers, 2000).

Depending on the species and environmental conditions, the new generation of infective juveniles emerge from the insect between 7 and 21 days after initial entry into the host. During this time, it is essential that the insect cadaver be protected from contamination by competing saprophytic organisms. Both *Photorhabdus* and *Xenorhabdus* produce antibiotics that are active against a wide spectrum of microbial species (Paul *et al*., 1981; Akhurst, 1982; Richardson *et al*., 1988). Hu and Webster (2000) reported that a strain of *Photorhabdus* produces high levels of 3,5-dihydroxy-4-ethylstilbene (ST) during the post-exponential phase of bacterial growth in *G. mellonella* larvae, and was active against the host's gut bacteria. Interestingly, Hu *et al.* (1999) also showed that ST has an inhibitory effect on some free-living, bacteriophorous nematodes such as *C. elegans*. ST produced by another strain of *Photorhabdus* also repelled some strains of *Steinernema*. Finally, *Photorhabdus* produces compounds that repel insect scavengers from the cadaver (Baur *et al*., 1998).

3.3.4. Stage III

Stage III is distinguished by the formation of the infective juvenile stage and the colonization of the infective juvenile by the partner bacteria.

3.3.4.1. Infective juvenile formation

The infective juvenile of entomopathogenic nematodes shares several characteristics with the dauer larvae of free-living nematodes such as *C. elegans*. Dauer formation in *C. elegans* is the result of an alternative route in nematode development that is taken in response to environmental cues. Therefore, in the presence of food, the nematodes develop through the normal juvenile stages (L1–L2–L3–L4) to adulthood. However, when food is scarce and nematode density is high the juvenile nematodes develop into an alternative L3 stage known as the dauer larva. Extensive genetic studies in *C. elegans* reveal that several different converging signalling pathways are involved in controlling the decision to form dauer larvae.

In entomopathogenic nematodes the formation of infective juveniles is critical for symbiosis. *In vivo* the infective juvenile always develops from the third generation of nematodes suggesting that infective juvenile formation is tightly controlled. *In vitro*

work suggests that food cues are involved in the decision to produce infective juveniles (Johnigk and Ehlers, 1999).

3.3.4.2. Colonization of the infective juvenile

Photorhabdus and *Xenorhabdus* are present at a high density when colonization of the infective juveniles commences so sophisticated enrichment strategies, as found in the *Vibrio*–squid model, are not required. Colonization requires specific interaction between the nematode gut and the bacterial cell surface. The nature of this specific interaction remains unsolved. When axenic *Heterorhabditis* are exposed to bacteria isolated from different nematode strains, the nematodes can, to varying degrees, grow and reproduce on the non-partner bacteria. However non-partner bacteria are rarely retained in the infective juvenile (Gerritsen *et al.*, 1997, 1998). Han and Ehlers (2000; R.-U. Ehlers, Kiel, 2000, personal communication) have shown that, although secondary cells can support low levels of growth and development of *H. bacteriophora* on artificial media, these variant cells are not retained by the nematode. The specificity of the bacteria–nematode symbiosis is therefore dependent on the form of the bacteria and presumably requires the expression of specific bacterial genes.

3.4. Phenotypic Variation: Primary and Secondary Cell Types

A remarkable feature of *Xenorhabdus* and *Photorhabdus* is the occurrence of variant cell types that arise during prolonged culture of the bacteria (Akhurst, 1980; Boemare *et al.*, 1997). The primary (or phase I) cell type is carried by the infective juvenile and is characterized by the presence of numerous phenotypic traits. In the variant cell type, referred to as the secondary (or phase II) form, the primary-specific traits are diminished or altered. The secondary cell type has not been isolated from naturally occurring nematodes.

Several phenotypic traits are consistently and simultaneously changed in the secondary cells of both *Photorhabdus* and *Xenorhabdus* (Boemare and Akhurst, 1988; Boemare *et al.*, 1997; Forst *et al.*, 1997a). These traits include the formation of non-mucoidy colonies, the loss of dye-binding ability, and a reduction in the amount of pigments, antibiotics and crystalline inclusion bodies produced. Other traits that are affected in many but not all strains include the ability to agglutinate red blood cells, to lyze red blood cells and to swarm on agar surfaces. Similarly, the production of fimbriae, the level of lipase, lecithinase and protease activity is reduced in many secondary cells of *Xenorhabdus* and *Photorhabdus*. In addition, secondary cells of *X. nematophila* do not make the stationary phase outer membrane protein, OpnB, while bioluminescence in secondary cells of *Photorhabdus* is barely detectable. Smiglielski *et al.* (1994) showed that secondary cells possess higher respiratory enzyme activity and incorporate proline faster than primary cells. The authors suggested that secondary cells represent a form of the bacteria that survives outside of the nematode. A free-living species of *Photorhabdus, P. asymbiotica*, has been isolated from human wounds (Farmer *et al.*, 1989; Peel *et al.*, 1999). This finding raises the intriguing possibility that *P. asymbiotica*, which lacks several primary-specific traits, exists in a free-living state in the soil. It should be noted that secondary cells are as virulent towards insects as the primary cells (Akhurst, 1980; Forst *et al.*, 1997a). Finally, low

osmolarity conditions induce the formation of secondary cells in some strains of *P. luminescens* (Krasomil-Osterfeld, 1995).

The phenomenon of secondary cell formation has been referred to as 'phase variation' (Boemare and Akhurst, 1988). Classical phase variation involves reversible genetic events such as DNA rearrangement or modification, and occurs at a significant frequency. Phase variation affects one or a small number of specific genes and confers a selective advantage such as avoidance of host immune systems or colonization of a new environmental niche. The formation of the secondary cell type in *Xenorhabdus* and *Photorhabdus* is different from classical phase variation in several respects. Secondary cell formation occurs at a low and unpredictable frequency and numerous phenotypic traits are altered simultaneously. Reversion occurs infrequently in *Xenorhabdus* and has not been reported for *Photorhabdus*. DNA rearrangement, inversion, recombination or loss of plasmids does not appear to be involved in secondary cell formation (Forst *et al.*, 1997a). Thus, we will refer to the phenomenon of secondary cell formation as 'phenotypic variation'.

Several important differences exist between the phenotypic variants of *Photorhabdus* and *Xenorhabdus*. First, intermediate and small colony variants have been reported for *Photorhabdus* but generally do not occur in *Xenorhabdus* (Hu and Webster, 1998). Second, as mentioned above, reversion of secondary to primary cells does occur in *Xenorhabdus* but has not been reported for secondary cells of *Photorhabdus* (Thaler *et al.*, 1998). Finally, the ability of nematodes to grow on the respective secondary cells is strikingly different. *Steinernema* can grow on secondary cells of *Xenorhabdus* plated on lipid-containing media (Volgyi *et al.*, 2000) while *Heterorhabditis* is unable to grow on secondary cells of *Photorhabdus* (Gerritsen and Smits, 1997; Hu and Webster, 1998; Bintrim and Ensign, 1998; A. Fodor, Budapest, 2000, personal communication). Similarly, when the bacteria and nematodes are grown together on kidney and liver enriched agar or in the insect haemocoel, *Steinernema* grows in the presence of secondary cells of *Xenorhabdus*, albeit at lower levels than the primary cells, while growth of *Heterorhabditis* on secondary cells is dramatically reduced (Akhurst, 1980). When *H. megidis* HSH was grown on a mixture of primary and secondary cells, the nematodes fed preferentially on the primary form and avoided the secondary form (Gerritsen and Smits, 1997). However, while *H. megidis* HSH could not grow on the secondary form of its own symbiont it could multiply at low levels on the secondary form of a different strain of *P. luminescens* (PE). Interestingly, when S. *carpocapsae* was grown on a 4:1 mixture of secondary to primary cells, the resulting infective juveniles carried only the primary cells (Akhurst, 1980). Taken together, these findings indicate that primary-specific products and properties are involved in the symbiotic interaction between the nematode and the bacterium. The primary cells may secrete as yet unidentified compounds, such as molecules that function as developmental signals for the nematode, that are absent in the secondary cells.

3.5. Primary-specific Genes and Gene Products

Isolating genes that encode primary-specific traits and understanding the genetic pathways by which they are regulated will provide insights into the molecular mechanisms governing bacteria–nematode symbiosis. Recent studies have characterized several primary-specific genes and gene products. The putative roles that the primary-specific

gene products play in symbiosis are discussed below. Individual primary-specific gene products have also been implicated in some aspects of pathogenicity.

3.5.1. Crystal proteins

Two types of proteinaceous crystalline inclusions are found in primary but not secondary cells. The function of the crystals is unknown but neither has insecticidal activity. The type I crystalline inclusions of *X. nematophila* are composed of a methionine-rich 26 kDa protein while the type II inclusions are composed of a 22 kDa protein (Couche and Gregson, 1987). The genes encoding these crystal proteins have not yet been isolated. On the other hand, the genes encoding the two crystal (Cip) proteins of *P. luminescens* NC1 have been sequenced (Bintrim and Ensign, 1998). The *cipA* and *cipB* genes encode the 11.6 kDa methionine-rich protein and the 11.3 kDa protein, respectively. The Cip proteins do not share amino acid sequence similarity with any other known protein but are 25% identical to each other. Northern blot analysis demonstrated that *cip* mRNAs were present in both primary and secondary cells, suggesting that the loss of crystals in secondary cells involves post-transcriptional events (J. Ensign, Madison, 2000, personal communication). Western blot analysis revealed that the crystal proteins of *Xenorhabdus* and *Photorhabdus* are synthesized during early stationary phase (Couche and Gregson, 1987; Bowen and Ensign, 2001).

Inactivation by gene disruption of either *cipA* or *cipB* created a variant cell type that resembled secondary cells in many respects (Bintrim and Ensign, 1998). Moreover, like the secondary cells, the *cip* strains were unable to support growth of *H. bacteriophora*, suggesting that the crystal proteins may represent a stored source of nutrients. Alternatively, it is possible that the *cip* mutations indirectly affected the production of other primary-specific molecules that are required for nematode growth. The *cip* strains were shown to be virulent towards *Manduca sexta*. These findings are consistent with the view that primary-specific products are essential for the growth of *Heterorhabditis* but are not directly involved in pathogenicity.

3.5.2. Exoenzymes: lecithinases, lipases and proteases

Exoenzymes are elaborated by many strains of *Xenorhabdus* and *Photorhabdus* although their production can be variable in individual strains. Lecithinase is produced by *X. nematophila* and *X. bovienii* but is absent in several strains of *Photorhabdus* examined (Thaler *et al.*, 1998). Production of lecithinase activity dramatically increases in *X. nematophila* as the cells enters stationary phase and is only weakly produced in most secondary cells, although in some strains lecithinase activity actually increases in secondary cells (Volgyi *et al.*, 2000). Thaler *et al.* (1998) have shown that enriched fractions of lecithinase do not exhibit either cytolytic or entomotoxic activity. It is believed that lecithinase is involved in the breakdown of insect phospholipids thereby providing a lipid source for the growth of *Steinernema*. On the other hand, Tn5-induced lecithinase mutants isolated in *X. bovienii* were found to require four times more bacterial cells than the wild-type strain to kill insect larvae (Pinyon *et al.*, 1996).

Lipase activity is expressed by most primary cells, but not secondary cells, of *Photorhabdus* (Thaler *et al.*, 1998). The reduced level of lipase activity in the secondary cells of *P. luminescens* K122 was shown to involve post-translational events (Wang and Dowds, 1993). The lipase gene, *lip-1*, was cloned from secondary cells and the level of

lip-1 mRNA present in secondary and primary cells was shown to be equivalent. The amount of Lip-1 protein secreted into the extracellular medium was also the same for secondary and primary cells even though the secondary cells produced low levels of lipase activity. The Lip-1 lipase of the secondary cells was shown to be secreted in an inactive form that could be activated by exposure to SDS. Clarke and Dowds (1995) showed that Lip-1 possesses entomotoxic activity towards *G. mellonella*. The production of lipase activity is more variable in *Xenorhabdus* and in fact some secondary cells of *Xenorhabdus* actually produce higher levels of lipase than the primary cells (Boemare and Akhurst, 1988; Volgyi *et al.*, 2000).

Dunphy *et al.* (1997) have shown that an avirulent mutant strain (AV1) of *X. nematophila* produced low levels of lipase activity and also secreted low levels of the quorum sensing autoinducer hydroxybutanoyl homoserine lactone (HBHL) relative to the parent strain. Addition of HBHL from *Vibrio harveyi* stimulated lipase production and restored the virulence properties in AV1. These findings, taken together with the well-established role of quorum sensing in the virulence in several other bacteria, suggest that quorum sensing may be involved in the pathogenicity of *Xenorhabdus*. Whether quorum sensing also plays a role in symbiosis remains to be determined.

Proteases are secreted by the primary cells of both *Photorhabdus* and *Xenorhabdus* while the secondary cells generally produce lower levels of protease activity. Wee *et al.* (2000) showed that secondary cells of *P. luminescens* produce a protease inhibitor which helps to explain why protease activity is detected in primary but not secondary cells. The number of different types of proteases produced, and the level of protease activity secreted, can vary considerably among individual strains (Bowen *et al.*, 2000). It is generally believed that protease activity is involved in the breakdown of insect proteins thereby providing nutrition for both bacterial and nematode growth. An alkaline metalloprotease of 61 kDa has been isolated from primary cells of *P. luminescens* Hm (Schmidt *et al.*, 1988). The protease was not produced by secondary cells. Three distinct protease fractions were isolated from the broth of *P. luminescens* W14 cultures (Bowen *et al.*, 2000). One fraction contained a single protein of 55 kDa while two fractions contained proteins of approximately 40 kDa. None of the secreted proteases were orally toxic towards *Manduca sexta* which further supports the conclusion that secreted oral toxicity is associated specifically with high molecular weight toxin complexes (ffrench-Constant and Bowen, 1999).

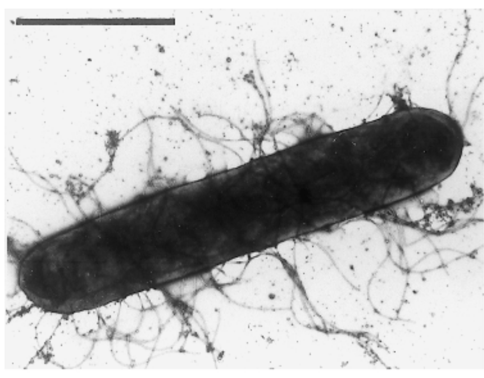
3.5.3. Outer membrane proteins

The outer membrane proteins (Opns) of *X. nematophila* have been characterized. OpnP is a pore-forming protein that constitutes 50% of the total protein content of the outer membrane and is homologous to the OmpF porin protein of *E. coli* (Forst *et al.*, 1995). The global regulatory protein, OmpR, controls the expression of *ompF* in *E. coli* and *opnP* in *X. nematophila* (Tabatabai and Forst, 1995; Forst *et al.*, 1997b). The outer membrane proteins, OpnB and OpnS, are induced as cells enter stationary phase and are not regulated by OmpR. Volgyi *et al.* (1998) have shown that OpnB is not produced in secondary form cells of several strains of *X. nematophila*. While the function of OpnB is not yet known, it is suspected to play a role in either nutrient uptake under stationary phase conditions or in specific interactions with the nematode intestinal surface.

3.5.4. Fimbriae and flagella

The primary form of *X. nematophila* produces cellular appendages called fimbriae that are thought to be involved in the specific association between the bacterium and the epithelial cells of the nematode gut. The fimbriae of *X. nematophila* are peritrichously arranged rigid structures of 6–7 nm diameter consisting of a major subunit of 17 kDa (Moureaux *et al.*, 1995). These fimbriae were shown to be mannose-resistant, resembling the *mrp* fimbriae of *Proteus mirabilis*. The secondary cells of *X. nematophila* do not produce fimbriae, which may account in part for their reduced ability to colonize or be retained in the gut of the infective juvenile nematode.

The motility behaviour of primary and secondary cells of various strains of *Xenorhabdus* has been analysed (Givaudan *et al.*, 1995). In most strains of *X. nematophila* examined, the primary cells exhibited both swimming and swarming behaviour while the secondary cells lacked flagella and were non-motile (Fig. 3.1). In one strain (ATCC 19061), the secondary form was able to swim in broth medium but was non-motile on agar surfaces (Volgyi *et al.*, 1998). This latter result demonstrates that the motility of primary and secondary cells can be variable (Givaudan *et al.*, 1995). In an attempt to elucidate the reason for the lack of flagellar production in the secondary cells, Givaudan *et al.* (1996) cloned the *fliC* and *fliD* flagellar genes of *X. nematophila*. They showed that *fliCD* operon was intact in the secondary cells but was not transcribed. Transcription of the *fliCD* genes is controlled by the master flagellar regulator *flhDC*. To determine whether the *flhDC* was defective in secondary cells, Givaudan and Lanois (2000) cloned this operon and showed that the structure of the *flhDC* operon was indistinguishable in the primary and secondary cells and that the amount of *flhDC* mRNA produced was identical in both cell types. These findings indicate that the lack of swarming and flagella synthesis in the secondary cells is not due to the absence of the FlhDC master regulatory complex. It is conceivable that a defect in the flagellar sigma factor *fliA*, which is required for the transcription of *fliCD*, accounts for the loss of motility in the secondary cells. Finally, it was shown that insertional inactivation of *flhDC* in the primary cell did not affect primary-specific traits but did cause a



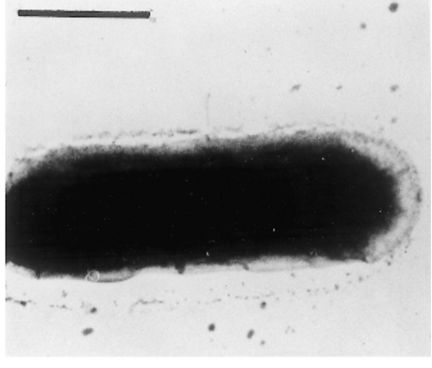


Fig. 3.1. Flagella are present in primary cells and are absent in secondary cells of *Xenorhabdus nematophila*. Bar equals 2 μm and 1 μm for the primary and secondary cells, respectively.

reduction in lipase and haemolysin production and a decrease in insect virulence (Givaudan and Lanois, 2000). These results suggest that lipase and haemolysins in *X. nematophila* are transported out of the cell using the flagellar secretion pathway.

3.5.5. *Lux* genes

Hosseini and Nealson (1995) showed that similar amounts of *lux* mRNA exists in the primary and secondary cells, suggesting that the lack of light production in the secondary cells is due to post-transcriptional events. Interestingly, *Photorhabdus* contains both luciferase and catalase activity which may work in concert to protect the bacteria and nematode from damage by reactive oxygen species generated in the insect haemocoel. *Xenorhabdus* lacks these activities suggesting that it may have evolved different mechanisms to protect itself from reactive oxygen damage. A more detailed description of bioluminescence in *Photorhabdus* has been presented in a previous review (Forst and Nealson, 1996).

3.6. Regulation of Primary-specific Traits

The finding that many of the primary-specific products are simultaneously expressed as cells enter stationary phase suggests that a network of coordinate regulation controls the expression of the primary-specific genes. In this regard, several questions arise: (i) What are the molecular mechanisms by which the primary-specific traits are regulated? (ii) Are the regulatory networks similar in *Xenorhabdus* and *Photorhabdus*? (iii) Can mutations in a single gene induce formation of secondary cells?

To address the question of whether a unique genetic locus can coordinately control primary-specific traits, a transposon mutagenesis approach was taken in *X. nematophila* (Volygi *et al.*, 2000). Three mutant strains, each containing a single transposon insertion in a different chromosomal location, displayed the secondary cell phenotype. These strains divided at the same rate as the parental strain and were fully virulent. In one strain, ANV2, the transposon was shown to have inserted into a novel gene termed *var1*. The *var1* gene encodes a protein of 121 amino acid residues that was not similar to any protein in Genbank. Introducing a *var1*-containing plasmid into ANV2 fully restored the primary-specific traits. In contrast, other transposon-induced secondary cells and a naturally isolated secondary variant were not complemented by the *var1* plasmid. These findings show that inactivation of a single gene is sufficient to simultaneously affect the expression of primary-specific traits and that several genes are involved in the regulation and production of primary-specific products. The ANV2 strain supported nematode growth and development but it emerged from the nematode later than the parent strain. When the *var1* plasmid was introduced into the ANV2 strain, emergence occurred at the same time as the wild-type strain. These results further support the idea that primary-specific traits are involved in the symbiotic interaction with the nematode.

Several studies have shown that the primary-specific traits in *Xenorhabdus* are not controlled by the global response regulator, OmpR (B. Boylan and S. Forst, unpublished data), the sigma factor, RpoS (Vivas and Goodrich-Blair, 2001), the master flagellar regulatory operon, *flhDC* (Givaudan and Lanois, 2000), the protein involved in homologous recombination, RecA (Pinyon *et al.*, 2000) or *hin* site-specific invertase (R. Akhurst, Canberra, 1996, personal communication).

As mentioned previously, inactivation of the *cip* genes in *P. luminescens* globally switched off many primary-specific genes. Mutant strains of *Photorhabdus* have been isolated in which the reverse phenotype was obtained: primary-specific genes were switched on in secondary cells (O'Neill, 1999). One such mutation, termed *kpv*, was isolated from ultraviolet-irradiated secondary cells that were screened for primary-like characteristics. The overall profile of total soluble and secreted proteins in the *kpv* and wild-type strains grown to stationary phase was similar, although not identical. Interestingly, the protein profiles in primary and naturally isolated secondary cells were identical during exponential growth, but dramatically different at stationary phase. Primary-specific proteins included a 58 kDa catalase, and proteins of 40 kDa and 12 kDa, each with > 60% identity to predicted proteins of unknown function from *C. elegans*. The *kpv* strain produced the 58 kDa catalase but not the 40 kDa protein. The isolation of this mutant implies the existence of a global negative regulator that was inactivated in the *kpv* strain, thereby switching the phenotypes of the secondary cell to that of the primary cell.

Another potential global regulator has been characterized in *Photorhabdus*. The *ner* gene encodes a DNA-binding protein found in many Gram-negative bacteria and their phages, and was first identified in phage Mu, where it negatively regulates the choice between lytic and lysogenic development. The *ner* gene was cloned from secondary cells of *P. luminescens* K122 (O'Neill, 1999). Introduction of a multi-copy plasmid containing the *ner* gene switched the phenotype of primary cells to that of secondary cells. Nucleotide sequence analysis showed that the *ner* genes of primary, secondary and *kpv* strains were identical. Thus, the *ner* gene was not involved in the formation of naturally isolated secondary cells and was not mutated in the *kpv* strain. These results suggest that, as in *Xenorhabdus*, phenotypic variation in *Photorhabdus* involves several different genetic loci.

It is apparent that mutations in a single gene can simultaneously affect a large number of phenotypic traits that convert primary cells to the secondary cell type or vice versa. The molecular mechanism by which this switch in phenotype occurs is not yet clear. It is also unknown whether the regulatory networks controlling primary-specific traits are similar in *Xenorhabdus* and *Photorhabdus*. Finally, the question of whether phenotypic variation has a biological function remains unanswered. One possibility is that this process represents a genetically controlled phenotypic switch that confers a selective advantage such that the secondary cell survives outside of the nematode host. In this case, it is not clear how the bacterium, especially *Photorhabdus* which does not appear to revert to the primary cell type, re-enters the symbiotic association. An alternative possibility is that phenotypic variation represents a process of spontaneous mutations in the regulatory network controlling primary-specific traits. These mutations may occur during prolonged incubation and this may explain why secondary cells have not yet been isolated from exponentially growing populations.

3.7. Genes Involved in Bacteria–Nematode Interactions

Mutations in genes that affect the interaction between *X. nematophila* and *S. carpocapsae* without altering primary-specific gene expression have recently been characterized. In enteric bacteria, OmpR is a global regulatory protein involved in the control of numerous cellular processes including porin gene expression and virulence. OmpR

also negatively regulates the *flhDC* operon of *E. coli*. Inactivation of *ompR* in *X. nematophila* resulted in a strain that supported nematode growth but was at a competitive disadvantage for retention by the nematode (B. Boylan and S. Forst, unpublished data). *OmpR* also appears to negatively regulate *flhDC* in *X. nematophila* since the *ompR* strain displayed hyperswarming behaviour. The sigma factor, RpoS, has been extensively studied for its role in the expression of stationary phase-induced genes and in virulence. It was found that inactivation of the *rpoS* gene did not alter the production of primary-specific genes, affect the ability of the bacterium to support nematode growth or diminish the virulence properties of *X. nematophila*. However, the *rpoS* mutant strain was unable to colonize the nematode intestinal vesicle (Vivas and Goodrich-Blair, 2001). It is surprising that inactivation of *ompR* and *rpoS* did not affect pathogenicity in *X. nematophila* since these genes positively regulate virulence factors in other enteric bacteria. Perhaps in symbiotic bacteria, *ompR* and *rpoS* have assumed new functions that do not exist in free-living bacteria.

Using a transposon mutagenesis approach, a mutant strain of *P. luminescens* that consistently failed to support nematode growth and reproduction was isolated (Ciche *et al*., 2001). This mutant was wild-type for most primary-specific traits but was defective in the production of iron sequestering molecules (siderophores) and for antibiotic activities. The transposon was inserted in an open reading frame homologous to *Escherichia coli* EntD, a 4′-phosphopantetheinyl (Ppant) transferase, which is required for the biosynthesis of the catechol siderophore enterobactin. Ppant transferases catalyse the transfer of the Ppant moiety from coenzyme A to a holo-acyl carrier or holo-peptidyl carrier protein(s) required for the synthesis of fatty acids, polyketides or non-ribosomal peptides. These results suggest Ppant transferase is involved in the synthesis of essential nutrients or developmental signalling factors that are required for *Heterorhabditis* growth and reproduction.

3.8. Genomics and Lateral Gene Transfer

The sequence of the genome of *P. luminescens* TT01 is anticipated to be completed in 2001 (www.tigr.org). The size of the *Photorhabdus* genome is 5.56 Mb, which is approximately 1.4 Mb larger than the average size of other gamma proteobacteria sequenced to date. Random genomic sequencing of *P. luminescens* W14 revealed that *Photorhabdus* contains a large class of genes encoding syringomycin and polyketide synthetases (ffrench-Constant *et al*., 2000). Syringomycins are cyclic lipodepsipeptide toxins with phytotoxic activity and a wide spectrum of antimicrobial activities that are secreted by many strains of *Pseudomonas syringae*. Polyketides are a diverse class of molecules produced by successive condensation of small carboxylic acids that have antimicrobial and antihelmintic activity. The polyketide synthetase genes of *Photorhabdus* were found to be most similar to genes found in Gram-positive soil organisms, suggesting that these genes were acquired by lateral gene transfer.

The *lux* operon provides a well documented example of lateral gene transfer. The *lux* operon is found in many genera of marine microorganisms. *P. luminescens* is the only terrestreial bacterium known to possess this operon. Phylogenetic analysis suggests that the *lux* operon of *Photorhabdus* was obtained from an avirulent strain of *Vibrio cholerae* (C. Wimpee, Milwaukee, personal communication, 2000). The toxin complex genes (*tc* genes) present in both *Xenorhabdus* and *Photorhabdus* represent

another class of molecules that were likely to have been obtained laterally (ffrench-Constant *et al.*, 2000). Genes encoding toxin complexes were shown to be carried on a 115-kb conjugative plasmid of *Serratia entomophila*, the causative agent of Amber disease of grass grubs (Hurst *et al.*, 2000). Interestingly, the biochemical properties of several of the stationary phase products (e.g. crystal proteins) are markedly different in *Photorhabdus* and *Xenorhabdus*, suggesting they were derived from disparate genetic origins. Finally, *Photorhabdus* and *Xenorhabdus* possess inducible defective phage particles and phage sequences that could function to transfer foreign DNA into the cell (Boemare *et al.*, 1992; ffrench-Constant *et al.*, 2000). Taken together, these findings suggest that numerous genes in *Photorhabdus* and *Xenorhabdus* were acquired by lateral gene transfer.

3.9. Conclusion

Xenorhabdus and *Photorhabdus* are unique in the bacterial world due to their ability to form a mutualistic symbiosis in one host and mount an aggressive pathogenic attack against a totally different phylum. Genetic studies have helped shed light on the roles that global regulatory systems and sigma factors play in these complex relationships. It is also clear that the control of primary-specific traits involves both complex networks of coordinate gene regulation and post-transcriptional and post-translational events. While recent advances have furthered our understanding of the bacteria–nematode association, several important questions remain unanswered. Of the two bacteria–nematode associations, why is the specificity of interactions more stringent for *Heterorhabditis-Photorhabdus* as compared to the *Steinernema-Xenorhabdus* symbiosis? What are the nutritional factors and putative developmental signalling molecules provided by the bacteria? What events control the transition from feeding on the bacteria and to retaining them as symbionts? What factors are involved in the species and tissue-specific colonization of the nematode and how are the bacteria retained in the respective nematode hosts? What is the mechanism by which the bacteria are released from the nematode into the haemolymph? What role did lateral gene transfer play in the evolution of this symbiotic-pathogenic life style? The advent of genomic sequencing provides new opportunities to address these questions. Comparative genomic analysis of different species and strains of *Xenorhabdus* and *Photorhabdus*, in combination with newly developed research tools (e.g. signature-tagged mutagenesis, microarray technology) will make these fascinating bacteria extremely attractive model systems in which to elucidate the molecular mechanisms governing the symbiotic interactions between the bacteria and the nematode.

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4 Virulence Mechanisms

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4.1. Introduction

Rhabditid nematodes have three pre-adaptations which have allowed them to evolve the lifestyles exhibited by the genera *Steinernema* and *Heterorhabditis* (Sudhaus, 1993). First, the order Rhabditida has evolved species that have a variety of associations with insects. Second, rhabditids produce the dauer juvenile stage which confers the capacity to enter an insect and persist in the absence of food. Third, rhabditids are bacterial feeders, and hence are pre-adapted to enter a mutualistic relationship with entomopathogenic bacteria inside the insect haemocoel.

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Microbes must be able to successfully infect their host in order to cause disease (pathogenicity), but infection is by no means sufficient to generate disease symptoms (virulence) (Finlay and Falkow, 1989). Toxins are the major determinants of virulence, but many toxins play only a minor role in the colonization process and their role in microbial ecology is little understood. In this chapter, virulence factors will encompass factors contributing to infection as well as disease with the exception of housekeeping functions required for growth on non-living substrates. It is likely that many of the bacterial genes involved in infection of the insect will be found to play a role also in the mutualistic interaction with the nematode, as recently highlighted by Hentschel *et al.* (2000) in a review of the similarities between symbionts and pathogens in bacterial–host interactions.

4.2. Entry and Establishment

4.2.1. Entering the insect host

An important function of the nematode is vectoring the entomopathogenic bacteria into the insect host. In doing so, they encounter various behavioural and mechanical forms of resistance. Grubs of *Popillia japonica* displayed grooming behaviour using their legs and the raster at the last segment as well as evasive reactions on sensing nematode attack (Gaugler *et al.*, 1994). Mechanical barriers include the sieve plates which protect the spiracles of many soil dwelling larvae (Fig. 4.1a), and entering the insect via the tracheal system is not possible in many grubs (Forschler and Gardner, 1991), leatherjackets (Peters and Ehlers, 1994) and maggots (Renn, 1998); however, in sawfly larvae the spiracles are the most important route of entry (Georgis and Hague, 1981).

A second route of entry is through the mouth opening or the anus. The width of both (e.g. in wireworms) may exclude infective juveniles (IJs) (Eidt and Thurston, 1995), and insects' mandibles may crush the nematodes to death (Gaugler and Molloy, 1981). Using the anus as an entry site avoids the latter problem and represents the main route in housefly maggots and leafminers (Renn, 1998). Still, frequent defecation may expel nematodes entering the anus, and in grubs and sawfly larvae invasion is more successful via the mouth than via the anus (Georgis and Hague, 1981; Cui *et al.*, 1993). When nematodes have reached the gastric caecae, malpighian tubules or the gap between the peritrophic membrane and midgut epithelium, expulsion with faeces is avoided. A general problem with parasitism via the intestinal tract is the host's gut fluid which may kill up to 40% of invading non-adapted nematodes and significantly reduce penetration via the intestine (Wang *et al.*, 1995).

After successfully entering the tracheal system or the intestine, the dauer larvae still must pass through the tracheole or the gut wall, respectively. The fragile tracheole wall might be penetrated just by the mechanical pressure of the pointed nematode head. The gut wall, however, is in part protected by the peritrophic membrane (Fig. 4.1b), which can be a serious obstacle for nematodes (Forschler and Gardner, 1991). *Steinernema glaseri* takes 4–6 h to penetrate the midgut of *P. japonica* (Cui *et al.*, 1993), the nematodes preferably penetrating in the midgut region and in the gastric caecae. Dauer larvae of *Heterorhabditis bacteriophora* were observed using their proximal tooth for rupturing the insect body wall (Bedding and Molyneux, 1982) and it was long believed that only *Heterorhabditis* could penetrate tissues like the insect's integument, since dauer larvae of *Steinernema* lack the tooth. Reports on a superior penetration of

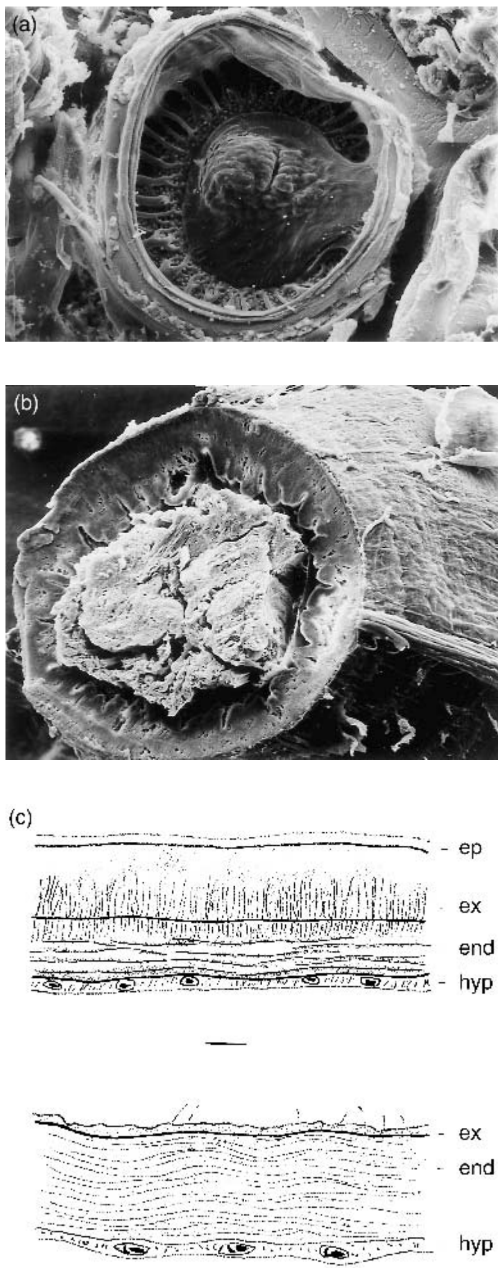

Fig. 4.1. Mechanical barriers protecting the insect from nematode penetration: (a) Sieve plate on the spiracle of the grub *Phyllophaga hirticola*, internal view showing central bulla and surrounding spiracular plate; (b) Midgut lumen of field collected *Phyllophaga hirticola* larva. Note the densely folded peritrophic membrane; (c) Schematic cross-section of the integument of *Tenebrio molitor* (upper) and *Tipula paludosa* larvae (lower). Note the absence of an epicuticle and the rudimentary exocuticle layer in *T. paludosa*. (After Forschler and Gardner, 1991 (a,b); Ghilarov and Semenova, 1957 (c)). ep, epicuticle; ex, exocuticle; end, endodermis; hyp, hypodermis.

S. glaseri compared with *H. bacteriophora* through gut tissues of grubs (Wang and Gaugler, 1998) and on the penetration of *S. feltiae* through the integument of leatherjackets (Peters and Ehlers, 1994) challenged this perspective. There is increasing evidence that nematode secretions are involved in penetration, at least in *Steinernema* species: protease inhibitors decrease penetration of *S. glaseri* through the gut wall of *P. japonica* (Abu Hatab *et al.*, 1995), and the midgut epithelium cells of *Galleria mellonella* showed a marked histolysis in response to secretions of axenic *S. carpocapsae* (Simoes, 1998). Penetration by *S. feltiae* through the cuticle of leatherjackets might be attributed to the absence of an epicuticular wax layer (Fig. 4.1c), which may block the activity of histolytic enzymes (Peters and Ehlers, 1994).

4.2.2. Steps in bacterial infection

The symbiotic bacteria enter the insect passively, carried within the nematode gut. Following entry, the bacteria must adhere to a surface within the host to establish infection. The fimbriae of *X. nematophilus* are a potential adhesin, having been found to be responsible for the *in vitro* agglutination by phase 1 bacteria of the haemocytes of *G. mellonella* (Moureaux *et al.*, 1995), though their *in vivo* role has not yet been elucidated. Random sequencing of the *P. luminescens* genome (ffrench-Constant *et al.*, 2000) has revealed the presence of sequences similar to the attachment invasion locus of *Yersinia* spp. These genes confer on *Yersinia* the ability to cross the gut epithelium of their animal hosts. *Xenorhabdus* and *Photorhabdus* inhabit several extracellular spaces which requires adaptations for resisting the host's immune system (section 4.3.3), as well as nutrient acquisition. Entomopathogenic bacteria may provide nutrients for themselves and their nematode symbiont by secreting a variety of extracellular enzymes which degrade host tissue. Organisms that spread via the bloodstream usually produce siderophores or have alternative means of removing iron that is tightly complexed to host proteins, and DNA sequences which probably specify siderophore production have been found in *P. luminescens* (ffrench-Constant *et al.*, 2000).

4.3. Interaction with the Insect Immune System

4.3.1. The insect immune system

The insect immune response (reviewed by Trenczek, 1998) consists of interactive cellular and humoral actions, the haemocytic mechanisms comprising phagocytosis, nodule formation and cellular encapsulation, and the humoral response including melanotic encapsulation, and the production of antimicrobial peptides, which may be induced (e.g. cecropins) or constitutive (e.g. lysozyme). The immediate response against nematodes is encapsulation, and against bacteria is phagocytosis, or nodulation in the case of a large load. Secondly, injury and microbe infection induce the production of antibacterial peptides. However, the details of the response vary with the species of insect and pathogen and their physiological states (e.g. Dunphy and Thurston, 1990; Dunphy and Bourchier, 1992).

4.3.2. Nematode encapsulation

After penetration of the dauer larva into the haemocoel, the insect's non-self response system initially deals only with the nematode. The symbiotic bacteria are carried in the

intestine and are released 30 min to 5 h postinfection (Dunphy and Thurston, 1990; Wang *et al.*, 1994). The nematode can be trapped in cellular capsules which are often hardened by melanin, or in cell-free (humoral) capsules consisting mainly of melanin. A prerequisite for this encapsulation is non-self recognition of the nematode.

Encapsulation of entomopathogenic nematodes has been reported in Orthoptera, Coleoptera, Diptera and Lepidoptera (Table 4.1). In each of these orders, there are also species which do not encapsulate invading nematodes. Whether encapsulation occurs depends on the particular nematode–insect combination. In *Acheta domesticus*, the nematodes *S. carpocapsae* and *H. bacteriophora* are encapsulated while *S. scapterisci* is not (Wang *et al.*, 1994). Interestingly, *S. scapterisci* has been found naturally associated with the orthopteran *Scapteriscus vicinus*. Similarly, *S. glaseri*, which is often found associated with scarabaeid larvae is not encapsulated in *P. japonica*, in contrast to *S. carpocapsae* and *H. bacteriophora*. These findings suggest that the nematodes avoid encapsulation in hosts similar to those with which they appear to be naturally associated.

Nematodes may resist encapsulation in insects by either avoidance of being recognized (evasion), by tolerating the encapsulation response (tolerance), or by actively suppressing the encapsulation response (suppression).

Table 4.1. Encapsulation of *Steinernema* and *Heterorhabditis* spp. in insect larvae.

Insect	Nematode	Reference
Dictyoptera		
Periplaneta americana	*H. bacteriophora*	Zervos and Webster, 1989
Orthoptera	*S. carpocapsae*	Wang *et al.*, 1994
Acheta domesticus	*S. glaseri*	
	H. bacteriophora	
Coleoptera		
Popillia japonica	*S. carpocapsae*	Wang *et al.*, 1994
	S. scapterisci	
	H. bacteriophora	
Hylobius abietis	*S. carpocapsae*	Pye and Burman, 1978
Otiorhynchus sulcatus	*S. kraussei*	Steiner, 1996
Diabrotica virgifera virgifera	*S. carpocapsae*	Jackson and Brooks, 1989
Leptinotarsa decemlineata	*S. carpocapsae*	Thurston *et al.*, 1994
Diptera		
Aedes sp. (5 species)	*S. carpocapsae*	Welch and Bronskill, 1962
Culex restuans	*S. carpocapsae*	Poinar and Leutenegger, 1971
Culex pipiens	*S. carpocapsae*	Poinar and Leutenegger, 1971
Tipula oleracea,	*S. anomali*	Peters and Ehlers, 1997;
T. paludosa	*S. carpocapsae*	A. Peters, Germany, 1994,
	S. feltiae	unpublished results
	S. glaseri	
	S. kraussei	
	S. intermedium	
Lepidoptera		
Mythimna unipuncta	*S. carpocapsae*	Simoes, 1998

4.3.2.1. Evasion

Evasion has been reported by Dunphy and Webster (1987), who discovered that a lack of non-self recognition prevents *S. carpocapsae* from being encapsulated in *G. mellonella*. When the IJs were treated with lipase before injection they were recognized and encapsulated. The authors concluded that lipid components on the nematode surface are responsible for protection against recognition. IJs may innately secrete these surface components or acquire them from the host during penetration. In *Heterorhabditis* spp., the second-stage cuticle plays an important role in evasion. Studies on *T. oleracea* suggest that *Heterorhabditis* spp. avoid non-self recognition by slipping off the second juvenile stage cuticle (J2-cuticle) just before or after entering the insect haemocoel (Peters *et al.*, 1997).

4.3.2.2. Tolerance

Tolerance of the encapsulation response may be achieved by overwhelming the insect's haemocoel with nematodes, and multiple infection is common for entomopathogenic nematodes and essential in the obligate bisexual genus *Steinernema*. An insect's potential to encapsulate nematodes may be limited. Welch and Bronskill (1962) estimated that the encapsulation potential of *A. aegypti* is six or seven. Mosquito larvae, however, were killed if only one or two nematodes invaded even if they had been encapsulated (Molta and Hominick, 1989). In *Leptinotarsa decemlineata* larvae a maximum of 21 encapsulated *S. carpocapsae* were found but at least one nematode escaped encapsulation when more than nine nematodes had invaded (Thurston *et al.*, 1994). Similar results were observed in larvae of *Tipula* spp. (Peters and Ehlers, 1997) and *P. japonica* (Wang *et al.*, 1994). In both instances the symbiotic bacteria were detected in the haemolymph of the infected larvae; they were either released before capsule formation was completed or escaped from the capsule (Fig. 4.2).

4.3.2.3. Suppression

Immune suppression was assessed in *G. mellonella* infected with *S. carpocapsae*, and the observed absence of suppression was rationalized by postulating the need for establishing a non-competitive environment for the symbiotic bacteria (Dunphy and Thurston, 1990). More recently, however, *S. carpocapsae* as well as their symbiont, *X. nematophilus* were shown to suppress encapsulation in the turnip moth, *Scotia segetum* (Yokoo *et al.*, 1992). Suppression of the encapsulation response was also found in *S. glaseri* invading *P. japonica* grubs (Wang and Gaugler, 1999). Two surface coat proteins were shown to reduce haemocyte numbers in *P. japonica*. One of these also markedly reduced melanization and the ability of haemocytes to phagocytose. The conversion of prophenoloxidase to phenoloxidase and the enzyme's activity itself, which is essential for wound healing, was not inhibited. Also, up to 4 h after nematode injection, encapsulation and haemocyte activity was only suppressed locally, which would allow for an elimination of contaminating bacteria entering through the penetration wound.

As soon as the symbiotic bacteria *Xenorhabdus* or *Photorhabdus* are released, the immune response is elicited. The presence of the symbiotic bacteria increased encapsulation of *Steinernema feltiae* in leatherjackets (Peters and Ehlers, 1997). At the same time, however, the bacteria suppress the immune response since they adhere to and kill the haemocytes (Dunphy and Webster, 1988). The period from nematode invasion to

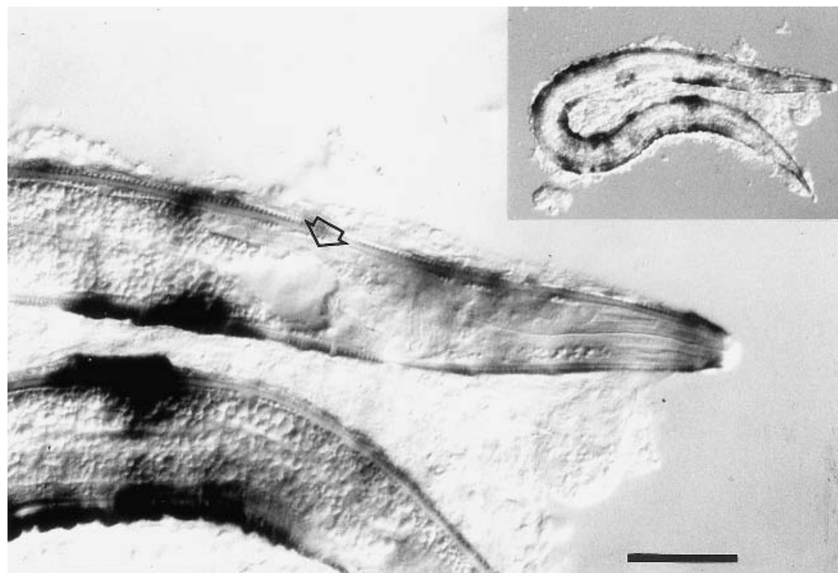

Fig. 4.2. Encapsulated *Steinernema feltiae* from the haemocoel of a *Tipula oleracea* larva 24 h post-inoculation. Attachment of haemocytes and melanization of the capsule has started. The enlarged section shows the expanded proximal part of the intestine (arrow). The symbiotic bacteria which, in the dauer juveniles, are harboured in a vesicle at this location have already been released.

bacterial release is hence crucial for counteracting the encapsulation response (see section 4.3.3.).

4.3.3. Immune response against the symbiotic bacteria

The effect of *Xenorhabdus* and *Photorhabdus* on the insect immune response has been most extensively studied for Lepidoptera, particularly *G. mellonella*, and a picture can be presented (Dunphy and Thurston, 1990; Forst and Nealson, 1996; Forst *et al.*, 1997), which nevertheless differs in detail between strains of pathogen and host. Bacteria are released from the nematode at various times postinfection depending on the strain (sections 4.3.2 and 4.3.4). Recent work with *P. luminescens* tagged with the green fluorescent protein gene shows that release is associated with peristalsis of the nematode gut, the bacteria moving towards the mouth and exiting at a fairly constant rate over a 3-h period (Ensign and Ciche, 2000). Within minutes of their appearance in the haemolymph, the bacteria are recognized by the insect's haemocytes and usually become sequestered into nodules (Dunphy and Thurston, 1990). The bacteria multiply within the nodules and then re-emerge into the haemolymph in a step that is independent of bacterial metabolism but dependent on the cell envelope. In cases where the bacteria are not trapped in nodules or where there is a delay in nodulation, the bacteria still proliferate and kill the host (Dunphy and Thurston, 1990; Dunphy and Bourchier, 1992). In *Spodoptera exigua*, recognition of non-self leading to nodulation was shown to be mediated by eicosanoids, and *X. nematophilus* inhibited this pathway (Park and Kim, 2000).

In parallel with their re-emergence into the haemolymph, the bacteria damage the haemocytes of Lepidoptera (Dunphy and Thurston, 1990; Dunphy, 1994, 1995) and Orthoptera species (van Sambeek and Wiesner, 1999) and then cause disintegration of the fat body (Dunphy and Bourchier, 1992), which is the main source of antimicrobial peptides (Trenczek, 1998). Host serum causes the release of lipopolysaccharide (LPS or endotoxin) from the outer membrane of the bacteria and the LPS generates a rapid increase in haemocyte counts, a phenomenon that is associated with haemocyte damage and is not indicative of haemocyte stimulation. LPS is cytotoxic via the lipid A moiety binding to glucosaminyl receptors on the haemocytes and the fatty acids of lipid A exerting a toxic effect (Dunphy and Thurston, 1990). LPS of *X. nematophilus* and *P. luminescens* (but not *Escherichia coli*) inhibits the activation of prophenoloxidase in both *G. mellonella* and *Lymantria dispar*; this presumably protects the nematodes, since *S. carpocapsae* is killed by the phenoloxidase cascade if it is activated (Dunphy and Webster, 1991; Dunphy and Bourchier, 1992). In a study with another lepidopteran, *Agrotis segetum*, living or dead cells of *X. nematophilus* also suppressed prophenoloxidase activation (Yokoo *et al.*, 1992). Haemolymph from *G. mellonella* contains LPS-binding proteins which inhibit the effects of *X. nematophilus* LPS on haemocytes and prophenoloxidase (Dunphy and Halwani, 1997), so apparently the insect is able to detoxify LPS until a critical level of the endotoxin is released. *In vitro* assays reveal that the *S. carpocapsae*–*X. nematophilus* complex produces a haemocyte unsticking activity and a cytotoxic protein, whereas LPS had neither effect on the haemocytes (Ribeiro *et al.*, 1999). This suggests that LPS acts in concert with other agents in the haemocoel to exert its *in vivo* effects in destroying haemocytes.

P. luminescens is insensitive to the lysozyme of *G. mellonella*, but in immunized insects the induced cecropins are thought to be the primary immune proteins responsible for eliminating bacteria from the haemocoel. This is prevented by a specific cecropin-degrading proteinase, which is secreted by the *H. bacteriophora*–*P. luminescens* complex and to a lesser extent by phase 1, though not phase 2 cells of the bacteria alone (Jarosz, 1998). This enzyme inhibited the cecropins of a number of lepidopteran larvae and pupae as well as larvae of one species of the order Hymenoptera.

There has been discussion about whether death of the insect is dependent on bacterial growth (septicaemia) or occurs before bacterial growth. Forst and Nealson (1996) showed that death of *M. sexta* occurred prior to (LT_{50} = 42 h) the proliferation of *X. nematophilus* in the haemolymph, and concluded that killing is mediated by a potent toxin. However, Clarke and Dowds (1995), measuring the total bacterial count in homogenized larvae, found that all *G. mellonella* larvae died after *P. luminescens* reached stationary phase, between 27 and 41 h after injection. In the former case, the bacteria may have proliferated within the nodules before larval death, but it is difficult to envisage a mechanism whereby this growth could mediate killing, since studies with virulence mutants showed that haemocyte damage is not an important factor in killing of *G. mellonella* by *X. nematophilus* or *P. luminescens* (Dunphy, 1994, 1995; Dunphy and Hurlbert, 1995). In summary, it appears that septicaemia may be important for killing in some bacterial–insect combinations, but not in others. Furthermore, damage to the immune system, while clearly important for the survival of the bacteria, is not significant as a virulence factor.

4.3.4. Additive and synergistic effects in symbiosis

Few *Xenorhabdus* and *Photorhabdus* strains are capable of infecting insects after being taken up orally. Hence, an obvious function of the nematode in the symbiosis is vectoring the bacteria inside the insect's haemocoel. The contribution of the nematode, however, goes beyond this.

Poinar and Thomas (1966) reported that even one axenic *S. carpocapsae* is sufficient to kill *G. mellonella*, though only within several weeks. Likewise, five injected axenic *S. feltiae* killed *G. mellonella* larvae within 4 weeks (Ehlers *et al.*, 1997). Burman (1982) discovered a secreted protein toxic to *G. mellonella* in axenic *in vitro* cultures of *S. carpocapsae*. A protein of 42 kDa was found in *G. mellonella* injected with axenic *S. carpocapsae* probably resembling the same toxin (Simoes *et al.*, 2000). Not all *Steinernema* species show axenic virulence. The nematode *S. glaseri* was not able to kill *G. mellonella* without its symbiotic bacteria (Akhurst, 1986). Likewise, axenic dauer larvae of *H. bacteriophora* and *H. megidis* do not kill *G. mellonella* larvae (Gerritsen *et al.*, 1998).

A solely additive effect of nematode and bacterial toxins does not explain the high virulence of the nematode–bacterial complex. There are a number of insect species, like the pine weevil, *Hylobius abietis,* or leatherjackets (*Tipula* spp.), which are resistant to *Xenorhabdus* and *Photorhabdus*, and investigations into these and other insects showed a synergistic increase in virulence when the associated bacteria and nematodes act together (Table 4.2).

The mechanisms of this synergism have been partly elucidated. Inhibitors of the host's antibacterial proteins were found in axenic cultures of *S. carpocapsae* (Götz *et al.*, 1981). The immune-inhibiting effect of *S. carpocapsae* secretions was confirmed in immunized larvae of *G. mellonella, Bombyx mori*, and the coleopteran *Tenebrio molitor* (Simoes, 1998). The active protein was characterized as a 58 kDa metallo-protease and it was found not to suppress the activity of lysozyme and the antibacterial proteins of *Phormia terranovae*. Inhibitors of antibacterial proteins of the cecropin group have been identified in *P. luminescens* (Jarosz, 1998), but whether *Heterorhabditis* spp. produce antibacterial proteins has not been tested, since axenic cultivation has not been successful.

Apart from the nematode's effect on antibacterial proteins, the cellular immune response can also be affected. When cellular capsules are formed around a nematode, many haemocytes attach to the capsule and die. This might explain the synergistic action of the *S. feltiae-X. bovienii* complex in *Tipula oleracea* (Peters and Ehlers, 1997). In *Agrotis segetum*, the prophenoloxidase cascade was inhibited by living and heat killed *S. carpocapsae* and by living and killed symbiotic bacteria (Yokoo *et al.*, 1992). This might decrease non-self recognition and sequestration of *X. nematophilus* from the haemocoel. Surface secretions of living axenic *S. glaseri* protected nematodes from melanotic encapsulation in *P. japonica* (Wang and Gaugler, 1999). By reducing haemocyte numbers and phagocytosis and by inhibiting nodule formation these secretions weaken the antibacterial defence of the host, which offers an explanation for the synergistic action of *S. glaseri* and *X. poinarii*.

While synergism is evident in *Steinernema* species, most studies suggest that pathogenicity of *Heterorhabditis* is mainly due to their symbiotic bacteria. Nevertheless, synergism between *Heterorhabditis* and *Photorhabdus* was shown in *Tipula oleracea* (Gerritsen *et al.*, 1998). Researchers should always be aware that generalization about

the interactions of the nematode–bacteria complex with the insect's immune system is impossible.

The timetable of bacterial release in the host probably reflects the role that each partner plays in symbiosis. *Heterorhabditis* spp. rely on their symbiotic bacteria to overcome the insect immune system. The symbionts of *H. megidis* suppress haemocyte phagocytosis in locusts as early as 12 h after oral nematode application and considerable haemocyte death occurs 12 h later (van Sambeek and Wiesner, 1999). The release of the symbiotic bacteria during or soon after invasion of *P. japonica* by *H. bacteriophora* (Wang *et al.*, 1994) might suppress nematode encapsulation. In *S. glaseri*, on the other hand, bacteria are released 12 h later. Nematode mediated inhibition of haemocyte phagocytosis and nodule formation initially only acts locally. Full system inhibition takes more than 4 h (Wang and Gaugler, 1999). A delayed release of the non-pathogenic *X. poinarii* is hence crucial for its establishment inside the insect host.

4.4. Bacterial Pathogenicity

4.4.1. Toxins

Bacterial proteins which are effective insecticides include the crystal proteins (δ-endotoxins) of *Bacillus thuringiensis* (Bt) and *B. sphaericus*, the Vip toxins of *B. thuringiensis*

Table 4.2. Synergism of the nematode and their symbiotically associated bacteria in pathogenicity against insects.

Nematode	Bacterium	Insect	Virulence of bacteria	Virulence of nematode	Virulence of nematode–bacteria combination	Reference
Steinernema glaseri	*Xenorhabdus poinarii*	*Galleria mellonella*	No mortality	No mortality	One nematode kills 100%	Akhurst, 1986
		Popillia japonica	No mortality	Not established	One nematode kills 100%	Yeh and Alm, 1992
S. carpocapsae	*X. nematophilus*	*Hylobius abietis*	LC_{50}: 3500[a]	Not established	One nematode kills 100%	Burman, 1982
		Hyalophora cecropia	LC_{50}: 500 N[b] LC_{50}: 500,000 I[c]	Approx. 500 (*N and* I)	LC_{50} < 10[a]	Götz *et al.*, 1981
S. feltiae	*X. bovienii*	*Tipula oleracea*	LC_{50}: 15,700	33% mortality at 20 injected nematodes per insect	90% mortality at 20 injected nematodes per insect	Ehlers *et al.*, 1997
Heterorhabditis megidis	*Photorhabdus luminescens*	*Tipula oleracea*	LC_{50}: > 100,000	No mortality at 300 applied nematodes per insect	70 to 91% mortality at 300 applied nematodes per insect	Gerritsen *et al.*, 1998
H. bacteriophora	*P. luminescens*		LC_{50}: > 100,000	No mortality at 300 applied nematodes per insect	37 to 54% mortality at 300 applied nematodes per insect	

[a] LC_{50} expressed in living cells or nematode injected per insect.
[b] N: non-immunized pupae.
[c] I: pre-immunized pupae producing antibacterial proteins.

and cholesterol oxidase of *Streptomyces* spp., all of which lyse the midgut epithelium of the insect host (references in Bowen *et al.*, 1998). Similar activities have been found in *P. luminescens* and *X. nematophilus*.

Bowen and Ensign (1998) assessed four strains of *P. luminescens* for secreted toxin production. Strains Hm, Hb, and NC-19 did not display lethal oral toxicity to the lepidopteran, *Manduca sexta*, despite being highly toxic by injection. However, a strain newly isolated from Florida, W-14 secreted a toxin that killed the larvae when administered orally or by injection. The W-14 toxin was similarly active against species from four orders of insects, unlike different Bt δ-endotoxins which often exhibit specificity for a given insect group. The W-14 toxin was independently isolated and the genes cloned by Guo *et al.* (1999) who found oral toxicity against the Southern corn rootworm. The toxin was detected in phase 2 as well as phase 1 cultures and was shown to be a protein complex with an estimated molecular weight of 10^6 composed of several subunits (Bowen and Ensign, 1998). Since *Photorhabdus* is generally delivered by *Heterorhabditis* to the insect haemocoel, it was surprising that the W-14 strain had an orally active toxin, which exerted a histopathological effect on the *M. sexta* midgut similar to the other insecticidal toxins listed above. Blackburn *et al.* (1998) observed apical swelling and blebbing of the midgut epithelium into the gut lumen and eventual lysis of the epithelium. Injection of the toxin into the haemocoel generated a similar histopathology to that of the orally administered toxin, implying that the toxin can affect the midgut from either the apical or the basal surfaces. The authors noted that other tissues (skeletal muscles, nervous system and malpighian tubules; D. Bowen, USA, personal communication) exposed to the haemocoel are unaffected, but acknowledge that the primary site of action of the toxin *in vivo* might not be the gut.

Four toxin complex-encoding loci, *tca, tcb, tcc* and *tdc* were cloned (Bowen *et al.*, 1998), each containing one or more open reading frames, corresponding to all but three of the polypeptides found in the toxin. There is considerable amino acid similarity between different predicted Tc proteins, and recently sequenced insect toxin genes from *Serratia entomophila* were shown to be homologous to the *tc* genes (Hurst *et al.*, 2000). Furthermore, two of the Tc polypeptides have regions similar to *Salmonella* virulence proteins required for growth and pathogenicity in mammalian macrophages, leading the authors to suggest that the *P. luminescens* homologue may attack insect haemocytes. The loci were deleted or disrupted singly or in pairs, showing that *tca* and *tcd* encode orally active toxins and that together they comprise most of the activity against *M. sexta*. However, deletion of either *tcb* or *tcc* alone also reduces mortality, suggesting that their products interact with Tca or Tcd in virulence against *M. sexta*, and of course they may play a more major role in virulence against other insect species. Random sequencing of the W-14 genome reveals the presence of other *tc*-like loci, as well as sequences similar to Rtx-like and haemolysin A-like toxins and their export systems (ffrench-Constant *et al.*, 2000).

Toxins and the genes encoding them have also been isolated from *X. nematophilus*, including one which is active following injection into *G. mellonella* (Smigielski and Akhurst, 1999), and one displaying oral toxicity towards *Pieris brassicae, Pieris rapae* and *Plutella xylostella* (Jarrett *et al.*, 1998). The DNA sequence coding for the latter activity displays strong identity to the *P. luminescens tc* loci (ffrench-Constant and Bowen, 1999).

Out of 200 *Photorhabdus* and *Xenorhabdus* strains sampled, some displayed high levels of oral toxin activity, most of these clustering in the *akhurstii* subgroup of

P. luminescens. The exact experimental methods determine the outcome of such a survey; thus, bacterial culture conditions and the identity of the host species must be varied extensively in attempting to rule out oral toxin production in a given strain of *Xenorhabdus* or *Photorhabdus* (D. Bowen, USA, personal communication). Whatever the range of strains producing oral toxins, it seems likely that most strains of *Xenorhabdus* and *Photorhabdus* secrete non-oral toxins that are active by injection, and indeed such activities have been reported for *P. luminescens* and *X. nematophilus* (Jarosz *et al.*, 1991; Clarke and Dowds, 1995).

4.4.2. Other potential virulence agents

A number of extracellular enzymes and other bacterial phenotypes have been evaluated for their role in virulence (reviewed by Dowds, 1998). One approach has been the injection of purified macromolecule or bacterial clone producing a specific phenotype into larvae, revealing their status as a toxin while acting alone (Table 4.3, column 1). A second technique involves the inactivation of a specific gene which reveals whether the gene plays a role in virulence in a wild-type background of other virulence factors (Table 4.3, column 2). Thus, injection studies showed that lecithinase by itself has no toxic activity, but mutagenesis work revealed that it contributes towards causing disease. A third approach involves screening a mutant bank (preferably generated by insertional mutagenesis) for avirulent mutants and assessing the biochemical properties of these strains. Unfortunately, the transposon mutants of Xu *et al.* (1991) had multiple genes inactivated, and all were highly pleiotropic (Hurlbert, 1994). However, by comparing virulent with avirulent mutants, certain properties can be eliminated as candidates for playing important roles in causing disease, at least in these mutant backgrounds (Table 4.3, column 3). For example, the avirulent mutants of Xu *et al.* (1991) were all deficient in haemolysis and motility, suggesting a possible role for these activities in virulence. However, other insertion mutants retained their virulence but lost their haemolysin and/or motility, showing that neither property is necessary for killing the host. Table 4.3 summarizes the results of such experiments and reveals that only lipase and LPS have toxic activities, while lecithinase plays a role in virulence but does not act alone as a toxin. Thus lipase may directly attack host tissue, while LPS has been shown to damage haemocytes (Dunphy and Thurston, 1990); lecithinase appears to affect mortality of the host only in the presence of other virulence factors.

Phase I bacteria express a variety of phenotypes which are absent or present in reduced amounts in phase II cells (e.g. degradative enzymes, antibiotics, flagella, pili, and glycocalyx) (Forst *et al.*, 1997), many of which would be expected to play a role in establishing an infection. It is therefore interesting that the phase II variant is usually highly virulent, though sometimes to a lesser extent than the primary form, and only a few strains tested had a phase II variant which had completely lost its virulence (Table 4.4). This suggests that many of the properties missing in virulent phase II cells may be needed at a stage in the infectious process that follows death of the host (e.g. for growth of the nematode and bacteria), and indeed the phase II form provides poor conditions for nematode growth (see Forst and Clarke, Chapter 3 this volume). The strains which have an avirulent phase II form may exhibit phase variation in toxin production, or might be strains that rely on septicaemia instead of toxin secretion for their virulence.

4.4.3. Environmental signals regulating virulence

Entomopathogenic bacteria can inhabit diverse environments including, the nematode gut, and the insect haemocoel, nodules and gut, all of which differ in their physical and chemical properties. Bacterial pathogens have evolved signal transduction systems for inducing expression of sets of genes needed to establish the culture in different locations (reviewed by Hentschel *et al.*, 2000), and a number of these regulatory systems have been identified in *Xenorhabdus* and *Photorhabdus*.

Quorum sensing systems link expression of target genes to bacterial population density, and many play a role in symbiont- or pathogen–host interactions. The intercellular signal, *N*-hydroxy butanoyl-*L*-homoserine lactone (HBHL) of *Vibrio harveyi*, which induces toxin (and light) production in this species, restored virulence to an avirulent mutant of *X. nematophilus*, as well as increasing lipase activity to a level near that of the wild-type (Dunphy *et al.*, 1997). Furthermore, bioactive HBHL was isolated from wild-type *X. nematophilus* and a second *Xenorhabdus* species, but was absent in three independent Tn5-induced avirulent mutants. These three mutants all had multiple and different insertions of Tn5 into their genomes (Xu *et al.*, 1991; Hurlbert, 1994), so it is noteworthy that all lacked the same autoinducer. This suggests a cascade of control in which a variety of regulatory genes higher up in the pathway are missing in the mutants, but that all ultimately affect production of HBHL, implying a key role for this molecule in virulence.

Table 4.3. Potential virulence properties.

		Effect on mortality in presence of other virulence factors	
Phenotype	Toxicity alone (injection study)	(w.t. background)	(mutant background)
Protease	No (a)		No (b)
Lecithinase	No (c)	Yes (d)	
Lipase	Yes (e)		No (b)
Antibiotics			No (b)
Motility			No (b)
Haemolysis			No (b)
Crystal proteins		No (f)	
LPS	Yes (g)/No (e,h)		
Bioluminescence	No (i)		

(a)–(h) references; pathogen; host.
(a) Bowen *et al.*, 2000; *Photorhabdus; Manduca sexta*;
(b) Xu *et al.*, 1991; *Xenorhabdus nematophilus; Galleria mellonella*;
(c) Thaler *et al.*, 1998; *X. nematophilus; G. mellonella, M. sexta, Spodoptera littoralis, Locusta migratoria*;
(d) Pinyon *et al.*, 1996; *X. bovienii; G. mellonella*;
(e) Clarke and Dowds, 1995; *Photorhabdus; G. mellonella*;
(f) Bintrim and Ensign, 1998; *Photorhabdus; M. sexta*;
(g) Dunphy, 1994; *X. nematophilus; G. mellonella*; Dunphy, 1995; *Photorhabdus; G. mellonella*;
(h) Clarke and Dowds, 1992; *Photorhabdus; G. mellonella*;
(i) Dunphy *et al.*, 1998; *X. nematophilus; G. mellonella*.
w.t. = wild-type.

EnvZ is the sensor of a two-component regulatory system known to contribute to virulence in Gram-negative bacteria, and an *env*Z mutant of *X. nematophilus* was found to be more pathogenic than the wild-type (Forst and Tabatabai, 1997). The *flh*DC operon of Enterobacteriaceae controls expression of about 50 genes involved in flagellum functioning, as well as certain stationary phase processes and type III secretion of proteins, including some associated with virulence. Since the *env*Z–*omp*R system negatively regulates *flh*DC, it is not surprising that Givaudan and Lanois (2000) found that disruption of the *flh*D gene of *X. nematophilus* led to attenuated virulence as well as loss of motility, lipase and haemolysin activities.

4.5. Evolution

4.5.1. Evolution of pathogenic bacteria

Virulence genes are often encoded by mobile genetic elements (transposons, phages, or plasmids) or on pathogenicity islands within the chromosome. These cassettes of genes are acquired by horizontal gene transfer, sometimes between widely divergent species, and can be gained or lost at high frequency because of their location on unstable DNA. Random DNA sequencing has revealed that *P. luminescens* has virulence factors previously only associated with the pathogens of vertebrates. This might be explained by the presence in its genome of a range of phage and insertion sequence elements which may have mediated the acquisition of the virulence genes by lateral transfer (ffrench-Constant *et al.*, 2000). There is evidence that the toxin genes recently isolated from *P. luminescens* and *X. nematophilus* have been acquired by horizontal transfer (ffrench-Constant and Bowen, 1999): only a subset of strains of these species are toxic to *M. sexta*; there are different genomic organizations of the toxin genes in the two species; and there are transposase-like sequences present on the cosmid clone (Jarrett *et al.*, 1998) containing the *X. nematophilus* toxin genes.

Table 4.4. Virulence of phase I and phase II variants.

Species	Strain	Virulence of phases	Reference
Xenorhabdus nematophilus	Agriotos	Equal	Akhurst, 1980
	DD136	Equal	Dunphy and Webster, 1984
	F1	Equal	Volgyi *et al.*, 1998
	19061	Equal	Volgyi *et al.*, 1998
	AN6	1° vir; 2° avir	Volgyi *et al.*, 1998
	N2–4	1° vir; 2° avir	Volgyi *et al.*, 1998
	N4	1° vir; 2° avir	Jarosz *et al.*, 1991
Photorhabdus	W14	Equal	Bowen and Ensign, 1998
	K122	2° vir but less so than 1°	Jackson *et al.*, 1995
	H8	2° low toxicity	Jarosz *et al.*, 1991
	Three clinical isolates (all phase 11)	All virulent to insects	Peel *et al.*, 1999

1° = phase I,
2° = phase II,
vir = virulent,
avir = avirulent.

4.5.2. Evolution of the nematode–bacterial partnership

Investigation of the virulence mechanisms of *Steinernema* and *Heterorhabditis* reveals fascinating insights into the interaction between the symbiotic partners and their victim, the insect host. There are clear differences between *Heterorhabditis* and *Steinernema* which corroborates the hypothesis on independent phylogeny. The phylogenetic ancestors of the now obligate insect pathogenic orders might have been saprozooic, feeding on an insect carcass with a bacterial flora rich in putative ancestors of *Xenorhabdus* and *Photorhabdus*. These might even have killed the insects, and the orally acting toxins found in some strains of *X. nematophilus* and *P. luminescens* might be a remnant from the time when these bacteria were not yet associated with nematodes but were facultative (like *Bacillus thuringiensis*) or obligate (like *Bacillus popilliae*) insect pathogens. The nematodes may then have adapted to act as transmitters of the pathogenic bacteria. Retention and transmission of a specific bacterial species by the putative ancestor was probably poor in the beginning. These non-specific associations with pathogenic bacteria might have been similar to those found in the slug nematode *Phasmarhabditis hermaphrodita* (Wilson *et al.*, 1995).

4.6. Conclusions

Many questions remain to be answered on the nature of the virulence of the nematode–bacterial complex. It is not clear how many toxins are produced by a given strain, whether separate toxins act within the gut and the haemocoel, whether these different activities characterize bacteria which enter the insect by different routes, how different strains compare in their range of toxins or host specificities etc. Assuming that only a group of bacteria produce oral toxins, are these strains which can or could recently live independently in the soil, or are they carried by nematodes that enter insects via the mouth? Are toxins that are active only by injection sufficient to kill the insect host, or do they act in concert with other virulence factors, and what is their mode of action? Do all strains produce a toxin of some sort, and if so, what is the role of septicaemia, if any, in the killing process? Finally, what is the role of the nematode in cases where both nematode and bacterial partners are needed for effective killing?

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5 Bacterial Metabolites

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5.1. Introduction

An outcome of research into the biology of the symbiosis of entomopathogenic nematodes and their associated bacteria has been the discovery of the diversity of chemical substances, some of them new to science, produced by the bacterial symbionts. This chapter focuses on these secondary metabolites, especially the organically soluble, small-molecule ones, and highlights their source, production and potential utility. The chapter builds upon the information reported previously (Nealson *et al.*, 1990).

Following the speculation of Dutky (1959) as to the antimicrobial activity of the bacterial symbiont of *Steinernema* (*Neoaplectana*), there was 'relative silence' on the subject for two decades until Paul *et al.* (1981) isolated and identified several new antibacterial compounds produced by *Xenorhabdus* spp. Thereafter, there has been a steady flow of publications reporting on the chemical nature and bioactivity of the products derived from both *Xenorhabdus* and *Photorhabdus* species (Frost and Nealson, 1996; Li *et al.*, 1998; Webster *et al.*, 1998). An important breakthrough was the discovery of a large-molecule, insecticidal toxin from cultures of *Photorhabdus* (Ensign *et al.*, 1990), and this is reported upon elsewhere (see Dowds and Peters, Chapter 4 this volume). However, it is the range of small-molecule compounds with antibiotic, insecticidal

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and nematicidal properties that has continued to intrigue researchers as they seek to identify a role for these substances in the biology of the symbiosis. Examination of *in vitro* cultures of the symbiotic bacteria has revealed secondary metabolites that have antibiotic activity against a wide range of bacterial and fungal species including human pathogenic fungi and yeasts (McInerney *et al.*, 1991a,b; Li *et al.*, 1997) and multi-drug resistant, human pathogenic bacteria (Chen, 1996). As well, antineoplastic activity has been reported (Webster *et al.*, 2000) and further medicinal potential is foreseen. A parallel consideration is the potential application of some of the secondary metabolites or their derivatives with antimycotic and nematicidal activity, in the agroforestry industry.

5.2. Structure and Bioactivity

More than 30 bioactive secondary metabolites, belonging to diverse chemical classes, have been reported from cultures of *Xenorhabdus* and *Photorhabdus* (Table 5.1). These include puromycin and madumycin II (unpublished), both of which were previously identified from cultures of *Streptomyces* (Suhadolnik, 1970; Tavares *et al.*, 1996). Most species of *Xenorhabdus* and *Photorhabdus* produce more than one group of bioactive secondary metabolites, and the metabolites from *Xenorhabdus* species are more diverse than those from *Photorhabdus*. Xenorhabdins are commonly produced by *Xenorhabdus bovienii*, xenocoumacins by *Xenorhabdus nematophilus* and hydroxystilbenes and anthraquinones by *Photorhabdus* spp. These metabolites not only have diverse chemical structures, but also a wide range of bioactivities of medicinal and agricultural interest, such as antibiotic, antimycotic, insecticidal, nematicidal, antiulcer, antineoplastic and antiviral. The structure and bioactivity of these metabolites is summarized in Table 5.1.

5.3. Antimicrobial Activity

The symbiotic bacteria produce both small- and large-molecule antibiotics. The small-molecule antibiotics inhibit the growth of a range of bacteria and fungi, many of which are of pharmaceutical and agricultural importance, such as *Escherichia, Staphylococcus, Aspergillus* and *Botrytis*. In contrast, macrobiomolecules, such as bacteriocin, from the symbiotic bacteria inhibit the growth of species or strains of closely related *Xenorhabdus* and *Photorhabdus* (Boemare *et al.*, 1992).

Akhurst (1982) demonstrated the antibiotic activity of cultures of *Xenorhabdus* spp. against a wide variety of microorganisms, including the Gram-positive *Micrococcus, Staphylococcus* and *Bacillus*, the Gram-negative *Escherichia, Shigella, Enterobacter, Serratia, Proteus, Erwinia, Flavobacterium* and *Pseudomonas*, and the yeasts *Candida* and *Saccharomyces*, although the chemical nature of the antibiotic substances in the cultures was not known. Chen *et al.* (1994) observed a strong antimycotic activity in cultures of the symbiotic bacteria on a range of fungi including *Botrytis cinerea, Fusarium oxysporum, Fusarium solani, Mucor piriformis, Pythium coloratum, Pythium ultimum, Penicillium* spp., *Rhizoctonia solani, Trichoderma pseudokingii* and *Verticillium dahliae*. Following the identification of hydroxystilbenes and indoles as antibiotics in cultures of *Photorhabdus* and *Xenorhabdus*, respectively, by Paul *et al.* (1981), more antimicrobial compounds were reported, such as xenorhabdins (McInerney *et al.*, 1991a), xenocoumacins (McInerney *et al.*, 1991b), xenorxides (Li *et al.*, 1998), xenomins (unpublished) and nematophin (Li *et al.*, 1997) (Table 5.1). Strain A2 of *X. bovienii* appears to be unusual

Table 5.1. Secondary metabolites and derivatives of *Xenorhabdus* and *Photorhabdus* species; their structure and bioactivities.

Groups	General structure	Identified metabolites[a]	Bioactivities[b]
Xenorhabdins		R_1 = H; R_2 = n-C_5H_{11}	1, 2, 6
		R_1 = H; R_2 = $(CH_2)_3CH(CH_3)_2$	1, 4
		R_1 = H; R_2 = n-C_7H_{15}	1
		R_1 = CH_3; R_2 = n-C_5H_{11}	1
		R_1 = CH_3; R_2 = $(CH_2)_3CH(CH_3)_2$	1, 2, 6
		R_1 = CH_3; R_2 = $CH_2CH(CH_3)_2$	1
		R_1 = CH_3; R_2 = $CH_2CH_2CH_3$	1
Xenorxides		R = n-C_5H_{11}	1, 2, 6
		R = $(CH_2)_3CH(CH_3)_2$	1, 2, 6
Xeno-coumacins		R = – $CH(NH_2)(CH_2)_3$-NH-$C(NH_2)NH$	1, 2, 3
		R = 2-pyrrolidinyl	1, 3
Indoles		R_1 = H; R_2 = CH_3	1, 2
		R_1 = AC; R_2 = CH_3	1, 2
		R_1 = H; R_2 = CH_2CH_3	1, 2
		R_1 = AC; R_2 = CH_2CH_3	1, 2
	Indole		5

Table 5.1. *Continued.*

Groups	General structure	Identified metabolites[a]	Bioactivities[b]
	Nematophin		1, 2
	Nematophin derivative		1
Isoflavonoids	Genistein		1
Hydroxy-stilbenes		R = – $CH(CH_3)_2$	1, 2, 5
		R = – CH_2CH_3	1
Anthra-quinones		R_1 = H; R_2 = H; R_3 = CH_3; R_4 = H; R_5 = H	1
		R_1 = H; R_2 = CH_3; R_3 = H; R_4 = H; R_5 = H	1
		R_1 = H; R_2 = H; R_3 = H; R_4 = H; R_5 = H	1
		R_1 = H; R_2 = CH_3; R_3 = CH_3; R_4 = H; R_5 = H	
		R_1 = CH_3; R_2 = H; R_3 = CH_3; R_4 = H; R_5 = H	
		R_1 = H; R_2 = CH_3; R_3 = CH_3; R_4 = H; R_5 = OCH_3	
		R_1 = CH_3; R_2 = H; R_3 = CH_3; R_4 = OH; R_5 = H.	

Table 5.1. *Continued.*

Groups	General structure	Identified metabolites[a]	Bioactivities[b]
Nucleosides	Puromycin		1, 2, 6, 7
Macrolides	Madumycin II		1

[a] The order of derivatives listed in this column represents the number given to the corresponding derivative for that same compound and is referred to in the text (e.g. the first xenorhabdin derivative in the table represents xenorhabdin 1 cited in the text).
[b] 1, antibiotic; 2, antimycotic; 3, antiulcer; 4, insecticidal; 5, nematicidal; 6, anticancer and 7, antiviral.

in the diversity of small-molecule, antimicrobial compounds since xenomins and xenorxides, several xenorhabdins, including three new ones, and four indoles have been isolated from this strain alone (Chen, 1996). These compounds showed strong activity against Gram-positive bacteria, yeast and many fungal species (Table 5.2). Of particular interest is the activity shown by nematophin and xenorxides with *in vitro* tests against multidrug-resistant strains of *Staphylococcus aureus*.

Xenorhabdins are dithiolopyrrolone derivatives, a class of compounds that was isolated initially from *Streptomyces* species, and are active against a variety of fungi, amoebae and bacteria (Celmer and Solomons, 1955). Xenorhabdins have significant antibacterial activity against Gram-positive bacteria but have little effect against Gram-negative bacteria (McInerney *et al.*, 1991a). The mechanism of antimicrobial action of dithiolopyrrolone derivatives, such as thiolutin, is primarily through inhibition of RNA and protein synthesis, as has been shown in yeast (Jimenez *et al.*, 1973; Tipper, 1973). Thiolutin reversibly inhibited the growth of *Saccharomyces cerevisiae* at low concentrations ($< 2\ \mu g\ ml^{-1}$) and completely inhibited RNA and protein synthesis at 2–4 $\mu g\ ml^{-1}$. RNA synthesis stopped immediately upon exposure to the thiolutin, and protein synthesis ceased within 20 min. The xenomins and xenorxides share a similar heterocyclic ring structure with the dithiolopyrrolones except that one of the two sulphate atoms is oxidized. All of these compounds exhibit somewhat similar

Table 5.2. Minimum inhibitory concentrations (MIC) of xenorxides (XO1, XO2), isolated from *Xenorhabdus bovienii* strain A21, and nematophin (NID), from *X. nematophilus* strain BC1, against bacteria, yeast and fungi.

	MIC (μg ml^{-1})		
Test species	XO1	XO2	NID
Bacillus subtilis	6	6	12
Escherichia coli ATCC 25922	> 100	> 100	> 100
Micrococcus luteus	25	6.0	> 100
Pseudomonas aeruginosa	> 100	> 100	> 100
Staphylococcus aureus ATCC 29213	6	6	0.75
S. aureus 0012[a]	3	3	1.50
S. aureus 0017[a]	3	1.50	0.75
Aspergillus fumigatus ATCC 13073	0.75	1.50	> 100
A. flavus ATCC 24133	0.75	1.50	> 100
Botrytis cinerea	12	25	12
Candida tropicalis CBS 94	> 100	> 100	> 100
Cryptococcus neoformans ATCC14117	6	6	> 100

[a] Clinical, methicillin-resistant strains.

antimicrobial activity *in vitro*, but they show substantial differences in their activity in mammals, and the precise antimicrobial mode of action of these oxidized compounds has not been described.

Interestingly, the indole and hydroxystilbene antibiotics produced by the bacterial symbionts are structurally very different from each other but appear to share the same mode of antimicrobial action (Sundar and Chang, 1992, 1993). They are all strongly active through their inhibition of bacterial RNA synthesis as seen by an accumulation of the regulatory nucleotide, guanosine-3′, 5′-bis-pyrophosphate (ppGpp) in susceptible bacteria (Sundar and Chang, 1992). ppGpp is generally considered to be a stress signal in microorganisms, and mediates macro-molecular regulation in many microorganisms including bacteria such as *Streptomyces*. The accumulation of ppGpp induces RNA synthesis inhibition within a few minutes of exposure to the antibiotics, and rapidly inhibits the growth of susceptible bacteria. However, the ppGpp regulatory effect is not the same in all organisms. In light of the rapid antimicrobial effect of the dithiolopyrrolones shown by Tipper (1973), it is understandable that these bacterial symbionts have evolved a strategy that helps them avoid or tolerate the inhibitory effect of these substances while the growth of other competing microorganisms is inhibited.

Xenocoumacins belong to the same class of compounds, with regard to structure and pharmacological activity, as the amicoumacins that were isolated initially from *Bacillus pumilus* (Itoh *et al.*, 1982). Compounds of this class are normally active against Gram-positive bacteria. Xenocoumacins are highly active against Gram-positive bacteria, including *Staphylococcus* and *Streptococcus* species, and some Gram-negative bacteria, such as *Escherichia coli* (McInerney *et al.*, 1991b). However, most enterobacteria and *Pseudomonas aeruginosa* are resistant to xenocoumacins, as are the drug-resistant strains of *S. aureus*. Xenocoumacin 1 (Table 5.1) is also active against the fungal species

Aspergillus and *Trichophyton* and the yeasts *Candida* and *Cryptococcus* (McInerney *et al.*, 1991b).

Large-molecule metabolites contribute also to the antimicrobial activity detectable in culture broths of the symbiotic bacteria. Poinar *et al.* (1980) showed first the existence of bacteriophage in *Xenorhabdus* and *Photorhabdus*. A bactericin, named xenorhabdicin, was purified and partially characterized on the basis of the protein particles, similar in shape to phage tail, consisting of two major subunits of 20 and 43 kDa (Thaler *et al.*, 1995). Bacteriacins have been detected in limited quantities in many *Xenorhabdus* and *Photorhabdus* cultures without any induction, and in increased quantities if induced by mitomycin (Boemare *et al.*, 1992). It is believed that the bacteriocins and phage elements from these bacteria are similar to those associated with lysogenic strains of *Photorhabdus* and *Erwinia* spp. These bacteriacins may contribute to the observed inhibitory effect between strains and species of *Xenorhabdus* reported by Akhurst (1982).

Chitinase isoforms of about 38.8 kDa are produced by several strains of both *Xenorhabdus* and *Photorhabdus* (Chen, 1996). Chitinases commonly contribute to the antimycotic activity of these bacteria through the destruction of fungal cell walls (Chen *et al.*, 1994; Isaacson, 2000). Some chitinases have lysozyme activity that hydrolyse peptidoglycans in bacterial cell walls and may also be antibacterial. The chitinases from *Xenorhabdus* and *Photorhabdus* inhibit conidial germination and mycelial growth but have no lysozyme activity (Chen, 1996). The levels of endochitinase and exochitinase activity differ among the strains of *Xenorhabdus* and *Photorhabdus* (Table 5.3), and vary qualitatively and quantitatively between the species and strains tested (Chen, 1996). The majority of the antimycotic activity of *Xenorhabdus* sp. RIO, the symbiont of *Steinernema riobrave*, is due to the water soluble rather than organically soluble metabolites. Of these proteinaceous metabolites the presence of exochitinase and significant endochitinase was demonstrated (Isaacson, 2000) by the release of *p*-nitrophenol from the appropriate respective substrates.

Proteinase and lipase activity has been identified from phase I *Xenorhabdus* species (Akhurst and Boemare, 1988; Boemare and Akhurst, 1988; Schmidt *et al.*, 1988) but rarely from phase II variants. Their role is unknown but speculation leads to the idea of these enzymes being used during bacterial breakdown of the insect tissues. An array of crystalline and proteinaceous products has been reported to be associated with these symbiotic bacteria and these are discussed in Chapter 2.

5.4. Insecticidal Activity

While screening metabolites for antibiotic properties McInerney *et al.* (1991a) identified five dithiolopyrrolone derivatives, xenorhabdins, from cultures of *Xenorhabdus* spp., and among them, xenorhabdin 2 (Table 5.1) was shown to be insecticidal also. In a larval feeding assay against *Heliothis punctigera*, xenorhabdin 2 caused 100% mortality at 150 $\mu g\ cm^{-2}$ with an LC_{50} of 59.5 $\mu g\ cm^{-2}$. It also decreased the weight of the surviving larvae. At a concentration of 37.5 $\mu g\ cm^{-2}$ there was only 18.8% larval mortality, but there was a 64.7% reduction in weight of the surviving larvae compared with that of the control larvae. The results suggest that xenorhabdin 2 interferes with larval feeding. Although the other xenorhabdins were not tested for insecticidal activity by McInerney *et al.* (1991a), a similar compound, thiolutin, had already been reported to

Table 5.3. Comparison of the final absorbance, total protein concentration, specific exo and endochitinase activities in 48 h tryptic soya broth cultures of three strains of *Xenorhabdus nematophilus* (ATC, BC1 and D1), and one strain each of *X. bovienii* (A21) and *Photorhabdus luminescens* (C9).

	Bacterial strains				
Characteristics	ATC	BC1	D1	A21	C9
Final absorbance (600 nm)	2.93	3.32	3.14	2.66	2.93
Total protein (mg ml^{-1})	0.13	0.95	0.24	0.63	0.46
Exochitinase (units mg protein^{-1})	29.64	10.32	35.18	12.51	8.03
Endochitinase (units mg protein^{-1})	32.14	9.07	17.75	7.01	8.82

have larvicidal activity against *Lucilia sericata* (Cole and Rolinson, 1972). The search for insecticidal metabolites from *Xenorhabdus* subsequently shifted to macrobiomolecules, such as proteinaceous toxins which are covered in another chapter (see Dowds and Peters, Chapter 4, this volume).

5.5. Nematicidal Activity

While examining interactions between the entomopathogenic nematode–bacterial symbionts and plant-parasitic nematodes, Hu *et al.* (1995) found that cell-free, culture filtrates of *Xenorhabdus* and *Photorhabdus*, cultured on tryptic soy broth (TSB), were toxic to second-stage juveniles of the root-knot nematode, *Meloidogyne incognita*, and to juveniles and adults of the pine wood nematode, *Bursaphelenchus xylophilus*. The ammonia component of the filtrate was considered a major contributing factor to this toxicity (Hu *et al.*, 1995; Hu, 1999). A toxic effect of the cell-free culture filtrates of *Xenorhabdus* spp. on the viability of second-stage juveniles and on egg hatch of *M. incognita* was also reported by Grewal *et al.* (1999). Further investigations showed that indole and 3,5-dihydroxy-4-isopropylstilbene (ST) from organic extracts of culture filtrates of *Photorhabdus luminescens* were contributing to the nematicidal effect (Hu *et al.*, 1996, 1999). At 100 μg ml^{-1} in test solutions ST caused nearly 100% mortality of fourth-stage juveniles and adults of the fungal-feeding nematodes, *Aphelenchoides rhytium* and *Bursaphelenchus* spp. and the bacterial-feeder, *Caenorhabditis elegans*. However, there was no mortality of second-stage juveniles of *M. incognita* or of infective juveniles of the entomopathogenic nematode, *H. megidis*, even at 200 μg ml^{-1} of ST. Indole was lethal to several nematode species at 300 μg ml^{-1}, and caused a high percentage of paralysis to *Bursaphelenchus* spp., *M. incognita* and *Heterorhabditis* spp. at 300, 100 and 400 μg ml^{-1}, respectively. Infective juveniles of the *H. megidis* 90 and *Heterorhabditis* sp. HMD strains were more resistant to indole than were other nematode species tested in that 100% mortality was not attained until 700 and 1000 μg ml^{-1} concentrations, respectively. ST and indole inhibited egg hatch of *M. incognita* at 100 and 25 μg ml^{-1}, respectively, in Petri dish assays (Hu *et al.*, 1999). In other experiments (Han and Ehlers, 1999), using culture filtrates of *P. luminescens* isolated from *H. bacteriophora* H06 and from *H. indica* LN2, it was shown that a mixture of the culture filtrates of the two isolates had a toxic effect on axenic, infective juvenile *H.*

bacteriophora H06 whereas the culture filtrate of each alone had no toxic effect on the juveniles.

The metabolites produced by some bacterial symbionts affected the chemosensory system of some nematode species. In a Petri dish bioassay, both ST and indole were shown to influence the nematode's dispersal behaviour (Hu *et al.*, 1999). Second-stage juveniles of *M. incognita* and infective juveniles of *Heterorhabditis*, the nematode symbiont of *Photorhabdus* that produced ST *in vitro* and *in vivo*, were not affected, but infective juveniles of some *Steinernema* species were repelled at a dosage as low as 0.1 μg. However, indole, which was produced by *Photorhabdus* and *Xenorhabdus* only under *in vitro* culture, repelled infective juveniles of some species of both *Heterorhabditis* and *Steinernema* (Hu *et al.*, 1999). Grewal *et al.* (1999) reported similar repelling effects of cell-free filtrates of *Xenorhabdus* on second-stage juvenile *M. incognita*.

The nematicidal properties of metabolites produced by *Xenorhabdus* and *Photorhabdus* are somewhat surprising in view of the close symbiotic relationship between the bacteria and entomopathogenic nematodes, and no biological role has been identified. Some of these metabolites may be utilized in behavioural strategies of the entomopathogenic nematodes in their interactions with other nematode species either in the insect cadaver or in the soil immediately following breakdown of the insect cuticle and emergence of the infective juveniles. By killing or repelling other nematode species that compete both for food and space the entomopathogenic nematodes increase their chances of survival. In particular, around plant roots to which they, other nematode species and potential insect hosts are attracted, the surviving entomopathogenic nematodes could search for and then invade and develop in an insect host with minimal competition (Hu, 1999; Hu *et al.*, 1999). Such survival strategies may be especially important for these entomopathogenic nematodes that have evolved obligate dependency on a particular species of bacterial symbiont.

5.6. Other Bioactivities

Xenocoumacins dosed orally in rats have, in addition to their antimicrobial activity, potent activity against stress-induced ulcers (McInerney *et al.*, 1991b). The pharmacological activity of xenocoumacins has not been tested, but it has been speculated to be similar to that of amicoumacins (McInerney *et al.*, 1991b), which have high acute toxicity to rats (Shimojima *et al.*, 1982).

In a large screening programme for chemo preventive agents conducted by the US National Cancer Institute, the dithiolopyrrolone derivative, thiolutin, was found to be a potent preventative of the effects of a carcinogen on normal rat tracheal epithelial cells (Arnold *et al.*, 1995). However, in a rat model this compound showed no efficacy in rat mammary glands that had been treated with a high dose of carcinogens (Steele *et al.*, 1994). Recently, several dithiolopyrrolone derivatives, derived originally from *Xenorhabdus*, showed potent cytotoxicity against a wide range of cancer cell lines, including lung, colon, prostate, skin, renal, breast and cervical, with a 50% inhibitory concentration in the micro molar range (Webster *et al.*, 2000) (Table 5.4).

5.7. Production of Antibiotics

The production of secondary metabolites with antibiotic properties is a characteristic common to many bacterial species. The study of *Xenorhabdus* spp. and *Photorhabdus*

spp. both *in vitro* (Paul *et al.*, 1981; Akhurst, 1982; Li *et al.*, 1995a,b, 1997) and *in vivo* (Maxwell *et al.*, 1994; Jarosz, 1996; Hu *et al.*, 1997, 1998, 1999; Hu and Webster, 2000) has increased our understanding not only of the chemistry of the metabolites and their possible role in the biology of the symbiosis, but also of their production techniques.

5.7.1. *In vitro* production

Antibiotic production by the symbiotic bacteria differs qualitatively and quantitatively depending on the strains and species of bacteria and their culture conditions. No antibiotic activity was detected, using an agar diffusion assay, from the metabolites of *Xenorhabdus* spp. cultured in 1% peptone water (Chen *et al.*, 1996). However, other media have been used successfully for antibiotic production, including yeast extract broth and its modifications (Akhurst, 1982; McInerney *et al.*, 1991a; Sztaricskai *et al.*, 1992; Sundar and Chang, 1993), Luria–Bertani broth (Sundar and Chang, 1993), sea water medium (Paul *et al.*, 1981) and TSB (Li *et al.*, 1995a,b, 1997).

Paul *et al.* (1981) showed that in cultures of *Xenorhabdus* sp. R strain the quantities of indole derivatives 1, 2 and 3 (Table 5.1) were about 1.3, 6.7 and 1 mg l^{-1}, respectively, and in cultures of *Photorhabdus* sp. Hb strain the quantities of hydroxystilbenes 1 and 2 (Table 5.1) were 7.3 and 2.2 mg l^{-1}, respectively. Yields of up to 300 and 100 mg l^{-1} of xenocoumacin 1 and 2, respectively, were obtained from *X. nematophilus* strain All (ATCC 53200) cultured in TSB (McInerney *et al.*, 1991b). Under continuous liquid culture in a yeast-extract medium, the production of xenorhabdins 1 and 2 (Table 5.1) by strain Q1 of *Xenorhabdus* sp. reached concentrations of 10.8 and 36.4 mg l^{-1}, respectively to give a total productivity of 2.4 mg l^{-1} h^{-1} (McInerney *et al.*, 1991a). In batch TSB cultures of *X. bovienii*, xenorhabdins were the dominant compounds produced, and xenomins and xenorxides were produced in only minute amounts (Chen, 1996).

Antibiotic production by the bacteria differs in culture over time. Li *et al.* (1997) showed that there was as much as a fivefold difference in nematophin concentration produced by different bacterial strains in TSB over time (Table 5.5). The concentration of nematophin in these bacterial cultures increased significantly from the first to the second day, and remained relatively high thereafter. In cultures of *X. bovienii* indole derivatives 2 and 4 (Table 5.1) had their maximum levels of 17.00 and 11.56 μg ml^{-1}, respectively on the first day of culture, but the concentrations decreased thereafter. In

Table 5.4. Cytotoxicity of xenorhabdin 2, measured as levels of inhibition against different cancer cell lines in culture.

Cancer cell lines	IC_{50} (μM)
Lung (NCI-H460)	0.11
Colon (HT29)	0.17
Skin (SK-MEL-28)	0.22
Prostate (Du-145)	0.33
Breast (MCF-7)	0.19
Leukaemia Moult-4	0.15

M = micromole; IC_{50} = 50% inhibitory concentration.

contrast, indole derivatives 1 and 3 (Table 5.1) had their lowest levels of 24.42 and 18.87 μg ml^{-1}, respectively on the first day, and the concentrations significantly increased thereafter. This phenomenon suggests that indole derivatives 2 and 4 were synthesized preferably in the early stage of bacterial growth and, subsequently, were hydrolysed to the corresponding derivatives 1 and 3. The biosynthesis of indole derivatives from tryptophan, a common amino acid, by the bacteria apparently involves methylene carbon retention and carboxyl carbon removal of the tryptophan since addition of tryptophan to the culture media enhances indole production (Sunder and Chang, 1993). The quantitative production of hydroxystilbene derivatives in cultures by *P. luminescens* differed significantly under different conditions, even for the same bacterial strain (Li *et al.*, 1995b; Hu *et al.*, 1999).

Phase variation of the bacteria, as well as temperature and aeration of the culture, influence bacterial growth and, consequently, affects antibiotic production (Akhurst, 1982; Chen, 1996). Phase I of the bacteria produce antibiotics whereas phase II do not (Akhurst, 1982). The change from phase I to phase II of the bacteria is a common occurrence in culture, and has been considered a problem in antibiotic production. As well, a much lower antibiotic activity was observed for *X. nematophilus* D1 strain cultured at 35°C than those at 15–30°C (Chen, 1996). *Xenorhabdus* spp. and *Photorhabdus* spp. are facultative anaerobes, but aeration is essential for antibiotic production. No antibiotic activity was detected in cultures in sealed vials that were not agitated (Akhurst, 1982; Chen *et al.*, 1996). Similar results have been observed for larval *G. mellonella* injected with entomopathogenic nematodes and sealed immediately in airtight containers (unpublished). The results indicate that antibiotic production of the symbiotic bacteria is influenced by many factors.

5.7.2. *In vivo* production

Three classes of antibiotics have been reported from nematode-infected *G. mellonella* cadavers: xenocoumacins, hydroxystilbenes and anthraquinones (Table 5.1).

Antibiotic production by the symbiotic bacteria differs qualitatively and quantitatively under *in vitro* and *in vivo* conditions (Hu *et al.*, 1998). 3,5-Dihydroxy-4-ethylstilbene was not detectable in broth cultures of *P. luminescens* C9 but was identified from larval *G. mellonella* infected by the *H. megidis* 90–*P. luminescens* C9 complex. A novel antibiotic (unpublished) was also identified from infected larvae in the same study (Hu *et al.*, 1998). A much greater quantity of ST is produced by *Photorhabdus* spp.

Table 5.5. Nematophin concentrations from three *Xenorhabdus nematophilus* strains, BC1, D1 and ATC, in tryptic soya broth cultured at 25°C over 5 days.

	Concentration of nematophin (μg ml^{-1})		
Time (days)	BC1	D1	ATC
1	116.83	54.84	11.25
2	605.34	200.67	148.04
3	478.48	39.93	88.72
4	369.28	36.14	36.69
5	564.20	108.00	70.00

in infected larval *G. mellonella* (665.2–4182.1 μg g^{-1} wet weight of insect) than in TSB culture (about 10–180 μg ml^{-1} wet insect) (Li *et al.*, 1995b; Hu *et al.*, 1997, 1999; Hu and Webster, 2000). This is coincident with the observation that much stronger antibiotic activity was detected in extracts of *in vivo* than in extracts of *in vitro* cultures of GI and SFU strains of *X. nematophilus* reported by Maxwell *et al.* (1994).

Antibiotics can be detected in entomopathogenic nematode-infected larval *G. mellonella* by the end of the growth phase of the bacterial symbionts, which is at about 24 h post-infection, as shown by the GI and SFU strains of *S. carpocapsae* (Maxwell *et al.*, 1994) and 90 and HMD strains of *Heterorhabditis* spp. (Hu *et al.*, 1999; Hu and Webster, 2000). The concentration of ST increased rapidly by 48 h postinfection and reached a peak at about the fifth day (Hu *et al.*, 1999; Hu and Webster, 2000). ST was produced in a much higher concentration in insects infected with *Heterorhabditis* spp. carrying *P. luminescens* (665.2–4182.1 μg g^{-1} wet weight of insect) (Hu *et al.*, 1999) than were xenocoumacins 1 and 2 (at a 1 : 1 ratio) in *G. mellonella* infected by *X. nematophilus* G1 (total concentration of 4 μg g^{-1} wet weight of insect) (Maxwell *et al.*, 1994). The concentration of ST was about 1500 and 4000 μg g^{-1} wet weight of *G. mellonella* larvae 2 and 5 days, respectively after infection by *Heterorhabditis* sp. (Hu *et al.*, 1997), and in five different *Heterorhabditis*–*Photorhabdus* complexes the concentration varied between 665.2 and 4182.1 μg g^{-1} wet weight of insect by the seventh day after infection (Hu *et al.*, 1999). A level of ST of about 3000 μg g^{-1} wet weight of *G. mellonella* cadaver infected with *P. luminescens* C9 was maintained in the cadaver for more than 21 days and over the entire period of nematode development (Hu *et al.*, 1999; Hu and Webster, 2000). Maxwell *et al.* (1994) reported that xenocoumacin 1 and 2 were still detectable 66 days post-infection of larval *G. mellonella* by GI and SFU strains of *S. carpocapsae*, respectively. Anthraquinones, some of which were reported to be antimicrobial (Sztaricskai *et al.*, 1992; Li *et al.*, 1995b), were produced in large quantities in larval *G. mellonella* infected by the *H. megidis* 90–*P. luminescens* C9 complex (Hu *et al.*, 1998).

In larval *G. mellonella* infected in the laboratory with the A21 and R strains of *Steinernema feltiae* Walsh (2000) showed the occurrence of two bacterial species in addition to the respective *Xenorhabdus* symbionts. A population of *Enterococcus*, which originated from the gut of the insect host, was eliminated in the first 48 h of infection, presumably due to the antibiotic activity of *Xenorhabdus* products. However, an *Acinetobacter* population, which originated from the nematode, appeared at about 100 h postinfection and attained a population level similar to that of the *Xenorhabdus* population. The *Acinetobacter* population continued at that level during the development of the *S. feltiae* population in the larval cadaver despite the presence of the large *Xenorhabdus* population and its antibacterial products. It was shown that *Enterococcus* was sensitive to small-molecule, organically soluble antibiotic metabolites of the *Xenorhabdus* but that *Acinetobacter* was not. A somewhat similar dixenic association was reported (Jackson *et al.*, 1995) for 10 out of 12 strains of *Heterorhabditis* where the *Photorhabdus* was associated with the bacterium *Provindencia* (*Proteus*) *rettgeri* after many years of laboratory culture.

These *in vivo* studies tend to support Dutky's hypothesis (1959) that antibiotics produced by the symbiotic bacteria prevent the putrefaction of cadavers infected by the nematode symbiont. The early and continued presence of antibiotics and their commonality across the different nematode–bacterium–insect interactions appears to have at least two functions. The antibiotics help to minimize competition from

non-symbiotic bacteria, especially early in the infection, and subsequently prevent microbial putrefaction of the nematode-infected insect cadavers. Nevertheless, there is substantial variation in the type of antibiotic metabolite produced by different species of bacterial symbiont. As well, bacterial species vary in their resistance to the antibiotics. As a result, a combination of the insect's initial immune response and the symbiont-derived antibiotics does not always lead to a monoxenic bacterial condition within the nematode-infected insect, and multiple bacterial associations with entomopathogenic nematodes have been reported (Poinar and Thomas, 1966; Lysenko and Weiser, 1974; Boemare, 1983; Jackson *et al.*, 1995; Babic *et al.*, 2000). Another hypothesis, proposed by Jarosz (1996) after studying the antibiotic production in *G. mellonella* by *S. carpocapsae* and *H. bacteriophora*, is that the lack of putrefaction of the infected insect is due rather to rapid growth of the bacterial symbiont which thereby prevents or minimizes competition by secondary invaders of the insect cadavers. It is apparent therefore, that the antibiotic substances are not the only factor influencing microbiological populations in these nematode-infected insects (Hu and Webster, 2000). More studies are necessary, especially of the antibiotic production in *Steinernema-Xenorhabdus*-insect interaction under *in vivo* conditions and in natural habitats, to help clarify the biological roles of these antibiotics in entomopathogenic nematode-infected insects.

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6 Biogeography

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6.1. Introduction

Biogeography is the study of living things in space and time (Cox and Moore, 2000). Biogeographers seek to answer such basic questions as why are there so many living things, why are they distributed in the way that they are, and are these patterns being affected by human activity. Linked to all questions is the spatial scale at which they are approached. Thus, two entomopathogenic nematode species could occupy the same continent, the same region and even the same square metre, but coexist because they occupy different soil depths or depend on different insects to maintain their populations.

Gaugler and Kaya's (1990) book was a milestone for entomopathogenic nematology. However, it is remarkable that of the 25 currently valid *Steinernema* species, 16 were described after 1989. Similarly, seven of the nine valid *Heterorhabditis* species were described after 1989. This reflects a dramatic increase in research on

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entomopathogenic nematodes, with more than 125 publications appearing annually from 1990 to 1998 compared with an average of about 30 per year from 1973 to 1979 (updated from Kerry and Hominick, 2001). Hence, an enormous amount of information has appeared since the book was published and our perceptions are changing at an exciting pace.

The biogeography of entomopathogenic nematodes was comprehensively assessed by Hominick *et al.* (1996) and the present contribution updates the information and consolidates and expands some of the concepts. Like the earlier one, this chapter is only a snapshot in time. Our understanding of the biogeography of entomopathogenic nematodes is in a fluid state.

6.2. Taxonomy

The nomenclature of entomopathogenic nematodes was elaborated and later summarized by Hominick *et al.* (1997, 2000) and the current status is discussed by Adams and Nguyen in Chapter 1 of this volume. Taxonomy is fundamental for biology – the name is the key to knowledge. If there is no name, there is no information and if the wrong name is used, wrong information is conveyed. The taxonomy of entomopathogenic nematodes has not been stable and many studies failed to identify species. Such studies contribute little to our knowledge of biogeography. Hominick *et al.* (1996) pointed out these limitations and also highlighted the fact that much of our present information relies on recent advances in molecular techniques, which provide accurate identification and a foundation for our knowledge. Recent molecular phylogenies confirm that the families Steinernematidae and Heterorhabditidae do not share a common ancestry but have evolved a similar lifestyle (Blaxter *et al.*, 1998). This is important to remember when considering the biogeography of the two genera, as some comparisons may not be appropriate.

6.3. Recovery from Soil

6.3.1. Techniques

Isolation of entomopathogenic nematodes from soil can be accomplished by standard extraction techniques for soil nematodes. Numerous nematodes are recovered, so entomopathogenic ones need to be separated and identified, a laborious procedure resulting in the processing of fewer samples compared with other methods (Curran and Heng, 1992). Also, identification of the juveniles requires particular taxonomic expertise so only a few scientists are capable of the task. The alternative is to use a bioassay. However, even a susceptible host recovers only a portion of a nematode population, and negative assays may reflect absence of nematodes or lack of infectivity of the resident population at the time of sampling (Hominick *et al.*, 1996).

There is some debate in the literature as to which methods are most efficient and appropriate for recovering entomopathogenic nematodes. For example, Spiridonov and Moens (1999) recovered two steinernematids from Belgian woodland soils by direct extraction although neither species had been recovered by three earlier surveys using *Galleria* baiting. Table 6.1 summarizes the expected results and limitations for the three basic techniques used to isolate entomopathogenic nematodes.

Table 6.1. Basic techniques for isolating entomopathogenic nematodes from soil, assessed for outcomes and limitations.

	Flotation	Baermann funnel	Bioassay
Nematode activity	Passive	Active	Infective
Efficiency	High	Lower	Lowest
Nematode populations recovered	Mixed	Mixed	One
Labour needed	High	High	Lower
Lab cultures established	No	Possible	Yes
Stages recovered	Dauers	Dauers	All
Taxonomic expertise needed	High	High	Moderate
Taxonomic work possible	No	No	Yes
Quantitative	Yes	Yes	Yes

In an effort to compare the efficiency of direct extraction methods with the *Galleria* bioassay, Sturhan and Mracek (2000) used both techniques on the same samples. Both methods recovered five species, but the baiting technique was felt to be less effective, especially at detecting mixtures of species at one site. In fact, the method of choice depends on the objectives of a study and no single method is without limitations or compromises. Also, removing soil from the environment is destructive, but this problem can be alleviated by adding *Galleria* larvae to the sampling site in an appropriate restraint that allows the nematodes access to the host after which it can be easily recovered.

6.3.2. Sampling effort

Entomopathogenic nematodes are aggregated rather than random in distribution. Spiridonov and Voronov (1995) demonstrated this particularly well. They used a modified Baermann funnel method to extract entomopathogenic nematodes from soil samples collected at 5-cm intervals along eight parallel transects in a cultivated plot in southern Estonia. They showed clearly that *S. feltiae* dauer juveniles were distributed in an aggregated fashion, with peaks of up to 80 nematodes occurring in 215 cm^3 of soil compared with normal levels of only a few nematodes per sample. Such spatial patchiness in populations has implications for sampling whether techniques use active or passive methods of extraction (Hominick *et al.*, 1996).

The prevalence (percentage of samples that are positive) and intensity (number of individuals in a sample) of entomopathogenic nematodes can vary with time (e.g. Spiridonov and Voronov, 1995). There are dominant species and comparatively rare ones; many samples are necessary to recover all species. For example, Sturhan (1999) sampled 1193 sites in Germany and recovered 11 different steinernematids and two heterorhabditids. Of 584 records for entomopathogenic nematodes, *S. affine* dominated and was recorded 139 times, while *S. carpocapsae* and a new species were each recorded only once and *H. bacteriophora* twice. The lesson is clear – regardless of the techniques used, it is difficult to state with confidence that a species does not exist in a locality, as it may be so rare that finding it is a function of sampling effort.

6.4. Biogeography

6.4.1. Baseline

In the broadest sense, entomopathogenic nematodes are widespread. Before 1990, there were a number of surveys that established that entomopathogenic nematodes were ubiquitous but many failed to identify isolates beyond genus. Also, the literature before 1990 is confusing, as identifications were frequently dubious and the identification and taxonomy of three of the most commonly isolated steinernematids (identified as *S. carpocapsae*, *S. feltiae*, or *S. bibionis*) was uncertain (Poinar, 1989). In the 1990s, molecular methods and better keys meant that identification to the species level was more reliable. Therefore, Poinar's (1990) contribution provides a baseline and the authoritative reference for the geographical origins of entomopathogenic nematode isolates at the time of his writing.

6.4.2. Studies since 1990

Table 6.2 documents key references for information relating to the biogeography of entomopathogenic nematode species since 1990. Studies that failed to identify the nematodes have been excluded. The information is organized by broad geographical areas to facilitate comparisons and shows the method of isolation and the species identified, together with cryptic biogeographic notes. Some of the studies listed are not surveys, but are reviews or taxonomic studies documenting the identity and

Table 6.2. Key references since 1990 for information relating to the biogeography of entomopathogenic nematode species.

Location	Method	Species identified	Comments	Reference
Europe				
Azores	GT	*H. bacteriophora, S. carpocapsae, S. glaseri*	1180 sites from 9 islands; 30 sites with hets., 16 with steiners	Rosa *et al.*, 2000
Belgium	DE GT	*S. feltiae, S. affine, S. kraussei*	19/36 samples from woodland positive; *Galleria* traps successful only for *S. feltiae*	Spiridonov and Moens, 1999
Belgium	GT	*S. feltiae, S. affine, H. megidis* (NW European type)	21/248 samples positive, 1 het., diverse habitats	Miduturi *et al.*, 1997
Belgium	GT	*S. feltiae, S. affine*	180 samples from 31 sites; 7 *S. feltiae*, 4 *S. affine* pops	Miduturi *et al.*, 1996a
Belgium	GT	*S. feltiae, S. affine, H. megidis* (NW European type)	130 samples from 21 sites; 9 *S. feltiae*, 5 *S. affine*, 1 *H. megidis* pop	Miduturi *et al.*, 1996b
Czech Republic	GT DE	*S. affine, S. intermedium, S. kraussei*	Compared efficiency of the two extraction methods	Sturhan and Mracek, 2000
Slovak Republic	DE	*S. affine, S. intermedium, S. feltiae, S. kraussei, S. carpocapsae, S. bicornutum, Heterorhabditis* sp.	36/111 samples positive, diverse habitats; *S. affine, S. intermedium* and *S. feltiae* make up 74% of records	Sturhan and Liskova, 1999
Czech Republic	GT	*S. kraussei, S. feltiae, S. intermedium, S. affine, S. bicornutum, 2 Steinernema* spp.	61/87 localities with insect aggregations positive for steinernematids	Mracek and Becvar, 2000

Table 6.2. *Continued*.

Location	Method	Species identified	Comments	Reference
Czech Republic	GT	*S. kraussei, S. feltiae, S. intermedium, S. affine, S. bicornutum, H. megidis*	342 samples; 54% positive; only one with heterorhabditid	Mracek *et al*., 1999
Denmark	GT	*H. megidis* (NW European type), *Heterorhabditis* (Irish type)	Targeted surveys for hets., 10/26 coastal sites positive	Griffin *et al*., 1999
Estonia	GT	*H. megidis* (NW European type)	Targeted surveys for hets., 6/22 coastal sites positive	Griffin *et al*., 1999
Germany	GT DE	*S. affine, S. intermedium, S. kraussei, S. feltiae*	Forest soils; compared efficiency of the two extraction methods	Sturhan and Mracek, 2000
Germany	DE	*S. affine, S. intermedium, S. feltiae, S. kraussei, S. carpocapsae, S. bicornutum, 5 undescribed S. spp., H. megidis* (NW European type), *H. bacteriophora*	437/1193 diverse habitats positive; some habitat specificity; hets. rare; *S. carpocapsae* isolated once	Sturhan, 1999
Germany	DE	*S. affine, S. feltiae*	1248 samples in experimental field, 94% with *Steinernema; S. affine* highly dominant	Sturhan, 1996
Germany	DE	*S. affine, S. feltiae, other steinernematids, H. megidis?*	26 samples from one plot	Sturhan, 1995
Germany	–	*H. bacteriophora*	Grassland, natural infection in *Amphimallon solstitialis* (Scarab.)	Glare *et al*., 1993
Germany	GT	*S. carpocapsae, S. feltiae, S. affine*	Farmland soil. 7/600 bait insects infected.	Ehlers *et al*., 1991
Greece	GT	*S. feltiae, H. megidis*	2/43 positive sites from various habitats	Menti *et al*., 1997
Hungary	GT	*H. bacteriophora, Heterorhabditis* (Irish type)	Targeted sandy soils for hets., 15/46 positive; *H. bacteriophora* dominant	Griffin *et al*., 1999
Italy, Southern	GT	*S. feltiae, S. anomali, S. affine, H. bacteriophora*	188 samples; 16 with steiners.; 10 with hets.	Tarasco and Triggiani, 1997
Italy	GT	*S. carpocapsae, S. feltiae*	697 localities, mainly farmland; 5% prevalence, half steiners.,half hets.	Ehlers *et al*., 1991
Netherlands	GT	*S. feltiae, S. affine, H. megidis* (NW European type)	48/100 sites positive, 13/100 with hets., 9 concurrent with steiners.	Hominick *et al*., 1995
Poland	GT	*S. feltiae, H. bacteriophora* (introduced previously)	Cultivated fields	Jaworska and Dudek, 1992
Republic of Ireland	GT	*S. feltiae, S. affine*	17/169 forest or grassland sites positive; *S. feltiae* from 14 sites	Dillon *et al*., 2000
Republic of Ireland	GT	Only Irish Group of *Heterorhabditis*	18/169 sites positive, all coastal; 40 inland sites negative	Griffin *et al*., 1994
Republic of Ireland	GT	*S. feltiae, S. affine, Heterorhabditis* sp.	551 samples; *S. feltiae* in 7%, *S. affine* in 3%, 1 sample with het.	Griffin *et al*., 1991
Spain	GT	*S. feltiae, S. carpocapsae, S. affine,* 2 unident. *S.* spp., *H. bacteriophora*	Catalonia (NE Spain). 35/150 sites positive; *S. feltiae* dominant (23 sites); *H. bacteriophora* in 2 sites	Garcia del Pino and Palomo, 1996a
Spain	GT	*H. bacteriophora, S. feltiae*	Pop dynamics at 6 sites for 14 months; 5 sites with *H. bacteriophora* one with *S. feltiae*	Garcia del Pino and Palomo, 1997

Table 6.2. *Continued*.

Location	Method	Species identified	Comments	Reference
Spain	GT	*S. glaseri, H. bacteriophora*	Catalonia; 60 soil samples yield 2 populations	De Doucet and Gabarra, 1994
Switzerland	GT	*S. intermedium, S. feltiae, S. kraussei, S. bicornutum, S. affine, S. carpocapsae, H. megidis, H. bacteriophora*	113/600 samples from lowland regions positive, 104 with steiners., listed in decreasing frequency; 6 *H. megidis*, one *H. bacteriophora*	Kramer *et al*., 2000
Switzerland	GT	*S. kraussei, S. affine, S. feltiae, S. intermedium, H. megidis* (NW European type)	473 samples, 27% positive. *S. kraussei* dominant in alpine environment, *S. feltiae* pre-dominant lower alps; others rare	Steiner, 1996
UK: England, Scotland, Wales	GT	*S. affine, S. feltiae, S. kraussei,* 3 new *Steinernema* spp.	Positive sites: 41/221 Scotland; 11/154 England; 1/39 Wales. *S. kraussei* dominant, cold-adapted?	Gwynn and Richard-son, 1996
UK: England, Scotland, Wales	GT	*S. feltiae, S. affine, 5 unident. S. spp., H. megidis* (NW European type), *Heterorhabditis* (Irish type)	Two surveys, 2nd one coastal; 197/403 and 60/157 sites positive; *S. feltiae* dominant, hets. rare	Hominick *et al*., 1995
UK: Scotland, S. Wales	GT	Only Irish Group of *Heterorhabditis*	2/51 Scottish and 9/20 Welsh sites positive, all coastal	Griffin *et al*., 1994
UK: Scotland	GT	*S. feltiae*	2.2% of 1014 samples positive, no other species	Boag *et al*., 1992
UK: England, Scotland, Wales	GT	*S. feltiae, Heterorhabditis* sp.	403 sites, 49% positive, only 1 with het. several *Steinernema* spp. unidentified	Hominick and Briscoe, 1990
North America, Caribbean				
Canada: West and North west	GT	*S. feltiae, S.* spp., *H. megidis*	125 sites, 18 with steinernematids, 7 with *H. megidis*, latter only in Southern BC	Mracek and Webster, 1993
Caribbean region	GT	*H. indica, H. bacteriophora, S. cubanum, S. biocornutum, S. puertoricense*	Exhaustive sampling of Guadeloupe islands; random samples from 7 other islands; molecular IDs of both nematodes and bacterial symbionts	Fischer-Le Saux *et al*., 1998
Guadeloupe and neighbouring islands	GT	*H. indica, H. bacteriophora, Steinernema* n.sp.	35/538 sites positive, mainly coastal; *H. indica* dominant; *Steinernema* in only one site	Constant *et al*., 1998
USA: California	GT	*S. carpocapsae, S. feltiae, S. kraussei, S. longicaudum, S. oregonense, H. marelatus, H. bacteriophora*	71/270 samples positive, natural habitats, 10 geographic regions; steiners. predominate, *S. kraussei* dominant	Stock *et al*., 1999
USA: New Jersey	GT	*H. bacteriophora, S. carpocapsae, S. feltiae*	Distn. along transects in turfgrass, endemic and released	Campbell *et al*., 1998
USA: New Jersey	GT	*H. bacteriophora?, S. glaseri, S. feltiae, S. carpocapsae*	Transects at 13 sites, 72/600 samples positive, heterorhabditids dominant	Stuart and Gaugler, 1994

Table 6.2. *Continued*.

Location	Method	Species identified	Comments	Reference
USA: New Jersey	GT	*S. glaseri, S. carpocapsae, S. feltiae, H. bacteriophora*	Sampled 304 diverse sites, 66 positive for epns, 42 with hets., 24 with steiners	Gaugler *et al*., 1992
USA: Tennessee	GT OI	*H. bacteriophora,* *S. carpocapsae*	113 nursery sites sampled; each species recovered from 17 samples. *Galleria*, housefly larvae, housecricket and lesser mealworm adults	Rueda *et al*., 1993
South America				
Argentina	–	*S. feltiae, S. scapterisci, S. carpocapsae, S. glaseri, S. rara, S. ritteri, H. bacteriophora, H. argentinensis*	Overview of nematodes in agriculture in continental Argentina	Doucet and De Doucet, 1997
Argentina, Pampean region	GT	*S. feltiae, S. carpocapsae, S. scapterisci, H. bacteriophora, H. argentinensis*	41/310 samples positive, 14 localities. Steiners. 66%, hets. 34% of samples	Stock, 1995
Colombia	GT	*H. bacteriophora*	Found in 3 of 8 sites	Caicedo and Bellotti, 1996
Venezuela	GT	*H. indica*	Mainly evaluate native vs. foreign epns	Rosales and Suarez, 1998
Asia				
China	–	*S. carpocapsae, S. glaseri, S. caudatum, S. longicaudum, H. bacteriophora, H. brevicaudis*	Overview of research on epns in China	Han, 1994
India: Andhra-Pradesh	OI	*S. feltiae?*	110 samples in 11 fields; *Corcyra cephalonica* (Lepidoptera) traps	Singh *et al*., 1992
India: Tamil Nadu	GT	*Steinernema* unidentified, *H. indica*	163 samples; 17 with *Steinernema*; 1 with *H. indica*	Josephrajkumar and Sivakumar, 1997
Indonesia	GT OI	*H. indica, 2 Steinernema* RFLP types	Sampled promising sites; 16/79 sites positive, all coastal; both genera equally prevalent. *Galleria*, *Tenebrio molitor* and bamboo insect traps	Griffin *et al*., 2000
Israel	GT	*H. bacteriophora, H. megidis*	Collected from irrigated Negev region	Glazer *et al*., 1993
Japan	GT	*S. kushidai plus 7 new Steinernema* spp., *H. indica, H. megidis*	1416 samples, 266 sites in 5 climatic regions; 142 positive sites, steiners. dominant	Yoshida *et al*., 1998
Malaysia: Peninsular	GT	*H. indica, H.* n. sp., two new steinernematids	10% of 425 samples positive; diverse habitats; steiners. dominant	Mason *et al*., 1996
Pakistan: Sindh	GT	*H. indica*, unident. steiners. & hets.	500 samples, 20 locations, 12% positive, mainly hets.	Shahina *et al*., 1998
Pakistan: Sindh & Balochistan	GT	*H. indica*, unident. steiners. & hets.	415 samples, 15 locations; 20 steiner and 31 het. Isolates, 23 *H. indica*.	Anis *et al*., 2000

Table 6.2. *Continued*.

Location	Method	Species identified	Comments	Reference
Palestine (Bethlehem)	?	*H. indica*	Molecular study	Sansour and Iraki, 2000
Republic of Korea	GT	*S. carpocapsae*, 2 unident. steiners., *H. bacteriophora*	23/499 samples positive, all 9 provinces sampled	Choo *et al*., 1995
Turkey	GT	*S. feltiae*	106 samples, 5 positive, one isolate identified	Ozer *et al*., 1995
Africa				
Kenya	GT	*S. karii, H. bacteriophora, H. indica*	Epns in 154/641 samples from central highlands and 7/200 from coastal lowlands; *H. indica* dominant at coast	Waturu, 1998
Australia/Pacific Ocean				
Hawaii	GT	Unidentified heterorhabditids and steinernematids	First to show association of hets. with coasts; 351 samples from 6 islands; 22 sites with hets., 2 with steiners.	Hara *et al*., 1991
New Zealand	GT	*S. feltiae, H. zealandica*	*S. feltiae* dominant	Barker and Barker, 1998
Global				
Global summary	TS	*S. affine, S. arenarium, S. carpocapsae, S. feltiae, S. glaseri, S. intermedium, S. kushidai, S. rara, S. scapterisci, H. bacteriophora, H. megidis, H. zealandica*	Comprehensive tables with data on *Steinernema* and *Heterorhabditis* strains	Poinar, 1990
Global summary	–	Various	Comprehensive review of natural hosts for epns	Peters, 1996
Europe, USA, Australia, New Zealand	TS	*H. bacteriophora, H. megidis* (NW European type), *Heterorhabditis* (Irish type)	Examined 46 *Heterorhabditis* isolates using RFLP patterns	Smits and Ehlers, 1991; Smits *et al*., 1991
India, Kenya, Indonesia, Cuba	TS	*H. indica* isolates	Examined 15 *H. indica* isolates using molecular and cross-breeding techniques	Stack *et al*., 2000
Azores, Egypt, South Africa, Caribbean, Europe, New Zealand, Australia	TS	*H. bacteriophora, H. indica*	Used satellite DNAs to identify 20 unassigned isolates and list a number of reference strains for both genera	Grenier *et al*., 1996, 1998
USA, Canada, Europe	TS	*S. kraussei* isolates	Examined 9 isolates using multivariate analysis	Stock *et al*., 2000

GT = *Galleria* traps; OI = other insect bait; DE = direct extraction; TS = taxonomic study.

distribution of a number of isolates collected widely and obtained from collaborators. These are at the end of the table and placed under a global heading.

The only continent where entomopathogenic nematodes have not been found is Antarctica (Griffin *et al.*, 1990). Table 6.2 has 36 references for European countries, eight for North America–Caribbean, four for South America, 12 for Asia, one for Africa and two for Australia–Pacific Ocean. Because of support from the European Union to encourage collaborative work on entomopathogenic nematodes, the biogeography and biodiversity of entomopathogenic nematodes is understood better in Europe than in any other geographical area of the world. Nevertheless, most of the world remains unexplored.

The most common method used to isolate the nematodes is a bioassay, usually based on the '*Galleria* trap'. Use of direct extraction methods is limited to a few workers with the specialized taxonomic expertise required to identify the nematodes that are recovered. Sample sizes and prevalences vary widely between different surveys, with prevalence ranging from around 2% to over 45%. While natural variability is to be expected, some can be attributed to methodology such as:

- Conducting targeted instead of random surveys. The 'discovery rate' can be increased substantially if likely habitats are surveyed preferentially. For example, Mracek and Becvar (2000) found 61 of 87 localities positive for steinernematids when they sampled sites with insect aggregations. Similarly, Griffin *et al.* (1999) took samples only from sites considered likely for heterorhabditids (sandy soils and especially coastal sites) and recorded a much higher prevalence than in non-targeted surveys conducted in neighbouring areas.
- Repeating bioassays on initially negative samples. This can significantly increase recovery as not all entomopathogenic nematodes infect bait insects at the same time (e.g. Hominick and Briscoe, 1990).
- Using direct extraction methods, which can recover inactive nematodes while bioassays recover only infective ones (e.g. Spiridonov and Moens, 1999).
- Applying different sampling methods. Some workers take one large sample per site, while other workers combine many small samples to make up a sample from a site (Griffin *et al.*, 1999). The low populations of entomopathogenic nematodes in soil, combined with their aggregated distributions, mean that absence in a sample could be a false negative, attributed to limitations of the sampling regime.

The surveys documented in Table 6.2 provide good geographical distribution information but do not provide comparable quantitative information. Provided that sample sizes are adequate, quantitative comparisons are best made within rather than between individual surveys.

Steinernematids are generally recovered more often than heterorhabditids during non-targeted surveys. Exceptions occurred in the surveys of the Azores, the Caribbean, New Jersey, Tennessee, Hawaii, Israel, Pakistan and the Kenyan coast. Heterorhabditids are rare in most European surveys, unless sampling is biased towards favourable habitats. Steinernematids may be recovered more often simply because there are many more species of steinernematids than heterorhabditids (see Tables 6.3 and 6.4) and they therefore occupy more niches. Also, the difference in life history of the two genera probably contributes to the unequal recovery. One heterorhabditid is sufficient to multiply after invasion while at least two steinernematids, a male and

female, must invade before reproduction can occur (Downes and Griffin, 1996). This means that *Steinernema* would be expected to have a higher characteristic abundance in the environment, and hence a higher probability of being recovered, an expectation supported by survey data.

Heterorhabditids are frequently found in sites adjacent to the sea (e.g. Hara *et al.*, 1991; Griffin *et al.*, 1994, 1999, 2000; Constant *et al.*, 1998; Waturu, 1998; Yoshida *et al.*, 1998; Stock *et al.*, 1999; Rosa *et al.*, 2000), an association that continues to intrigue. The frequent occurrence of the preferred sandy soils in these habitats may provide part of an explanation (Griffin *et al.*, 1999, 2000). However, Rosa *et al.* (2000) noted that in their samples from the Azores, most *H. bacteriophora* isolates were recovered from soil sampled at sea level but that the soils had a low sand content and were similar to soils where steinernematids were recovered. While the association with coastal areas remains unexplained, there are also many studies that show that heterorhabditids are not restricted to these habitats. It is likely that the extent of the preference of heterorhabditids for sandy soils varies between species (Griffin *et al.* (2000). Thus, species such as *H. bacteriophora*, which occurs in silt loam, may depend less on open textured soil than either *H. indica* or the Irish type of *Heterorhabditis*. Steinernematids may also be found at coastal sites rather than inland ones during surveys (e.g. Constant *et al.*, 1998; Griffin *et al.*, 2000). Hominick *et al.* (1996) speculated that washed-up marine detritus is a rich source of nutrients and supports large populations of insects, which could support populations of entomopathogenic nematodes. In any case, there is a great deal of evidence to support the notion that coastal zones provide excellent sources for entomopathogenic nematodes.

6.4.3. Geographical distribution

Tables 6.3 and 6.4 document the geographical distribution for steinernematid and heterorhabditid species. Adams and Nguyen (Chapter 1, this volume) or Hominick *et al.* (1997) should be consulted for the references for the taxonomic authorities for species. The localities in the tables are arranged in the same geographical blocks as in Table 6.2, and countries within these areas are listed alphabetically. The baseline for the tables is Poinar's (1990) authoritative contribution. The references in Tables 6.3 and 6.4 do not always reflect new isolations. Sometimes, a reference refers to an authoritative identification of a nematode previously isolated and provided from a reference collection.

Undescribed species are frequently encountered during surveys, but species without formal descriptions are not listed in the tables because accurate identification is fundamental to understanding geographical distribution and habitat specificity (Reid *et al.*, 1997). Hominick *et al.* (2000) list 23 undescribed steinernematids out of a total of 43 that were included in an analysis for a phylogenetic tree of the genus. A large number of undescribed species exist in laboratories around the world.

Adams *et al.* (1998) compared the molecular phylogeny of heterorhabditids and concluded that *H. indica* is probably conspecific with *H. hawaiiensis* and *H. argentinensis* with *H. bacteriophora*, so there are probably fewer *Heterorhabditis* species than listed in Table 6.4. Smits and Ehlers (1991) proposed that there are three groups of *Heterorhabditis* in Europe, namely *H. bacteriophora, H. megidis* (NW European Group) and an undescribed species similar to *H. megidis* and termed

Table 6.3. Recognized species of Steinernematidae with geographical records.

Species in chronological order	Locality	Reference
Steinernema kraussei (Steiner, 1923) Travassos, 1927	Egge Mountains, Warburg county near Nevenherrsee Westphalia, Germany	Type locality
	Austria	Peters, 1996
	Belgium	Spiridonov and Moens, 1999
	Czech Republic	Grenier *et al*., 1996; Fischer-Le Saux *et al*., 1998; Mracek, *et al*., 1999; Mracek and Becvar, 2000; Sturhan and Mracek, 2000
	Germany	Mracek *et al*., 1992; Peters, 1996; Sturhan, 1999; Stock *et al*., 2000; Sturhan and Mracek, 2000
	Netherlands	Hominick *et al*., 1995
	Russia	A.P. Reid, Egham, 2000, personal communication
	Slovak Republic	Sturhan and Liskova, 1999
	Switzerland	Steiner, 1996; Kramer *et al*., 2000
	UK	Hominick *et al*., 1995; Gwynn and Richardson, 1996; Stock *et al*., 2000
	Canada: British Columbia and Alberta	Stock *et al*., 2000
	USA: California	Stock *et al*., 1999, 2000
	USA: New York	Stock *et al*., 2000
S. glaseri (Steiner, 1929) Wouts, Mracek, Gerdin and Bedding, 1982	Tavistock Country Club, Haddonfield, New Jersey, USA	Type locality
	Azores	Rosa *et al*., 2000
	Spain	De Doucet and Gabarra, 1994
	USA: Florida	Poinar, 1990; Fischer-Le Saux *et al*., 1998
	USA: New Jersey	Gaugler *et al*., 1992; Stuart and Gaugler, 1994
	USA: North Carolina	Poinar, 1990; Grenier *et al*., 1996; Fischer-Le Saux *et al*., 1998
	Argentina	Doucet and De Doucet, 1997
	Brazil	Poinar, 1990
	China: Hainan and Guangdong Provinces	Li and Wang, 1989; Wang *et al*., 1991; Han, 1994; Liu *et al*., 1998
	Republic of Korea	Stock *et al*., 1997
S. feltiae (Filipjev, 1934) Wouts, Mracek, Gerdin and Bedding, 1982	Eastern Russia	Type locality
	Austria	Peters, 1996
	Belgium	Miduturi *et al*., 1996a,b, 1997; Spiridonov and Moens, 1999
	Czech Republic	Poinar, 1990; Mracek *et al*., 1999; Mracek and Becvar, 2000
	Denmark	Poinar and Lindhardt, 1971
	Estonia	A.P. Reid, Egham, 2000, personal communication
	Finland	Poinar, 1990; Peters, 1996
	France	Poinar, 1990; Fischer-Le Saux *et al*., 1998

Table 6.3. *Continued.*

Species in chronological order	Locality	Reference
	Germany	Ehlers *et al.*, 1991; Sturhan, 1995, 1996, 1999; Sturhan and Mracek, 2000
	Greece	Menti *et al.*, 1997
	Hungary	Mracek and Jenser, 1988
	Italy	Ehlers *et al.*, 1991; Tarasco and Triggiani, 1997
	Netherlands	Poinar, 1990; Hominick *et al.*, 1995
	Poland	Sandner and Bednarek, 1987; Jaworska and Dudek, 1992
	Rep of Ireland	Griffin *et al.*, 1991; Dillon *et al.*, 2000
	Russia	Spiridonov and Voronov, 1995; Peters, 1996
	Slovak Republic	Sturhan and Liskova, 1999
	Spain	Garcia del Pino and Palomo 1996a, 1997
	Sweden	Burman *et al.*, 1986
	Switzerland	Steiner, 1996; Kramer *et al.*, 2000
	UK	Hominick and Briscoe, 1990; Hominick *et al.*, 1995; Gwynn and Richardson 1996
	Northern Ireland	Blackshaw, 1988
	UK, Scotland	Boag *et al.*, 1992
	Ukraine	Peters, 1996
	Canada: British Columbia, Alberta, Yukon	Mracek and Webster, 1993
	USA: California	Poinar, 1992; Stock *et al.*, 1999
	USA: Florida	Fischer-Le Saux *et al.*, 1998
	USA: New Jersey	Gaugler *et al.*, 1992; Stuart and Gaugler, 1994; Campbell *et al.*, 1998
	Argentina	Stock, 1993, 1995; Doucet and De Doucet, 1997
	India: Andhra Pradesh?	Singh *et al.*, 1992
	Turkey	Ozer *et al.*, 1995
	Egypt	Peters, 1996
	Australia: New South Wales, Canberra, Victoria, Queensland	Poinar, 1990
	Australia: Tasmania	Poinar, 1990; Grenier *et al.*, 1996; Fischer-Le Saux *et al.*, 1998
	Hawaii	A.P. Reid, Egham, 2000, personal communication
	New Zealand	Poinar, 1990; Peters, 1996; Barker and Barker, 1998
S. affine (Bovien, 1937) Wouts, Mracek, Gerdin and Bedding, 1982	Denmark	Type locality
	Belgium	Miduturi *et al.*, 1996a,b, 1997; Spiridonov and Moens, 1999
	Czech Republic	Mracek *et al.*, 1999; Mracek and Becvar, 2000; Sturhan and Mracek, 2000
	Denmark	Poinar, 1990
	France	Fischer-Le Saux *et al.*, 1998
	Germany	Poinar, 1990; Ehlers *et al.*, 1991; Sturhan, 1995, 1996, 1999; Peters, 1996; Sturhan and Mracek, 2000
	Italy (Southern)	Tarasco and Triggiani, 1997
	Netherlands	Hominick *et al.*, 1995

Table 6.3. *Continued*.

Species in chronological order	Locality	Reference
	Rep. of Ireland	Griffin *et al*., 1991; Dillon *et al*., 2000
	Slovak Republic	Sturhan and Liskova, 1999
	Spain	Garcia del Pino and Palomo, 1996a
	Switzerland	Kramer *et al*., 2000; Steiner, 1996
	UK	Hominick *et al*., 1995; Gwynn and Richardson, 1996
S. carpocapsae (Weiser, 1955) Wouts, Mracek, Gerdin and Bedding, 1982	Czechoslovakia	Type locality
	Austria	Ehlers *et al*., 1991
	Azores	Grenier *et al*., 1996; Rosa *et al*., 2000
	Czech Republic	Poinar, 1990
	France	Poinar, 1990; Grenier *et al*., 1996; Fischer-Le Saux *et al*., 1998
	Georgia	Fischer-Le Saux *et al*., 1998
	Germany	Ehlers *et al*.,1991; Sturhan, 1999
	Italy	Ehlers *et al*., 1991
	Poland	Poinar, 1990
	Russia, Leningrad	Poinar, 1990; Fischer-Le Saux *et al*., 1998
	Slovak Republic	Sturhan and Liskova, 1999
	Spain	Garcia del Pino and Palomo, 1996a
	Sweden	Poinar, 1990
	Switzerland	Kramer *et al*., 2000
	UK, England	Georgis and Hague, 1981
	Canada, Prov Quebec	Poinar, 1990
	Mexico	Poinar, 1990
	USA: California	Poinar, 1990; Stock *et al*., 1999
	USA: Florida	Parkman and Smart, 1996
	USA: Georgia	Poinar, 1990; Grenier *et al*., 1996
	USA: Massachusetts	Poinar, 1990
	USA: New Jersey	Gaugler *et al*., 1992; Stuart and Gaugler, 1994; Campbell *et al*., 1998
	USA: North Carolina	Poinar, 1990
	USA: Tennessee	Rueda *et al*., 1993
	USA: Virginia	Poinar, 1990; Grenier *et al*., 1996
	Argentina	Poinar, 1990; Stock, 1995; Doucet and De Doucet, 1997
	Brazil	A.P. Reid, Egham, 2000, personal communication
	China: Beijing	Han, 1994
	Rep of Korea	Choo *et al*., 1995
	Taiwan	Hsiao and All, 1998
	Australia: New South Wales, Tasmania	Poinar, 1990
S. arenarium (Artyukhovsky, 1967) Wouts, Mracek, Gerdin and Bedding, 1982	Usmanski Forest, Voronezh Region, Russia	Type locality
	Central Russia	Artyukhovsky *et al*., 1997
	Italy (Southern)	Tarasco and Triggiani, 1997

Table 6.3. *Continued*.

Species in chronological order	Locality	Reference
	Russia	Poinar, 1990; Fischer-Le Saux *et al*., 1998
	Spain	Garcia del Pino and Palomo, 1995
S. intermedium (Poinar, 1985) Mamiya, 1988	Charleston, South Carolina, USA	Type locality
	Czech Republic	Mracek *et al*., 1999; Mracek and Becvar, 2000; Sturhan and Mracek, 2000
	Germany	Sturhan, 1999; Sturhan and Mracek, 2000
	Slovak Republic	Sturhan and Liskova, 1999
	Switzerland	Steiner, 1996; Kramer *et al*., 2000
	USA: South Carolina	Fischer-Le Saux *et al*., 1998
S. rarum (de Doucet, 1986) Mamiya, 1988	Rio Cuarto, Prov of Cordoba, Argentina	Type locality
	Argentina	Doucet and De Doucet, 1997; Fischer-Le Saux *et al*., 1998
S. kushidai Mamiya, 1988	Shizuoka, Japan	Type locality
	Japan	Yoshida *et al*., 1998
S. ritteri de Doucet and Doucet, 1990	Rio Cuarto, Prov Cordoba, Argentina	Type locality
	Argentina	Doucet and De Doucet, 1997
S. scapterisci Nguyen and Smart, 1990	Rivera, Uruguay	Type locality
	USA: Florida (introduced)	Parkman and Smart, 1996
	Argentina	Stock, 1992, 1995; Doucet and De Doucet, 1997
	Uruguay	Fischer-Le Saux *et al*., 1998
S. caudatum Xu, Wang and Li, 1991	Guangdong Prov, China	Type locality
	China: Guangdong	Han, 1994
S. longicaudum Shen and Wang, 1992	Shandong Prov, China	Type locality
	USA: California	Stock *et al*., 1999
	Shandong Prov, China	Han, 1994
	Australia	R.Bedding, Malente, 1995, personal communication
S. neocurtillae Nguyen and Smart, 1992	La Crosse, Alachua County, Florida, USA	Type locality
S. cubanum Mracek, Hernandez and Boemare, 1994	Citrus plantations, Cuba	Type locality
	Cuba	Fischer-Le Saux *et al*., 1998
S. puertoricense Román and Figueroa, 1994	Loiza, Puerto Rico	Type locality

Table 6.3. *Continued*.

Species in chronological order	Locality	Reference
	Puerto Rico	Fischer-Le Saux *et al*., 1998
S. riobrave Cabanillas, Poinar and Raulston, 1994	Lower Rio Grande Valley, Texas, USA	Type locality
	USA, Texas	Fischer-Le Saux *et al*., 1998
S. bicornutum Tallosi, Peters and Ehlers, 1995	Strazilovo, Vojvodina, Serbia	Type locality
	Czech Republic	Mracek *et al*., 1999; Mracek and Becvar, 2000
	Denmark	A.P. Reid, Egham, 2000, personal communication
	Germany	Sturhan, 1999
	Slovak Republic	Sturhan and Liskova, 1999
	Switzerland	Kramer *et al*., 2000
	Jamaica	Fischer-Le Saux *et al*., 1998
	Canary Islands	Garcia del Pino and Palomo, 1996b
S. oregonense Liu and Berry, 1996	Grant's Pass, Oregon, USA	Type locality
	USA: California	Stock *et al*., 1999
S. abbasi Elawad, Ahmad and Reid, 1997	Lat 16°N, Long 54°E, Sultanate of Oman	Type locality
S. ceratophorum Jian, Reid and Hunt, 1997	Liaoning Prov, NE China	Type locality
S. karii Waturu, Hunt and Reid, 1997	Central Province, Kenya	Type locality
	Kenya	Waturu, 1998
S. monticolum Stock, Choo and Kaya, 1997	Gyeongnam Province, Rep of Korea	Type locality
	Korea	Fischer-Le Saux *et al*., 1998
S. siamkayai Stock, Somsook and Reid, 1998	Lohmsak District, Petchabun Prov, Thailand	Type locality
S. tami Van Luc, Khuong, Reid and Spiridonov, 2000	Cat Tien National Park, Vietnam	Type locality
Neosteinernema longicurvicauda Nguyen and Smart, 1994	Palm Beach County, Florida, USA	Type locality

the Irish Group. *Heterorhabditis bacteriophora* seems to occur in warmer regions such as the Mediterranean and Central Europe while the other two are found in more temperate zones. Griffin *et al.* (1999) recently analysed the status of this interesting complex of species. The Irish Group of *H. megidis* is being described as a new species.

Table 6.4. Recognized species of Heterorhabditidae with geographical records.

Species in chronological order	Locality	Reference
Heterorhabditis bacteriophora Poinar, 1976	Brecon, South Australia	Type locality
	Azores	Grenier *et al.*, 1996, 1998; Rosa *et al.*, 2000
	France	Grenier *et al.*, 1996, 1998
	Germany	Smits and Ehlers, 1991; Smits *et al.*, 1991; Glare *et al.*, 1993; Sturhan, 1999
	Hungary	Mracek and Jenser, 1988; Griffin *et al.*, 1999
	Italy	Akhurst, 1987; Poinar, 1990; Smits and Ehlers, 1991; Smits *et al.*, 1991; Tarasco and Triggiani, 1997
	Moldavia	Smits and Ehlers, 1991; Smits *et al.*, 1991
	Poland (introduced)	Jaworska and Dudek, 1992
	Spain	Smits and Ehlers, 1991; Smits *et al.*, 1991; De Doucet and Gabarra, 1994; Garcia del Pino and Palomo, 1996a, 1997
	Switzerland	Kramer *et al.*, 2000
	USA: California	Poinar, 1990; Smits and Ehlers, 1991; Smits *et al.*, 1991; Fischer-Le Saux *et al.*, 1998; Stock *et al.*, 1999
	USA: Florida, Georgia, Kentucky, South Carolina, Texas	Poinar, 1990
	USA: New Jersey	Gaugler *et al.*, 1992; Stuart and Gaugler, 1994; Campbell *et al.*, 1998
	USA: North Carolina	Poinar, 1990; Smits and Ehlers, 1991; Smits *et al.*, 1991
	USA: Ohio	Fischer-Le Saux *et al.*, 1998
	USA: Tennessee	Rueda *et al.*, 1993
	USA: Utah	Poinar, 1990; Smits and Ehlers, 1991; Smits *et al.*, 1991; Grenier *et al.*, 1996; Stack *et al.*, 2000
	Dominican Republic	Grenier *et al.*, 1996, 1998; Fischer-Le Saux *et al.*, 1998
	Guadeloupe	Grenier *et al.*, 1996, 1998; Constant *et al.*, 1998; Fischer-Le Saux *et al.*, 1998
	Puerto Rico	Fischer-Le Saux *et al.*, 1998
	Trinidad	Grenier *et al.*, 1996, 1998; Fischer-Le Saux *et al.*, 1998
	Argentina	Poinar, 1990; Stock, 1995; De Doucet *et al.*, 1996, 2000; Grenier *et al.*, 1996; Doucet and De Doucet, 1997
	Brazil	Poinar, 1990
	Colombia	Caicedo and Bellotti, 1996
	China: Guangdong, Shandong	Akhurst, 1987; Poinar, 1990; Han, 1994
	Israel	Glazer *et al.*, 1993
	Rep of Korea	Choo *et al.*, 1995
	Kenya	Waturu, 1998
	South Africa	Grenier *et al.*, 1996, 1998
	Australia	Akhurst, 1987; Poinar, 1990; Smits and Ehlers, 1991; Smits *et al.*, 1991; Fischer-Le Saux *et al.*, 1998; Grenier *et al.*, 1996
	New Zealand	Smits and Ehlers, 1991; Smits *et al.*, 1991; Grenier *et al.*, 1996, 1998

Table 6.4. *Continued.*

Species in chronological order	Locality	Reference
H. megidis Poinar, Jackson and Klein, 1987	Jeromesville, Ohio, USA	Type locality
	Czech Republic	Mracek *et al.*, 1999
	Greece	Menti *et al.*, 1997
	Switzerland	Kramer *et al.*, 2000
	Canada: British Columbia	Mracek and Webster, 1993
	USA: Ohio	Fischer-Le Saux *et al.*, 1998
	Israel	Glazer *et al.*, 1993
	Japan	Yoshida *et al.*, 1998
NW European group	Belgium	Miduturi *et al.*, 1996b, 1997
NW European group	Denmark	Griffin *et al.*, 1999
NW European group	Estonia	Griffin *et al.*, 1999
NW European group	Germany	Smits and Ehlers, 1991; Smits *et al.*, 1991; Sturhan, 1999
NW European group	Netherlands	Smits and Ehlers, 1991; Smits *et al.*, 1991; Hominick *et al.*, 1995
NW European group	Norway	A.P. Reid, Egham, 2000, personal communication
NW European group	Poland	Smits and Ehlers, 1991; Smits *et al.*, 1991
NW European group	Russia	Fischer-Le Saux *et al.*, 1998
NW European group	Switzerland	Steiner, 1996
NW European group	UK (England)	Hominick *et al.*, 1995
NW European group	USA: Ohio	Smits and Ehlers, 1991
Irish group	Denmark	Griffin *et al.*, 1999
Irish group	Hungary	Griffin *et al.*, 1999
Irish group	Rep. of Ireland	Smits and Ehlers, 1991; Smits *et al.*, 1991; Griffin *et al.*, 1994
Irish group	UK (England)	Hominick *et al.*, 1995
Irish group	UK (Scotland and Wales)	Griffin *et al.*, 1994
H. zealandica Poinar, 1990	Near Auckland, New Zealand	Type locality
	Lithuania	Poinar, 1990
	Russia	Poinar, 1990
	Australia: Tasmania, Queensland	Poinar, 1990
	New Zealand	Akhurst, 1987; Poinar, 1990; Grenier *et al.*, 1996; Barker and Barker, 1998; Fischer-Le Saux *et al.*, 1998
H. indica Poinar, Karunakar and David, 1992	Coimbatore, Tamil Nadu, India	Type locality
	USA: Florida	Stack *et al.*, 2000
	Cuba	Grenier *et al.*, 1996, 1998; Fischer-Le Saux *et al.*, Stack *et al.*, 2000
	Dominican Republic	Grenier *et al.*, 1996, 1998; Fischer-Le Saux *et al.*, 1998
	Guadeloupe	Constant *et al.*, 1998; Fischer-Le Saux *et al.*, 1998
	Jamaica	Fischer-Le Saux *et al.*, 1998; Stack *et al.*, 2000
	Martinique	Fischer-Le Saux *et al.*, 1998
	Puerto Rico	Fischer-Le Saux *et al.*, 1998
	Trinidad	A.P. Reid, Egham, 2000, personal communication
	Virgin Islands	Stack *et al.*, 2000
	Venezuela	Rosales and Suarez, 1998

Table 6.4. *Continued*.

Species in chronological order	Locality	Reference
	India: Coimbatore	Grenier *et al*., 1996; Stack *et al*., 2000
	India: Tamil Nadu	Josephrajkumar and Sivakumar, 1997
	Indonesia	Griffin *et al*., 2000; Stack *et al*., 2000
	Israel	Fischer-Le Saux *et al*., 1998
	Japan	Yoshida *et al*., 1998
	Malaysia (Peninsular)	Mason *et al*., 1996
	Pakistan: Sindh & Balochistan	Shahina *et al*., 1998; Anis *et al*., 2000
	Palestine (Bethlehem)	Sansour and Iraki, 2000
	Sri Lanka	A.P. Reid, Egham, 2000, personal communication
	Egypt	Grenier *et al*., 1996, 1998; Stack *et al*., 2000
	Kenya	Waturu, 1998; Stack *et al*., 2000
	Australia	Akhurst, 1987; Fischer-Le Saux *et al*., 1998; Stack *et al*., 2000
H. argentinensis Stock,1993	Rafaela, Santa Fe Prov, Argentina	Type locality
	Argentina	Stock, 1995; Doucet and De Doucet, 1997
H. brevicaudis Liu, 1994	Fujian Prov, China	Type locality
	China: Fujian	Han, 1994
H. hawaiiensis Gardner, Stock and Kaya, 1994	Kauai, Hawaii, USA	Type locality
H. marelatus Liu and Berry, 1996	Seaside, Oregon, USA	Type locality
	USA: California	Stock *et al*., 1999
	USA: Oregon	Stack *et al*., 2000
H. poinari Kakulia and Mikaia, 1997 (sp. *inquirenda*)	Eastern Georgia	Type locality

Since differences related to taxonomic status have been acknowledged, the groups are listed separately in Table 6.4 whenever authors distinguished them.

It is striking that many more steinernematids than heterorhabditids have been described and many more are awaiting formal description. The discrepancy in species numbers in the two genera may simply be an artifact, reflecting the practical difficulty of distinguishing species in the morphologically conservative heterorhabditids. I believe that it is a real phenomenon and that the biodiversity of steinernematids is greater than that of heterorhabditids. Downes and Griffin (1996) speculated that genetic variation within populations of *Heterorhabditis* would be extremely low due to hermaphroditism. They therefore suggested that the nematodes might be clonal, or nearly clonal, organisms. A relatively limited dispersal combined with low genetic variability would result in populations that are highly adapted to local environmental

conditions. The existence of groups within the *H. megidis* taxon and the apparent similarities of described taxa leading to synonymies would support the hypothesis. Additional support comes from recent work by Stack *et al.* (2000), which suggests that gene flow within the species *H. indica* may be restricted. They also noted that reproductive incompatibility exists between isolates of *H. bacteriophora*. In any case, on a global scale, many more steinernematid than heterorhabditid species exist and these may show a greater biological diversity with attendant diverse characters useful for particular biological control programmes (Hominick *et al.*, 1996). For heterorhabditids, diversity seems to exist at the sub-specific level, with populations selected and adapted to local conditions.

As more surveys are performed and identifications become more reliable, the known range of species continues to expand. Germany has the highest recorded number of indigenous entomopathogenic nematode species, with 13 distinguished so far, five of them being undescribed steinernematids. Extensive sampling and use of direct extraction methods at least partly explains how such biodiversity is discovered (Sturhan, 1999). There is no reason to suppose that Germany is unique in having such a diverse entomopathogenic nematode community.

For all organisms, even amongst well known species, discoveries in unusual places are constantly modifying known distribution patterns, thereby demanding changes in the explanations that biologists give for these patterns (Cox and Moore, 2000). Some of the most notable discoveries listed in Tables 6.3 and 6.4 that lead to an increased understanding of the biogeography of entomopathogenic nematodes or allow us to speculate, are highlighted in the following points:

- Though *S. feltiae* is common in many parts of the world, it was unknown in the continental USA until Poinar (1992) discovered a strain in fungus gnats in California. Since then, it has been recovered in New Jersey and Florida as well.
- *Steinernema kraussei* was first isolated in Germany, and then was reported from a number of European countries. Hence, it would be considered to have a Palearctic distribution. However, its recent discovery from several sites in North America suggests a Holarctic distribution (Stock *et al.*, 2000).
- *Steinernema feltiae* and *S. carpocapsae* can be said to have a wide distribution in temperate regions. This may be related to their wide host range, documented by Peters (1996), allowing them to pursue a generalist life strategy.
- *Steinernema carpocapsae* is rare in central and northern European localities. Originally described as indigenous to the Czech Republic, it was not recovered by Mracek and Becvar (2000) even though they sampled intensively in suitable habitats, including the type locality. Sturhan and Liskova (1999), however, did recover *S. carpocapsae* in 2 of 40 samples with entomopathogenic nematodes in the neighbouring Slovak Republic. The species seems to favour more temperate regions.
- *Steinernema affine* and *S. feltiae* are the most commonly encountered species in Europe (Sturhan and Liskova, 1999).
- *Steinernema affine* naturally infects dipterans (Peters, 1996) and seems to be common but Palaearctic in distribution, while *S. glaseri* is less common, appears to be restricted to scarabaeid larvae for hosts (Peters, 1996) and is geographically widespread.

- *Steinernema biocornutum* was initially isolated in Serbia and recent surveys report that it exists in neighbouring countries. Its discovery in the tropical climates of Jamaica and the Canary Islands is unexpected and needs to be confirmed and explained.
- *S. intermedium* was known only from the USA but has now been recorded from several European countries. A study of morphological variability amongst these geographically distinct populations, such as that of Stock *et al.* (2000) for *S. kraussei*, would be informative from a taxonomic perspective.
- Geographically, the most widespread heterorhabditid is *H. bacteriophora*, which occurs in regions with continental and Mediterranean climates, while *H. indica* is widespread in the tropics and sub-tropics (Burnell and Stock, 2000; Stack *et al.*, 2000). So far, *H. megidis* has been discovered only in the northern hemisphere, where it typically has a more northerly and more restricted distribution than *H. bacteriophora* (Stack *et al.*, 2000).
- Griffin *et al.* (1999) provided evidence of a geographic separation in Europe between the *H. megidis* complex of nematodes (the putative new Irish species and NWE type) and the *H. bacteriophora* group. However, they noted that the occurrence of the Irish type in Hungary and of *H. megidis* in Greece clearly indicates that a strict division between a northern '*megidis*' group and a southern *H. bacteriophora* group is too simplistic.
- The early literature alleged that steinernematids were generally temperate or cold-adapted species while heterorhabditids were more tropical. However, the data do not support such a generalization. Adaptation to climate is more a species than a generic character. While most steinernematids have been described from temperate regions, this probably reflects the distribution of entomopathogenic nematode specialists rather than the nematodes. Many tropical steinernematids exist but are undescribed (Hominick *et al.*, 2000).

Species such as *S. feltiae, S. carpocapsae, H. bacteriophora* and *H. indica* are essentially ubiquitous. This implies that dispersal for at least some species is highly efficient and probably occurs by a variety of means, including active methods by hosts and passive ones such as by wind, water and human activity. Soil has been moved around the globe as a by-product of trade, thus inadvertently spreading soil nematodes. One of the best known examples concerns the potato cyst nematode, which co-evolved with potatoes in the Andes and was spread to Europe in the 16th century when the Spanish Conquistadors returned with the curious new plants. It is highly likely that entomopathogenic nematodes have also been dispersed in soil associated with planting material. For example, Rosa *et al.* (2000) noted that entomopathogenic nematodes were found only on the eastern and central groups of the Azores, and that most of the plant and animal species found on the archipelago were introduced from Europe and Africa.

Use of entomopathogenic nematodes in biological control programmes is another human activity that complicates interpretation of natural dispersal. Introductions could occur in areas where the nematodes do not occur naturally, but subsequently become established. In New Jersey, *S. glaseri* was widely released to control the Japanese beetle, but assessment 50 years later showed that colonization was largely unsuccessful and restricted to southern parts of the state, perhaps near the

lower temperature limits for the species (Gaugler *et al.*, 1992). On the other hand, *S. scapterisci* from Uruguay was released into Florida for control of the mole cricket, and became established to exert significant control (Parkman and Smart, 1996). We must assume that dispersal events occur continually, and that a proportion of these events will result in successful establishment of 'exotic' species or strains (Downes and Griffin, 1996).

When Poinar and Kozodoi (1988) pondered whether *S. glaseri* and *S. anomali* (= *S. arenarium*) were sibling species, they noted that *S. glaseri* had not been isolated in Europe. They therefore presumed that it is a New World species that had evolved in South America and entered eastern North America when the two Americas were connected. The final link between North and South America did not form until the late Pliocene, about 3 million years ago (Cox and Moore, 2000). Poinar and Kozodoi (1988) concluded that the two species evolved as a result of parallelism after the parent stock split long ago in the mid-Jurassic. However, *S. glaseri* has now been isolated from Spain, the Azores, China and the Republic of Korea as well as North and South America. It can now be argued that both *S. glaseri* and *S. arenarium* arose while Europe, Asia and North America were part of one land mass during the late Cretaceous, about 90 million years ago. By the Eocene, about 40 million years ago, North America had separated (Cox and Moore, 2000). Hence, the presence of *S. glaseri* in South America would be a recent event in geological time, a conclusion opposite to the one proposed by Poinar and Kozodoi (1988). The two species probably existed before the separation of North America from Europe and hence it may be that *S. arenarium* will eventually be found in the New World. Both species occur in Spain, so they presumably can coexist.

When Tables 6.3 and 6.4 are compared with the table in Hominick *et al.* (1996), it is clear that our biogeographic knowledge of entomopathogenic nematodes is increasing rapidly. Nevertheless, the comparatively few studies and small sample sizes, combined with limited geographical coverage of the surveys, mean that huge sampling efforts are required before distribution ranges for individual species are determined.

6.4.4. Habitat preferences

The literature on habitat preference for entomopathogenic nematodes before 1995 is contradictory. Most surveys have insufficient data to test for correlations. As more surveys occur, providing large sample sizes, and accurate identification is made to the species level, some habitat preferences are becoming apparent (Hominick *et al.*, 1996; Sturhan, 1999). This should not be surprising as all organisms have specific niche requirements that will be satisfied only in particular habitats. Also, the soil habitat has three dimensions, so preferences may extend to occurrence at particular depths in the soil. For example, Campbell *et al.* (1998) found that *S. carpocapsae* was recovered primarily near the soil surface in turfgrass and that recovery varied during the day, while *H. bacteriophora* was recovered uniformly throughout the upper 8 cm of soil.

Habitat preferences are likely to reflect the distribution of suitable hosts. There is now general support for the opinion that entomopathogenic nematodes are more restricted in their host range than laboratory infections suggest (Peters, 1996). Differences in host preference could help to explain coexistence of species (Sturhan, 1999). In addition, each species will be physiologically and behaviourally adapted

for survival only in particular habitats. Thus, *H. indica* occurred mainly in calcareous sands in Guadeloupe while *H. bacteriophora* was found in more acidic conditions (Constant *et al.*, 1998). Other results suggest that *H. indica* is not restricted by vegetation type (Griffin *et al.*, 2000). The association with sandy coastal soils remains the most robust correlation between habitat and presence of heterorhabditids. However, *H. bacteriophora* depends less on coastal regions than either *H. indica* or the *H. megidis* complex and is widely distributed in turf and weedy habitats (Stuart and Gaugler, 1994; Stock *et al.*, 1996). In the Azores, *H. bacteriophora* was found at low altitudes and displayed no habitat preference as it was recorded from cropland, woodland, pasture, orchard and native vegetation (Rosa *et al.*, 2000).

The extensive and intensive surveys in Germany have revealed habitat preferences for several steinernematids (Sturhan, 1999). *Steinernema affine* prevails in cultivated fields and grassland while *Species B, S. intermedium* and *S. kraussei* are mainly forest species. *Steinernema feltiae* prefers fields and grassland but also occurs in woodlands. *Steinernema affine* and *S. feltiae* were virtually the only species found in arable soil. Hominick *et al.* (1995) combined results from surveys of the UK and the Netherlands and also found *S. feltiae* and *S. affine* mainly in fields and verges. In the Slovak Republic, Sturhan and Liskova (1999) found *S. affine* principally in arable soils and *S. intermedium* in forest biotopes while *S. feltiae* showed no distinct preferences. Spiridonov and Moens (1999) found *S. kraussei* mainly in woodlands in Belgium, especially under coniferous trees, while *S. feltiae* was present inside woodlands and on the edges. Sampling natural habitats in California, Stock *et al.* (1999) found that coniferous forests harboured the largest biodiversity of entomopathogenic nematodes while oak woodlands provided maximum prevalence. The most abundant and widely distributed entomopathogenic nematode species was *S. kraussei*, which existed mainly as a forest species. They also recorded *S. feltiae* from grassland and *S. carpocapsae* from woodlands, while noting that it had been reported from an apple orchard and golf courses in California. As a broad generalization, prevalence of steinernematids seems to be highest in woodlands, which support the largest steinernematid biodiversity (Stock *et al.*, 1999; Sturhan, 1999; Sturhan and Liskova, 1999). Soil type is essential for the existence of some *Heterorhabditis* species but is apparently of less importance for steinernematids (Sturhan, 1999).

6.5. Legislation Affecting Surveys

Biologists wishing to collect and characterize organisms must be aware that many rules and regulations govern their actions (Hominick *et al.*, 1996; Smith, 2000). The principles are embodied in the Convention on Biological Diversity (CBD), ratified by more than 140 countries. The objectives of the Convention are the conservation of biodiversity, the sustainable use of its components and the equitable sharing of benefits arising from the utilization of genetic resources. The CBD gives sovereign rights over genetic resources to the country of origin. Smith (2000) has elaborated the topic and draws attention to legislation and requirements relevant to collecting, handling and exchanging biological organisms and to aspects of intellectual property rights relating to their use. He provides a suggested code of conduct for collecting biodiversity. No matter where a collection is made, it must be done legally, following national and international law. The essential elements are to obtain prior informed

consent from the country to be surveyed and to maintain good documentation to clarify the status of not only material collected but also organisms provided to or received from other collections. This assumes that an appropriate authority to provide consent exists, which may not always be the case. The CBD has been signed into law, but in many countries the funds and official mechanisms to implement and regulate the articles do not exist. Nevertheless, if organisms are to be exploited, then the country of origin must be taken into account.

The Convention has an important role to play in ensuring continuing, global access to biological diversity for control of alien pests. In Article 15.2, Parties to the Convention are asked to 'Endeavour to create conditions to facilitate access to genetic resources for environmentally sound uses by other Contracting Parties.' Entomopathogenic nematodes are genetic resources and the Convention stipulates that collection should be done by mutual agreement between Parties. Also, the Party providing them should do so in a context of prior informed consent. In practice, this means close cooperation between countries providing and receiving biological control agents, including entomopathogenic nematodes. If agreements are in place before surveys occur, including permission to collect, the process of compliance with the law is made much simpler.

As entomopathogenic nematodes are ubiquitous but largely unstudied in most parts of the globe, the initial strategy for insect control programmes should be to prioritize the search for, and utilization of, indigenous isolates. This will not only minimize environmental, regulatory and CBD concerns, but also contribute to our understanding of the biogeography of these remarkable organisms.

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7 Physiology and Biochemistry

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7.1. Introduction

The aim of this chapter is to supplement knowledge of the physiology and biochemistry of entomopathogenic nematodes with information on other nematodes, particularly *Caenorhabditis elegans*. Two areas of research are of particular importance.

First, advances in *in vitro* production on solid and liquid media have enabled entomopathogenic species to be mass-produced. Attention has focused on improving yields and nematode quality but comparisons with *in vivo* production suggest that further progress is possible through knowledge of nematode developmental processes, nutritional requirements and environmental physiology.

Second, the efficacy of entomopathogenic nematodes depends on various abiotic and biotic factors and the selection of the best pre-adapted isolate(s) is important. The physiological and biochemical attributes that increase the efficacy of entomopathogenic nematodes are critical to this selection process.

7.2. Reproduction

The specialized third stage 'dauer' juvenile of *Steinernema* and *Heterorhabditis* spp. is the infective stage. Once in the host, symbiotic bacteria are released from the nematode and feeding begins inside the host haemocoel.

7.2.1. Amphimixis and hermaphroditism

Infective juveniles of *Steinernema* spp. develop into amphimictic females or males whilst *Heterorhabditis* develop into self-fertilizing hermaphrodites with a female phenotype. In both genera, the second generation consists of amphimictic females and males, which mate to provide the third generation, or infective juveniles.

The *Heterorhabditis* hermaphrodite deposits some eggs externally. However, most second generation *Heterorhabditis* hatch and develop inside the hermaphrodite, on which they feed, resulting in the death of the adult and exit of the infective juveniles from the carcass, a phenomenon known as 'endotokia matricida' (Johnigk and Ehlers, 1999). This also occurs in second generation amphimictic females of *Heterorhabditis*. Initially, *Steinernema* females lay some eggs but later endotokia matricida occurs.

The host insect dies within 48 h of infection but symbiotic bacteria allow the nematodes to continue to multiply. Hui and Webster (2000) considered that production of the antibiotic, 3,5-dihydroxy-4-isopropylstilbene, by the symbiont *Photorhabdus luminescens*, minimized competition from other microorganisms and prevented putrefaction of cadavers of *Galleria mellonella* infected with *Heterorhabditis megidis*.

7.2.2. Evolutionary and ecological aspects

Although *Steinernema* and *Heterorhabditis* spp. share the same life cycle strategy, they do not share a common ancestry. Blaxter *et al.* (1998) consider the Rhabditida to be a paraphyletic group, with *Steinernema* and *Heterorhabditis* belonging to different clades (the latter more closely related to *C. elegans*). The independent evolution of the two groups is supported by them having different symbionts, *Photorhabdus* for *Heterorhabditis* and *Xenorhabdus* for *Steinernema*.

The genus *Xenorhabdus* contains five species, yet *Photorhabdus* has only one described species, although biochemical and molecular characterization indicates that

P. luminescens comprises many strains (Han and Ehlers, 1999). This difference in the species richness of symbionts may correlate with nematode diversity, there being more species of *Steinernema* than *Heterorhabditis*. Individual *Heterorhabditis* species have a global distribution and this may be related to their hermaphroditic mode of reproduction in the first generation, infection by a single juvenile being sufficient to establish a population (Hominick *et al.*, 1996). This has the concomitant effect of reducing genetic mixing.

The requirement for rapid reproduction in an infected host insect, typical of *r*-selected life history strategies, appears to be satisfied by hermaphroditism in *Heterorhabditis*, yet neither this method nor parthenogenesis is used by *Steinernema*. The advantages of using a non-amphimictic method of reproduction may thus be slight. The survival strategies of entomopathogenic nematodes are discussed by Glazer (Chapter 8, this volume). One strategy is worth comment here. Endotokia matricida is an effective method of ensuring development during adverse environmental conditions. It enables the formation of infective juveniles at a time when external food supply is reducing and results in juveniles with sufficient energy reserves for prolonged survival (Johnigk and Ehlers, 1999).

7.3. Developmental Biology

Post-embryonic and embryonic cell lineages of *C. elegans* are described by Sulston and Horvitz (1977) and Sulston *et al.* (1983), respectively. Little is known of the developmental biology of other nematodes.

7.3.1. Embryonic

Information on embryonic development in entomopathogenic nematodes is lacking. In *C. elegans*, embryogenesis is largely determinate, with an essentially invariant lineage pattern. The first cleavage is asymmetric and early divisions produce six 'founder cells' (blastomeres). The descendants of three of these cells are each of essentially one type; descendants of the others are of mixed cell types.

Nuclear receptors play an essential role in metazoan development and *C. elegans* has > 200 putative nuclear receptor genes (transcription factors) (Asahina *et al.*, 2000). Several proteins have been identified that are required for cell specification in *C. elegans* (Schnabel and Priess, 1997) but little is known about the molecular bases of most cell–cell interactions during embryogenesis (Leroi and Jones, 1998). Jones and Candido (2000) have demonstrated that the ubiquitin-like protein NED–8 conjugating system is required for normal embryonic and postembryonic development in *C. elegans*.

Comparative studies suggest that considerable variations in embryogenesis can exist in some nematodes (Leroi and Jones, 1998; Schierenberg, 2000). In the Enoplida, early cleavages are symmetrical with variably positioned blastomeres until the eight-cell stage, and bilateral symmetry appears later than in other nematodes (Malakhov, 1998). The rhabditid *Acrobeloides nanus* expresses a regulative potential for somatic cell specification; cell specification is thought to be assigned by specific reciprocal inhibitory cell–cell interactions, absent in *C. elegans* (Wiegner and Schierenberg, 1999).

7.3.2. Post-embryonic

Nematodes were regarded as classic eutelic organisms, lacking post-embryonic cell proliferation, but this is now known to occur in some tissues in the Rhabditida and Enoplida (Leroi and Jones, 1998). With few exceptions nematodes, including entomopathogenic species, have four juvenile stages and moult four times, although variations in post-embryonic development are apparent between species. The molecular biology of post-embryonic development in *C. elegans* is the subject of extensive research. For example, RNAi studies have shown that the putative nuclear receptor gene *nhr-25* involved in embryogenesis has additional roles in moulting and sexual differentiation (Asahina *et al.*, 2000).

Two aspects of post-embryonic development will be considered: (i) the dauer stage, as the infective juvenile of entomopathogenic nematodes is the key stage in their production and application; (ii) sex determination, which is of fundamental importance for strain selection and production.

The formation of a non-feeding, developmentally-arrested, dauer stage is a survival strategy common to many rhabditid species (see Glazer, Chapter 8, this volume). The developmental switch for dauer formation in *C. elegans* is based on an amphidial neuronal response to overcrowding or starvation determined by the ratio, at a given temperature, between a dauer-inducing pheromone (hydroxylated short-chain fatty acid-like compounds), produced by all life stages, and a dauer-inhibiting food signal. The latter also enhances the recovery of the dauer to a normal, fourth stage juvenile. Higher temperatures favour dauer formation. The decision to produce a dauer usually occurs at the first juvenile stage but second-stage juveniles can be induced to form a dauer under extreme conditions. Second-stage juveniles programmed to form a dauer can develop into normal third-stage juveniles if environmental conditions improve (Riddle and Albert, 1997).

In *C. elegans*, an insulin receptor-signalling pathway regulates adult lifespan and dauer formation (Ailion *et al.*, 1999). More than 30 (*daf*) genes are involved in the control of dauer formation, and the proposed genetic pathway has parallel branches with separate groups of cilium structure genes (Fig. 7.1). This may enable integration of signals from two independent sources, allowing a more robust response to environmental conditions (Riddle and Albert, 1997).

Mutations in cilium structure genes result in abnormal endings to sensory cilia. Juveniles with this phenotype are dauer-defective (Daf-d). Two other Daf-d genes, *daf-22* and *daf-6*, appear to work upstream and be involved in pheromone production and access of amphidial neurons to the environment, respectively.

Dauer constitutive (Daf-c) mutations induce dauer stage formation, even in the absence of pheromone. Sequencing of Daf-c genes suggests a role in protein growth factor-mediated intercellular signal transduction; *daf-11* encodes a predicted transmembrane guanyl cyclase thought to be involved directly in signal transduction at an amphidial neuron.

Other Daf-c genes act downstream of chemoreception. *daf-1, daf-4* and *daf-7* are involved in a transforming growth factor-β (TGF-β) signal transduction pathway; *daf-7* is required for transducing environmental cues that support continuous development with plentiful food. Dauer-inducing pheromone inhibits *daf-7* expression in amphidial neurons, promoting dauer formation, the food signal reactivates its

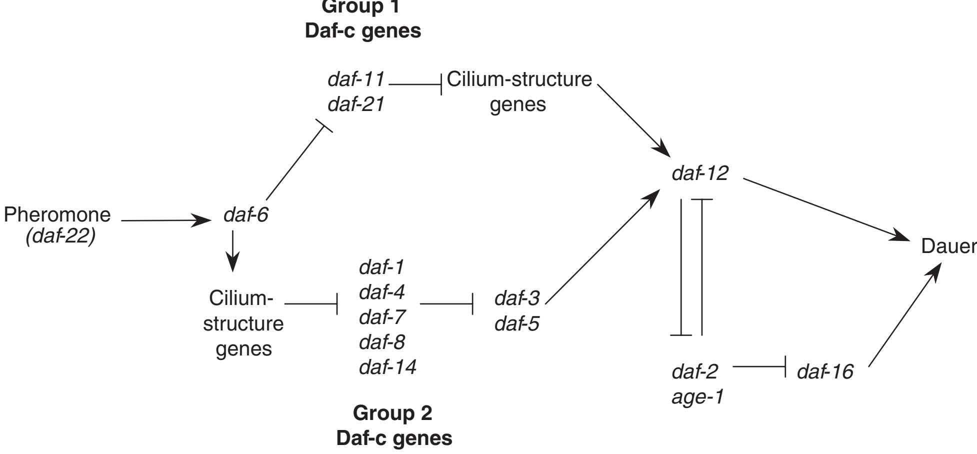

Fig. 7.1. Genetic pathway for dauer formation (after Schackwitz *et al.*, 1996 by M.N. Patel). Arrows indicate positive regulatory interactions; lines ending in bars indicate negative interactions. Daf-constitutive (Daf-c) genes are *daf-11, daf-21, daf-1, daf-4, daf-7, daf-8, daf-14* and *daf-2*. Daf-defective (Daf-d) genes are *daf-22, daf-6, daf-3, daf-5, daf-12* and *daf-16*.

expression and promotes recovery from the dauer stage (Ren *et al.*, 1996). *daf-8* and *daf-14* encode intracellular proteins (Smads) and downstream of these, and other group 2 genes, are *daf-3* and *daf-5*, which are thought to be antagonized by receptor-activated Smads.

The Daf-d gene *daf-12* is located at the convergence of putative genetic pathways that regulate dauer formation, developmental age and adult longevity in *C. elegans* (Antebi *et al.*, 2000). It encodes a nuclear protein with homology to the steroid-thyroid hormone receptor superfamily, which it is proposed integrates hormone signals in cellular targets to coordinate major life history traits.

Dauer formation, aging and neutral lipid accumulation in *C. elegans* are regulated by DAF-2, a homologue of mammalian insulin/insulin-like growth factor I (IGF-I) receptors, one of whose ligands may be a hybrid molecule of insulin and IGF (Kawano *et al.*, 2000). DAF-2 functions via AGE-1, a phosphatidylinositol-3-OH kinase catalytic subunit of the type that is usually activated by tyrosine kinase signalling pathways. At high pheromone levels, *daf-12* is active and *daf-2* is inactive. Downstream from *daf-2* and *age-1*, the Daf-d gene *daf-16* is a member of the Fork head family of transcription factors. The DAF-2/AGE-1 signalling cascade modifies the transcriptional activity of DAF-16.

Tomalak and Mracek (1998) have observed that the morphology of infective juvenile-specific lateral fields in dumpy *Sfdpy-1*, segmented *Sfseg-1*, and double, dumpy-segmented, mutants of *S. feltiae* differed markedly compared with wild-type individuals, suggesting that both genes are involved in cuticle formation in dauer juveniles.

Studies on the infective juvenile (dauer) genetic pathway in entomopathogenic nematodes are now required. Knowledge of the genetic pathways for infective juvenile recovery in entomopathogenic nematodes would be of particular value. Liquid media are inoculated with infective juveniles and major process instability is caused by low

and unsynchronized recovery. *In vivo*, recovery is approximately 95% within 1–2 days whereas in liquid cultures of *Heterorhabditis* recovery is spread over several days and can vary markedly. Ehlers *et al.* (1998) reported that the maximum production of *H. megidis* in monoxenic culture in laboratory-scale bioreactors could vary by more than three-fold and the time taken to reach maximum density by twofold due to variability in infective juvenile recovery (18–90%).

Recovery of infective juveniles is triggered by a bacterial food signal, which is more effective in liquid culture than *in vivo* (Strauch and Ehlers, 1998). Grewal *et al.* (1997) were the first to demonstrate symbiont-specific recovery, using infective juveniles of *S. scapterisci* in monoxenic combinations with its symbiont, *Xenorhabdus* sp. 'S' and with the symbionts of *S. carpocapsae* and *S. riobravis*. Although development occurred on all *Xenorhabdus* spp., infective juvenile recovery was delayed significantly and reduced with the symbionts of *S. carpocapsae* and *S. riobravis*.

Jessen *et al.* (2000) showed that CO_2 enhanced infective juvenile recovery in *Heterorhabditis*. This was not due to increased acidity caused by increasing CO_2 concentrations. In one case a marked recovery occurred in the absence of a food signal, which, together with a variable threshold response of infective juveniles, suggests that they are differently pre-disposed to respond to recovery-inducing signals. If these effects occur in large-scale bioreactors, the control of CO_2 could improve bioreactor process technology (Jessen *et al.*, 2000).

The mechanisms for sex determination in nematodes have been studied only in *C. elegans*. In contrast with some other nematodes, sex determination is entirely genotypic and occurs early in embryogenesis (Leroi and Jones, 1998). The primary mechanism is based on the ratio of sex (X) chromosomes to autosomes. Individuals with a ratio of 1 : 1 develop into self-fertilizing hermaphrodites, those with a ratio of 0.5 : 1 develop as males. Dosage compensation mechanisms ensure that similar levels of expression of X-linked genes are maintained irrespective of the number of sex chromosomes (Parkhurst and Meneely, 1994; Leroi and Jones, 1998). For an individual to survive, the dosage compensation pathway must be expressed very early in embryogenesis. Since this pathway is linked to the pathway for sex determination, the latter must also be expressed early in development. It will be of interest to know whether sex determination among entomopathogenic species is similar to *C. elegans*, or if sex chromosomes and, thus, dosage compensation are absent and expression of genes determining sex can be delayed until post-embryonic development (Leroi and Jones, 1998).

Kahel-Raifer and Glazer (2000) found that injection of individual *H. bacteriophora* into *G. mellonella* gave a similar number of females and hermaphrodites (35–40%) and 20–25% males. Increasing the number injected per insect did not significantly alter these proportions. However, the proportion of hermaphrodites was greater in small host cadavers. When individual hermaphrodites were cultured on NGM agar, temperature (21–30°C) did not affect sex determination. However, rearing on a richer medium reduced the proportion of hermaphrodites, suggesting that the main factor affecting sex differentiation is the nutrition source.

7.4. Musculature and Neurobiology

Information on the musculature and nervous system of entomopathogenic nematodes is lacking. The assumption is that the basic features are the same as those described for other nematodes (see Bird and Bird, 1991; Wright and Perry, 1998). For *C. elegans*,

these systems have been described at the ultrastructural and molecular level in the books edited by Wood *et al.* (1988) and Riddle *et al.* (1997).

Nematode somatic muscles are obliquely striated and comprise the spindle (with contractile sarcomeres), a non-contractile cell body, and the arm(s), which extends from the cell body to the longitudinal nerve cord or to the nerve ring. Before the nerve cord, each arm divides into several 'fingers', which subdivide into multiple fine processes that receive synaptic input from excitatory and inhibitory motor neurons. Nematode specialized muscles are non-striated (single sarcomere).

Somatic muscles are arranged in longitudinal rows in a single layer divided by the epidermal cords into four quadrants. Individual motor neurons synapse with dorsal or ventral muscles, thus restricting body flexures to the dorso-ventral plane only; the more complex innervation of the anterior end permits lateral and dorso-ventral movements of the head. The usual method of locomotion, a sinusoidal wave-form, is produced by alternate contraction of the dorsal and ventral musculature antagonized, in the majority of nematodes, by the turgor pressure of the pseudocoelomic hydrostatic skeleton.

Body waving, nictating and jumping movements of entomopathogenic infective juveniles enhance the chance of host location. Body waving occurs when > 30% of the body is raised off the substrate, typically for a few seconds (Campbell and Gaugler, 1997). Body waving is a feature of all entomopathogenic species examined but nictating is found in only a few species. Nictating nematodes adopt a straight posture while balancing on a bend in their tail; this can be maintained for extended periods. Jumping is an unusual attribute of infective juveniles of some species. When standing on their tails, the jumping response is triggered by a range of cues, including host volatiles. In *S. carpocapsae*, the juvenile quickly bends the anterior half of its body until its head region contacts the ventral side of the body, where it is held by the film of water covering the nematode (Campbell and Kaya, 1999a,b). Its body slides in a posterior direction, causing the bend to become more acute. The forces generated by the stretched cuticle and, presumably, musculature on one side and the compressed cuticle and musculature on the other are sufficient to break the surface tension holding the two body parts together. As the body rapidly straightens, the surface tension holding the nematode is also broken, thus propelling the nematode through the air. It is not known whether body waving, nictating and jumping require adaptations to the musculature similar to those found in the insect-parasitic species, *Mermis nigrescens*, which enable it to perform a unique looping locomotion (Gans and Burr, 1994). Specialized movement also may involve a more complex arrangement of motor neurons than the classical nematode model described below.

The nervous system in nematodes is essentially conservative. In the *C. elegans* hermaphrodite it consists of 302 neurons; the adult male has 381 neurons, the additional ones being associated with secondary sexual structures in the tail region. The neurons have a simple, relatively unbranched morphology and a single gap junction is sufficient for functional coupling between neurons. The majority of nerve processes run longitudinally, as ventral and dorsal nerve cords, or circumferentially, as commissures. The nervous system in *C. elegans* comprises two largely independent parts, the pharyngeal system (20 neurons) and the much larger somatic system.

The circumpharyngeal nerve ring is the main mass of the central nervous system. Neuronal processes from the cephalic sensilla run posteriorly as six nerve bundles and

synapse at the nerve ring. Processes from cells in the tail ganglia connect with the caudal sensilla. In the head region, the first four sets of somatic muscles connect to motor neurons from the nerve ring, the next four connect to motor neurons in both the nerve ring and the ventral nerve cord, and the remaining muscles connect to motor neurons in the ventral or dorsal nerve cords. The majority of the nerve processes leaving the nerve ring form the ventral nerve cord, whose interneuronal processes synapse either with the pre-anal ganglion or with excitatory motor neurons in the nerve cord. The motor neurons are arranged in a linear sequence; some have processes that run under the epidermis as commissures to the dorsal mid-line, where they pass anteriorly or posteriorly forming the dorsal nerve cord. The latter has few interneurons and no cell bodies.

Acetylcholine and γ-amino butyric acid (GABA) are putative nematode excitatory and inhibitory neurotransmitters, respectively (Rand and Nonet, 1997; Wright and Perry, 1998). Other putative neurotransmitters in nematodes include L-glutamate, dopamine, 5-hydroxytryptamine and various neuropeptides. The enzyme responsible for the removal of acetylcholine from the synaptic cleft, acetylcholinesterase, is the target site for organophosphate and carbamate nematicides (Wright, 1981). Different molecular forms of this enzyme have been found in several species of nematodes, including *S. carpocapsae* (Arpagaus *et al.*, 1992; Opperman and Chang, 1992). There is some indication of the adverse effects of nematicides on nictating behaviour and infectivity. Treatment with oxamyl (100 and 500 ppm) initially increased the number of nictating *S. carpocapsae* compared with untreated controls but, after 48 h, nictation declined markedly (Ishibashi and Takii, 1993). Incubation in 50 ppm oxamyl or fenamiphos significantly reduced penetration of *G. mellonella* larvae by *S. carpocapsae* and *S. feltiae* in sand-tube bioassays (Patel and Wright, 1996).

7.5. Sensory Physiology

Perry and Aumann (1998) and Jones (2002) reviewed information on nematode sensilla and sensory responses, including details of sensilla function at the molecular level, derived primarily from work on *C. elegans*. There is extensive information on the behaviour of entomopathogenic nematodes, reviewed in the context of behavioural ecology by Lewis (see Chapter 10, this volume). Understanding behavioural attributes of a species is one of the prerequisites for its efficient use in insect control strategies.

Chemicals that are associated with interactions between organisms are termed semiochemicals, which include allelochemicals and pheromones mediating inter- and intra-specific interactions, respectively. Entomopathogenic nematodes are likely to respond to host semiochemicals and, especially for species that move through the soil environment (‘cruisers’ or ‘cruise foragers’) in search of a host, both volatile and non-volatile chemical cues are likely to be involved. Unoriented searching, or ranging, behaviour comprises random, often linear, movements and occurs in the absence of resource cues, such as food, sex partners or vectors; oriented, or localized, searching behaviour occurs when information on the spatial position of resources is available for an animal (Perry and Aumann, 1998).

Carbon dioxide is a non-specific attractant, or kairomone (eliciting a favourable response by the receiver), that is involved in attraction of entomopathogenic nematodes to hosts (Gaugler *et al.*, 1980). Robinson (1995) found that *S. glaseri* was attracted

to CO_2. The positive movement towards CO_2 is the basis of investigations on the use of entomopathogenic nematodes as a prophylactic soil treatment to coat the surface of plant roots and prevent invasion by plant-endoparasitic nematodes (e.g. Ishibashi and Choi, 1991; Perry *et al.*, 1998). However, the adverse effects on host plant invasion may not be caused by this physical barrier but by ammonia released by the *Xenorhabdus* bacteria (Grewal *et al.*, 1999). The response of insects to CO_2 appears to be modulated by additional cues, and there is evidence of modulatory effects in the response of *C. elegans* to volatiles from bacteria (Grewal and Wright, 1992); synergistic and antagonistic effects of combinations of allelochemicals on entomopathogenic nematodes require investigation.

As with CO_2, temperature is probably a universal cue eliciting nematode responses and may be involved in host location. Using a 3-D assay system with temperature gradient fluctuations that mimicked those occurring naturally, Robinson (1994) found that movement of *S. glaseri* and *H. bacteriophora* was rapid and largely random. He suggested that responses of these nematodes to natural patterns of temperature change may be unimportant compared with the effects of CO_2 and chemicals released by the insect host.

Plant roots have an important influence on nematode migration and host-finding. For example, there was no attraction of entomopathogenic nematodes to the black vine weevil (*Otiorhynchus sulcatus*), and Van Tol and Schepman (1999) considered that *Heterorhabditis* sp. used the plant roots as a 'highway' to locate the insects feeding on the roots. Attraction to roots is complex and mediated by several cues from the soil rhizosphere. Infective juveniles of *S. feltiae* responded positively to surface sterilized and unsterilized larvae of *G. mellonella* and tomato seedling roots but negatively to radish seedling roots; the negative response to radish seedling roots changed following surface sterilization (Hui and Webster, 2000). Hui and Webster (2000) considered that cues from the larval cuticle and seedling roots, such as those associated with their surface microflora, may significantly influence the host finding ability of entomopathogenic nematodes.

It is probable that short distance cues, including insect-specific chemicals, are involved in host location. However, research has not led to the characterization of insect-produced allelochemicals to which infective nematodes respond. The response of entomopathogenic nematodes to some host insect components has been studied. *Steinernema carpocapsae* were strongly attracted to nine insect species from diverse ecological niches and movement towards aqueous surface washes from *G. mellonella* larvae whose heads and anuses were sealed indicated that the chemoattractant(s) may be a component of cuticular secretions (Schmidt and All, 1978). Nematodes were also attracted to insect faeces but tests with individual components and combinations of components of faeces of *G. mellonella* resulted in a variety of responses by *S. carpocapsae*, including positive and negative taxis and, with uric acid, aggregation behaviour (Schmidt and All, 1979). However, these studies have been criticised by Lewis *et al.* (1992), who considered that the target of attraction was too close to the inoculation zone, and the bioassays were too long to differentiate between attraction and arrestment. Ammonia present in *Blatella germanica* faeces was considered by Grewal *et al.* (1993) to initiate avoidance behaviour.

Plasma from three species of host insects was an attractant for *S. carpocapsae*, and differences in the percentage attraction corresponded to host susceptibility, with

plasma from the most susceptible host, *Pieris rapae crucivora*, being the most attractive (Khlibsuwan *et al.*, 1992a). Carbohydrate moieties on the nematode cuticle and/or the amphids may play a role in detection of the allelochemicals (Khlibsuwan *et al.*, 1992b). Purification and characterization of the plasma components of the haemolymph may identify the active agent(s), provide more information on host specificity, and determine how the agent(s) get to the insect surface.

Although semiochemicals ensure that juveniles locate a suitable host, several studies have shown that the majority of free-living juveniles of entomopathogenic nematodes are not infective. One hypothesis that has been advanced to explain this apparent paradox proposes that some part of the nematode–host complex produces an inhibitory cue that results in infective juveniles in the surrounding environment not infecting hosts (Glazer, 1997). Using the *G. mellonella*-*S. feltiae* system, Fairbairn *et al.* (2000) have provided evidence that infected *G. mellonella* release a chemical cue, or allomone (eliciting a negative response by the receiver), into the environment that inhibits subsequent infection. As concomitant infections by more than one nematode species can result in the death of all nematodes in the insect due to incompatibilities between *Xenorhabdus* symbionts, the release of an allomone is an effective method of avoiding such detrimental interspecific competition. Another allomone that has been identified is the nematicidal compound α-terthienyl. Roots of marigolds (*Tagetes* spp.) produce α-terthienyl, which has been used to suppress populations of certain plant-parasitic nematodes; α-terthienyl also adversely affected infectivity of *S. carpocapsae* (Kanagy and Kaya, 1996).

Clearly, information on insect allelochemicals to which entomopathogenic nematodes respond is fragmentary. Comparative studies of chemicals, both volatile and non-volatile, from host and non-host insects are needed to determine if there are host-specific attractants. Similarly, the responses of different species to chemicals from individual species of insect needs to be compared, to determine if certain attractants can be correlated with nematode pathogenicity. Concentration-dependent effects of non-volatile chemicals require evaluation and behavioural assays need to be complemented by other techniques such as electrophysiological analysis of neuronal responses, which has been used with other nematodes (Perry, 2001). In addition, examination of the function of genes associated with sensory behaviour will require more extensive information on nematode physiology and biochemistry than presently is available

7.6. Biosynthesis

With the exception of some lipids and carbohydrates, information on biosynthetic processes in entomopathogenic nematodes is extremely limited.

7.6.1. Amino acids and related compounds

Knowledge of which amino acids cannot be synthesized by nematodes (‘essential’ amino acids) has been based partly on nutritional studies on species that have been grown axenically in fully defined culture media (Vanfleteren, 1980). For example, *C. elegans* and *C. briggsae* require arginine plus nine amino acids that are usually essential in mammals. The requirement for entomopathogenic nematodes to be produced monoxenically has precluded such studies.

Radiolabelled [^{14}C]acetate has been used to investigate directly the *de novo* synthesis of amino acids and some plant-parasitic species have been found to biosynthesize compounds that are essential in mammals (Chitwood, 1998). Little is known about the pathways involved in amino acid biosynthesis in nematodes. A novel cystathionine β-synthase, catalysing the synthesis of cystathionine from homocysteine plus serine or cysteine, has been purified from the rhabditid *Panagrellus redivivus*. This differs markedly from its mammalian equivalent and suggests distinctive differences in sulphur amino acid metabolism in nematodes (Papadopoulos *et al.*, 1996). A gene encoding S-adenosylhomocysteine hydrolase, which synthesizes homocysteine has been identified in *C. elegans* (Prasad *et al.*, 1993).

A number of amino acids and related compounds, such as L-glutamate, GABA, dopamine and 5-hydroxytryptamine, are putative neurotransmitters in nematodes. Evidence for their synthesis in *C. elegans* and other nematodes is reviewed by Rand and Nonet (1997) and Wright and Perry (1998), respectively. The biosynthesis of other compounds related to amino acids is reviewed by Chitwood (1998).

7.6.2. Nucleic acids and proteins

Like most organisms, nematodes can synthesize purine and pyrimidine bases but studies on the biosynthesis of nucleoside monophosphates are lacking. There is also limited evidence for the biosynthesis of cyclic nucleotides from their nucleoside triphosphates (Chitwood, 1998). Studies on DNA and RNA polymerases in nematodes have focused on *C. elegans* (Riddle *et al.*, 1997). Many transcription factors have been identified in *C. elegans* (section 7.3). Anderson and Kimble (1997) have reviewed the translational mechanisms involved in protein synthesis in *C. elegans*.

7.6.3. Carbohydrates

Glycogen is the principal carbohydrate energy store in nematodes (section 7.7). Specific sugars, such as trehalose, and related compounds, such as inositol and glycerol, can be synthesized by nematodes during dehydration or freezing (see Glazer, Chapter 8, this volume). Trehalose also acts as an energy reserve in some nematodes (Behm, 1997) and in many species it is important in hatching (Perry, 2002). The synthesis of these compounds is usually at the expense of glycogen and/or neutral lipid reserves; synthesis of trehalose from the latter appears to involve the glyoxylate cycle (see section 7.7). The enzymes involved in trehalose synthesis from glucose in most organisms, trehalose 6-phosphate synthase and trehalose 6-phosphate phosphatase, have been detected in nematodes (Behm, 1997). In the mycophagous species *Aphelenchus avenae*, the rate-limiting step appears to be the formation of trehalose 6-phosphate (Madin and Crowe, 1975).

Qiu and Bedding (1999) showed that infective juveniles of *S. carpocapsae* synthesized trehalose but not glycerol at low temperatures. The cold-induced increase in trehalose in several other entomopathogenic species suggests that this is a common survival mechanism. The changes in lipid, glycogen and protein levels of nematodes during cold induction and subsequent recovery indicated that trehalose was not synthesized from glycogen, but from lipids and/or proteins. Qiu *et al.* (2000a) found that *S. carpocapsae* exposed to osmotic dehydration synthesized both glycerol and trehalose. Glycogen reserves appear to act as a buffer reservoir for these protectants

during both dehydration and rehydration but their principal biosynthetic pathways during dehydration are more likely to involve lipids and proteins than glycogen.

7.6.4. Lipids

Neutral lipids include triacylglycerols (major energy reserves, see section 7.7), diacylglycerols, free fatty acids, sterol esters of fatty acids, and free sterols. Phospholipids, which provide the major proportion of cellular membranes, are usually derived from a molecule of glycerol, two fatty acids, phosphoric acid and a second alcohol (e.g. choline, ethanolamine or inositol). In freshly-emerged infective juveniles of *Steinernema* species, phosphatidylethanolamine and phosphatidylcholine accounted for about 40 and 30%, respectively, of total phospholipids, phosphatidylserine and phosphatidylinositol accounting collectively for a further 25% (Patel and Wright, 1997c). Other lipids include glycolipids, lipoproteins and proteolipids (Chitwood, 1998). For entomopathogenic nematodes, the quality and quantity of lipids in infective juveniles produced on a commercial scale is paramount since this has a critical influence on nematode viability and infectivity (see section 7.7.2).

In most free-living, plant-parasitic and insect-parasitic nematodes C18 fatty acids predominate but the ratio of saturated to unsaturated fatty acids differs between species. In *P. redivivus* and most plant-parasitic nematodes, unsaturated fatty acids predominate (75–92%). In entomopathogenic nematodes unsaturated fatty acids are more variable, with reported levels of 35–62% (Selvan *et al.*, 1993a,b) and 26–37% (Fodor *et al.*, 1994; Patel and Wright, 1997b), differences that could be due to variations in dietary lipids.

Manipulation of nematode lipid content and quality through their diet has considerable potential for the enhancement of nematode viability and infectivity during storage. Abu Hatab *et al.* (1998) found *S. glaseri* grown in *Popillia japonica* (a natural host) accumulated greater amounts of total lipids, phospholipids and sterols compared with nematodes in *G. mellonella* (a factitious host) or in solid or liquid media. C18 fatty acids predominated irrespective of the production method but in *in vivo*-produced nematodes, oleic (18 : 1) acid was the major fatty acid, whereas in *in vitro* a mixture of oleic (18 : 1) and linoleic (18 : 2) fatty acids predominated. Similarly, Abu Hatab and Gaugler (1999) found that the greatest accumulation of lipids was in *H. bacteriophora* grown in *P. japonica* or solid culture. Fractionation of the polar lipids revealed greater levels of phosphatidylglycerol in nematodes from liquid cultures, whereas, in solid culture the major polar lipid fractions were phosphatidylcholine and phosphatidylethanolamine. The ratio of saturated to unsaturated fatty acids was lower in nematodes produced *in vitro*.

Culture temperature can also influence lipid composition. Abu Hatab and Gaugler (1997) found that *S. riobravis* accumulated greater proportions of saturated fatty acids when grown at high temperature (30°C) and suggested that this could contribute to thermal tolerance. Jagdale and Gordon (1997) compared the fatty acids in *S. carpocapsae, S. riobravis* and *S. feltiae* strains that had been recycled or stored at 5–25°C. In all strains, the unsaturation indices (UI) for total lipids were greater at low temperatures. The UI for phospholipids were also greater in all strains except *S. riobravis*, which had a lower degree of physiological adaptation to cold temperatures compared with other strains. Increased UI was due to an increase in polyunsaturated

fatty acids with a concomitant decline in the proportion of saturated fatty acids, especially palmitic (16 : 0) and/or stearic (18 : 0).

Studies on *C. briggsae, P. redivivus* and *Turbatrix aceti* suggest that, unlike most metazoans, nematodes are capable of *de novo* biosynthesis of fatty acids (Chitwood, 1998). Watts and Browse (2000) have shown that biosynthesis of polyunsaturated fatty acids by *C. elegans* is initiated by the introduction of a double bond at the Delta 9 position of a saturated fatty acid. They identified three fatty acid desaturase genes: FAT-6 and FAT-7 readily desaturated stearic acid (18 : 0) with less activity on palmitic acid (16 : 0), while FAT-B is a novel palmitoyl-CoA-specific membrane desaturase with minimal activity on the common Delta 9 substrate stearic acid. Isotopic experiments have indicated that lipogenesis is not functional in infective juveniles of *S. carpocapsae* (Qiu *et al.*, 2000b).

7.7. Intermediary Metabolism

Neutral lipids are the major energy reserves in nematodes except for parasitic stages in vertebrates, where glycogen generally predominates (Barrett and Wright, 1998). Significant amounts of trehalose (see section 7.6.3) and glucose have also been reported.

7.7.1. Carbohydrate catabolism

Aerobic and anaerobic utilization of glycogen has been demonstrated in all groups of nematodes. In infective juveniles of *S. carpocapsae*, differential rates of decline for glycogen and lipids under aerobic conditions result in glycogen becoming the principal energy store in aged individuals (Wright *et al.*, 1997; Qiu and Bedding, 2000a; Qiu *et al.*, 2000b). Under anaerobic conditions, *S. carpocapsae* are inactive but can survive for several days, utilizing glycogen and trehalose (Qiu and Bedding, 2000b). Glycolytic enzymes have been demonstrated in many nematodes, including entomopathogenic and other rhabditid species (Table 7.1). Iodoacetamide, a glycolytic inhibitor, is toxic to mixed stages of *P. redivivus* (Butterworth *et al.*, 1989), and reversibly reduced infectivity in aged infective juveniles of *S. carpocapsae*, when glycogen was the primary energy store (Patel and Wright, 1997a). Studies on glycolytic and oxidative enzymes in *S. carpocapsae* indicate that it is a typical facultative aerobe (Shih *et al.*, 1996).

Evidence for the pentose pathway (involved in nucleotide synthesis in mammals) in nematodes is limited to the first two enzymes: glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. The latter has been found in *S. carpocapsae* and *S. feltiae* (Barrett and Wright, 1998).

Several tricarboxylic acid (TCA) cycle enzymes have been demonstrated in rhabditids, including *S. carpocapsae* and *S. feltiae.* The distribution of label after incubation with ^{14}C substrates in *C. briggsae, Rhabditis anomola, P. redivivus* and *T. aceti* is consistent with a functional TCA cycle (Barrett and Wright, 1998). In *C. elegans* juveniles committed to development to the adult stage, an increase in TCA cycle activity has been observed (Wadsworth and Riddle, 1989).

The glyoxylate cycle enables acetylCoA from the β-oxidation of fatty acids to be used for gluconeogenesis. Among metazoa it has only been found in nematodes. The cycle enzymes, isocitrate lyase and malate synthase, bypass the decarboxylation steps in the TCA cycle, catalysing the net conversion of isocitrate and acetylCoA to succinate and malate. In *C. elegans*, these enzymes are encoded by a single gene and are expressed

as a single bifunctional polypeptide (Liu *et al.*, 1995). Evidence that the cycle is functional exists for *A. avenae, P. redivivus, T. aceti, R. anomala* and *C. briggsae* (Barrett and Wright, 1998) and for infective juveniles of *S. carpocapsae*, where trehalose and glycogen were found to be resynthesized mainly from lipids (Qiu *et al.*, 2000b; Qiu and Bedding, 2000b). Glyoxylate cycle activity is developmentally regulated. In *C. elegans* activity is greatest in embryos and first-stage juveniles and is localized mainly in the gut and muscles (Liu *et al.*, 1997). The cycle may be important in the conversion of lipid to carbohydrate in anhydrobiotic nematodes (see section 7.6.3).

Free-living and parasitic nematodes produce a limited range of anaerobic end-products. In organisms that are periodically exposed to anaerobic conditions, resynthesis of carbohydrate from end-products is important in substrate conservation. *Caenorhabditis* spp. and *P. redivivus* produce various compounds, including ethanol, glycerol and acetate (Barrett and Wright, 1998). In *P. redivivus*, ethanol production involves a partial reaction of the pyruvate dehydrogenase complex. Under anaerobic conditions, *P. redivivus* mitochondria convert pyruvate to acetaldehyde and the latter is reduced to ethanol by an NADH-linked alcohol dehydrogenase. Acetaldehyde formation is modulated by a number of physiological effectors including ATP and NAD^+ (Barrett and Butterworth, 1984).

The advantages to free-living and plant-parasitic nematodes of producing neutral end-products such as ethanol or glycerol are unclear. Glycerol can be resynthesized easily into carbohydrate and glycerol production may therefore represent 'overflow' metabolism when substrate levels are high (Barrett, 1984). The resynthesis of carbohydrate from ethanol is more complex and involves the glyoxylate cycle. *Steinernema carpocapsae* is similar to vertebrate-parasitic nematodes in excreting succinate under anerobic conditions, whilst succinate, propionate, acetate and lactate accumulate in the tissues (Thompson *et al.*, 1991). The pathway of succinate production is unknown, but is probably the same as in vertebrate-parasitic species involving carbon dioxide fixation and a partial reversed TCA cycle (Barrett, 1981).

7.7.2. Lipid catabolism

Aerobic utilization of lipids has been demonstrated in free-living, plant-parasitic, insect-parasitic (Barrett and Wright, 1998) and entomopathogenic nematodes (Patel *et al.*, 1997). Where examined, it is the triacylglycerol fraction (typically 70–80% of total lipid) being utilized and there is some indication that saturated fatty acids are preferentially metabolized (Selvan *et al.*, 1993a,b; Holz *et al.*, 1998). Patel and Wright (1997b) reported that *S. carpocapsae, S. feltiae, S. riobravis* and *S. glaseri* infective juveniles preferentially utilized C18 : 1n9, C16 : 0 and C18 : 0 during storage. Except for *S. carpocapsae* (see section 7.7.1), the decline in infectivity in *Steinernema* spp. is correlated with the decline in neutral lipid reserves (Patel *et al.*, 1997). Fitters *et al.* (1999) assessed the fatty acid composition of north-west European isolates of *Heterorhabditis* sp. during storage and found that the UI increased during storage, again suggesting preferential use of saturated fatty acids.

Following hydrolysis of acylglycerols by lipase, fatty acids are broken down by β-oxidation to give acetylCoA, NADH and reduced flavoprotein. β-oxidation enzymes have been demonstrated in whole or in part in a number of species, including *C.*

Table 7.1. Glycolytic enzymes in rhabditids (after Barrett and Wright, 1998).

Species	Enzyme
Caenorhabditis briggsae	1, 3–7, 13, 14
Caenorhabditis elegans (adult)	1, 4, 7, 13, 15
(dauer)	1, 4, 7, 11, 13
Panagrellus redivivus	5–7, 11–14
Steinernema carpocapsae (dauer)	2–4, 11–14, 16
Steinernema feltidae (dauer)	2, 3, 14, 16
Turbatrix aceti	3, 5, 6, 8–13

[1] Hexokinase; [2]phosphoglucomutase; [3]glucosephosphate isomerase; [4]phosphofructokinase; [5]aldolase; [6]triosephosphate isomerase; [7]glyceraldehyde-3-phosphate dehydrogenase; [8]phosphoglycerate kinase; [9]phosphoglyceromutase; [10]phosphopyruvate hydratase; [11]pyruvate kinase; [12]lactate dehydrogenase; [13]fructose-1, 6-bisphosphatase; [14]glycerol-1-phosphate dehydrogenase; [15]alcohol dehydrogenase, [16]mannose-6-phosphate isomerase.

elegans, *P. redivivus* and *T. aceti*; a functional pathway has been demonstrated in *P. redivivus* and two other nematode species (Barrett and Wright, 1998).

7.7.3. Amino acid catabolism

Caenorhabditis briggsae and *Ditylenchus triformis* have been shown to use protein as an energy source during starvation and there is some evidence for protein utilization during starvation in infective juveniles of plant-parasitic species (Barrett and Wright, 1998). The first step in amino acid catabolism involves the removal of the amino group by transamination or deamination. *Panagrellus redivivus* is the only free-living nematode to be investigated in any detail (Grantham and Barrett, 1986a). The main transaminase present is the 2-oxoglutarate/glutamate system, with a few amino acids acting as donors for pyruvate/alanine and oxaloacetate/aspartate systems. It can oxidatively deaminate a range of L-amino acids, but D-amino acid oxidase activity is low. Specific, non-oxidative deaminases, such as serine and threonine hydratase, are also present. In general, the ability to transaminate and deaminate amino acids is similar to many other organsims. Alanine amino transferase has been characterized in *P. redivivus* and *S. feltiae* (Walker and Barrett, 1991) and aspartate amino transferase activity has been reported in *S. feltiae* and *S. carpocapsae* (Jagdale *et al.*, 1996).

The carbon skeletons of amino acids are converted to glycolytic or TCA cycle intermediates and, eventually, to CO_2. All three of the enzymes involved in proline catabolism have been demonstrated in *P. redivivus* (Grantham and Barrett, 1986b). This species can also catabolize the branched chain amino acids leucine, isoleucine and valine by similar pathways to those found in mammalian liver.

Nematodes are typical of aquatic animals, and 40–90% of non-protein nitrogen is excreted as ammonia (Wright, 1998; Thaden and Reis, 2000). Nothing is known about entomopathogenic species but like other nematode stages with a non-functional gut, the major route for nitrogenous excretion in infective juveniles would be expected to be via the body wall, although excretion might also occur via the excretory pore

(Wright, 1998). The retention of the second-stage sheath by the infective juvenile would be unlikely to prevent egress of ammonia.

7.7.4. Cytochrome chains

Within mitochondria, NADH and reduced flavoprotein are oxidized by cytochrome chains resulting in the reduction of oxygen to water and the formation of ATP. Adenylate nucleotide levels (ATP, ADP, AMP) have been measured in a wide range of nematodes, including *S. carpocapsae* (Thompson *et al.*, 1991, 1992). In addition, arginine phosphate and/or arginine kinase have been found in several species, including *S. carpocapsae, S. feltiae* and *S. riobravis* (Thompson *et al.*, 1992; Jagdale *et al.*, 1996).

Cytochromes *a+a3*, and various other cytochromes, have been demonstrated in several free-living and plant-parasitic nematodes (Barrett and Wright, 1998). Mitochondrial fractions from *A. avenae* show an ADP : O ratio of 1.4–2.2, which is similar to values normally found in mammals, and substrate and inhibitor responses in *C. elegans* appear similar to mammalian cytochrome chains. Large amounts of a *b*-type cytochrome have been described in *C. briggsae* and there may be a cyanide insensitive terminal oxidase in this species in addition to a mammalian-type cytochrome oxidase. Alternative oxidases are probably not cytochrome-linked but may be membrane-bound NADH flavoprotein oxidases (Barrett and Wright, 1998).

7.7.5. Regulation of metabolism

A key feature of dauer juveniles of *C. elegans* is their greatly enhanced longevity compared with non-dauers. They also have a much slower metabolism and possess larger energy reserves than normal juveniles (Albert and Riddle, 1988). In the non-dauer, DAF-2 signalling allows reproductive growth associated with utilization of food and relatively small energy reserves. In *daf-2* mutants, there is an increased accumulation of lipids and glycogen (Kimura *et al.*, 1997). Weak *daf-2* and *age-1* mutants that do not form dauers still live much longer than the wild-type (Morris *et al.*, 1996), suggesting lifespan regulation by an insulin-like metabolic control analogous to longevity enhancement in mammals by caloric restriction (Kimura *et al.*, 1997). Ogg *et al.* (1997) showed that mutants lacking *daf-16*, which is downstream of *daf-2* and *age-1*, bypass the need for the above pathway. Thus, the main role of DAF-2/AGE-1 signalling is to antagonize DAF-16, a factor involved in regulating the transcription of key metabolic and developmental control genes.

Mutations in the clock genes *clk-1* and *gro-1* mildly affect energy production in *C. elegans*, but repress energy consumption dramatically, thereby reducing the rate of anabolic metabolism and lengthening life span (Braeckman *et al.*, 2000).

Environmental conditions that reduce the metabolic rate of *C. elegans* also extend longevity. Van Voorhies and Ward (1999) found that the metabolic rate of long-lived *C. elegans* mutants was reduced compared with the wild-type and that a genetic suppressor that restored normal longevity to long-lived mutants restored normal metabolic rate. Thus, the increased longevity of some mutants may be due to a reduction in their metabolic rate, rather than to an alteration in a genetic pathway that leads to enhanced longevity *per se*.

7.8. Osmotic and Ionic Regulation

Various nematodes are known to osmoregulate in hypo-osmotic and/or hyper-osmotic environments (Wright, 1998). This is of particular importance to nematodes because of their hydrostatic skeleton; changes in water content will impair movement if resulting changes in body length exceed *c.* 15%. Within soil solutions, fluctuations in ion content and osmotic pressure occur due to both natural variation and agricultural inputs (Bednarek and Gaugler, 1997). Fluctuations can be marked in the upper layers of the soil where changes in the net evaporative rate also have the greatest impact. On foliage, infective juveniles of entomopathogenic nematodes are applied in aqueous solutions and are exposed to increasing osmotic stress through evaporative water loss.

There have been few studies on osmoregulation in entomopathogenic nematodes although hyper-osmotic regulation is implied by the ability of many species to remain infective in distilled water (Thurston *et al.*, 1994; Patel *et al.*, 1997). Some ability to regulate hypo-osmotically would also be expected for infective juveniles and other life stages within the insect host, and *Heterorhabditis* spp. are found frequently in coastal sandy soils where they may experience high salinity.

Piggott *et al.* (2000) measured the water content of infective juveniles of *H. indicus* and two *Steinernema* spp. isolates from Malaysia in a balanced salt solution supplemented with varying concentrations of sodium chloride. Hypo-osmotic regulation by *H. indicus* and *Steinernema* sp. SSL85 occurred at 1000 mOsm kg^{-1}, indicating an osmoregulatory ability sufficient to maintain infectivity at the high solute conditions that can occur in soils and on foliage. Regulation of water content by *Steinernema* sp. M1 was less marked. These differences may relate to the habitats from which they were isolated. *Steinernema* sp. M1 was found in heavy, relatively moist soil in central Malaysia; the others were collected from sandy coastal soils that are likely to show greater fluctuation in osmotic pressure due to drainage, evaporation and influences of seawater.

The ability of infective juveniles to remain hyper-osmotic to soil water may be due principally to passive mechanisms (Womersley *et al.*, 1998). However, *H. bacteriophora* juveniles show a reduced ability to regulate their body length, and thus volume, when incubated in a hyper-osmotic sodium chloride solution compared with an osmotically equivalent balanced salt solution (S.J. Piggott, unpublished data, Ascot, UK, 2000), indicating the involvement of specific ionic mechanisms. Reports of a putative Na^+/K^+ antiporter sequence (Marra *et al.*, 1993), characterization of cDNA for a water channel (Kuwahara *et al.*, 1997) and a gene (*flr-1*) encoding a novel Na^+ channel (Take-uchi *et al.*, 1998) in *C. elegans* should stimulate interest in how nematodes regulate water and ions actively.

Cuticle permeability is a key factor in determining the ability of nematodes, including infective juveniles, to survive evaporative water loss (see Glazer, Chapter 8, this volume). An isolate of *S. feltiae* with marked desiccation tolerance has been shown to have a marked ability to osmoregulate. However, a *Heterorhabditis* isolate with poor desiccation tolerance could osmoregulate while an isolate with moderate desiccation tolerance could not (S.J. Piggott, unpublished data, Ascot, UK, 2000). This indicates that there is not necessarily a link between the mechanism(s) that control water loss due to osmosis and the mechanisms(s) that determine desiccation survival. Comparison of wild-type *H. megidis* with a desiccation-tolerant mutant, whose greater tolerance

to evaporative water loss has been related to a greater negative charge on its sheath (O'Leary *et al.*, 1998), also failed to show a correlation with ability to regulate water content in a hyper-osmotic solution (Piggott *et al.*, 2000).

7.9. Summary

Information on the physiology and biochemistry of entomopathogenic nematodes is, at best, fragmentary. Fundamental research is required to extend our knowledge of their biology, and this will be important in relation to commercial applications. For many aspects, inferences about entomopathogenic nematodes are drawn from research on *C. elegans. Caenorhabditis* genomics and post-genomics will increasingly underpin research to determine the function of a wide range of genes and genetic pathways in entomopathogenic nematodes. Of particular interest will be the molecular genetics of infective juvenile formation and recovery, biosynthesis of energy stores and metabolic regulation, and the various physiological and biochemical processes concerned with the survival of infective juveniles.

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8 Survival Biology

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8.1. Introduction

The natural habitat for entomopathogenic nematodes, the soil, is a difficult environment for persistence of any organism considering its complexity of physical, chemical and biological components. Nevertheless, entomopathogenic nematodes have been isolated from soils throughout the world in ecosystems ranging from sub-arctic to arid and temperate to tropical climates (Poinar, 1990; Hominick *et al.*, 1996). It is assumed that along the course of evolution entomopathogenic nematodes, just like other terrestrial organisms, adopted unique survival mechanisms to resist environmental extremes.

Nematodes, like bacteria, fungi, and plants can survive unfavourable environmental conditions in a dormant state which considerably prolongs their life span and enables them to withstand the rigours of a fluctuating regime (Barrett, 1991). Dormancy can be separated into diapause and quiescence. Diapause is a state of arrested development and development does not recommence until specific

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requirements have been satisfied, even if suitable environmental conditions return. Quiescence is a facultative response, involving lowered metabolism, to unpredictable unfavourable environmental conditions and is readily reversible when favourable conditions return; if the stress persists, some organisms can enter a state of cryptobiosis where there is no measurable metabolism. Unfavourable environmental conditions include absence of water, extreme temperatures, lack of oxygen and osmotic stress; the types of quiescence induced in organisms by these conditions are termed anhydrobiosis, thermobiosis, cryobiosis, anoxybiosis and osmobiosis, respectively (Barrett, 1991).

While each one of these factors may effect nematode survival, it is important to realize that in the soil these elements interact. For example, soil temperature is determined by factors that control heat transfer. Moist soil has greater conductance and lower increase in temperature than dry soil when exposed to the same heat source. Another example is that solute concentrations increase upon drying of the soil. Therefore, desiccation stress of nematodes is commonly accompanied by pre-exposure to osmotic stress. It is likely that physiological and biochemical mechanisms involved in tolerance to different stresses also interact.

Substantial information has accumulated on survival mechanisms of plant-parasitic and free-living nematodes, but little is known about these aspects in relation to entomopathogenic nematodes. In the present chapter I will characterize the 'surviving stage' of the entomopathogenic nematodes and then describe survival mechanisms in relation to the main environmental factors listed above. I will also address the implications of survival strategies on utilization of steinernematid and heterorhabditid nematodes as biological control agents.

8.2. The 'Surviving Stage'

In many parasitic nematodes, which spend part of their life cycle in a protected and favourable environment (i.e. the host), only free-living stages have the capability to survive the harsh environment outside the host. The cereal nematode *Heterodera avenae* survives the dry season in a cyst form (Cooper and Van Gundy, 1971) and new second-stage juveniles emerge from the eggs as soon as environmental conditions (moisture and presence of host root) (Brown, 1984) are favourable. The root-knot nematode *Meloidogyne* spp. survives the dry season as eggs in a protective sack (Cooper and Van Gundy, 1971). The juvenile and the adult stages of *Dytylenchus dipsaci* oversummer in a form of dormancy (Cooper and Van Gundy, 1971; Norton, 1978). Among several rhabditid families, environmental stress (lack of food, high population density, heat, etc.) induce the development of a unique juvenile stage, the 'dauer juvenile' (Riddle, 1988). This stage is adapted morphologically and physiologically, to remain in the environment without feeding while searching for a new food source. In the entomopathogenic rhabditid nematodes, steinernematids and heterorhabditids, the dauer stage is also the infective stage that locates and invades insect hosts (Lewis *et al.*, 1992). This infective stage also provides protection to symbiotic bacteria carried in its intestine (Akhurst, 1995). It is equipped with two layers of external membrane, the cuticles of the third stage and of the second moult, which is retained to provide additional protection (Campbell and Gaugler, 1991a,b; Timper and Kaya, 1989).

The nematode cuticle or integument is a highly ordered extracellular multilayered structure, which varies in different species and developmental stages (Inglis, 1983; Wright, 1987, 1991; Bird and Bird, 1991). Patel and Wright (1998) emphasized the important role of the cuticle in entomopathogenic nematode desiccation survival. They suggested that differences found in survival and the rate of water loss during desiccation at 80% relative humidity (RH) between *Steinernema carpocapsae* and the three other species might be related to the cuticle ultrastructure. Analysis of scanning electron micrographs (Fig. 8.1) of infective juveniles surface reveal that external openings (mouth and anus) are closed (Mracek *et al.*, 1981), thus preventing penetration by microbial antagonists and toxic chemicals.

Infective juveniles do not feed and so are dependent on internal sources for energy which enable them to survive until a host is located. Quantitative and qualitative analysis of biochemical reserves of entomopathogenic nematodes has been conducted by Selvan *et al.* (1993) and Abu Hatab *et al.* (1998) showing that lipid content and fatty acid composition are highly dependent on media components. Several studies have shown (Lewis *et al.*, 1995; Patel *et al.*, 1997b; Patel and Wright, 1997a,b) that nematode infectivity declines as energy reserves are depleted in storage. For instance, a decline in neutral lipids was closely correlated with the observed decline in infectivity and survivorship of three steinernematids (Patel *et al.*, 1997a). In this study, *S. riobrave* survived less well (120–135 days) than *S. feltiae* and *S. glaseri* (> 450 days).

The other significant energy reserve found in nematodes is glycogen (Wright, 1998; Barrett and Wright, 1998). This storage compound occurs in appreciable amounts in some entomopathogenic nematodes (Selvan *et al.*, 1993), but its functional significance has been less clear. Utilization of glycogen by infective juveniles of *S. carpocapsae, S. glaseri* and *Heterorhabditis bacteriophora* can be inferred from the data of Lewis *et al.* (1995). Subsequent work confirmed that the glycogen content of *S. carpocapsae, S. glaseri, S. feltiae* and *S. riobrave* declined during storage and suggested that glycogen may play a significant if secondary role to neutral lipids in the maintenance of infectivity in these species (Patel and Wright, 1997b).

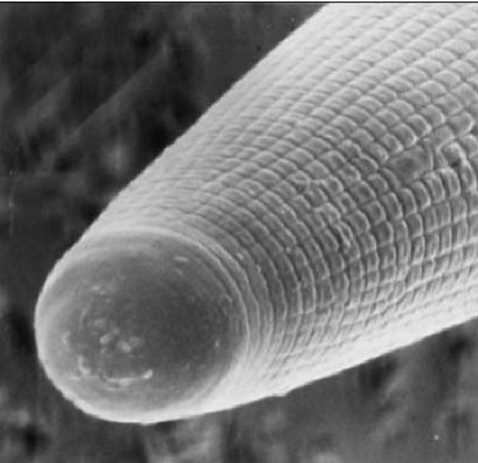

Fig. 8.1. Scanning electron micrograph of head region of an infective juvenile of the entomopathogenic nematode *Heterorhabditis bacteriophora*.

From the above studies on energy stores, it is clear that survivorship of infective juveniles of entomopathogenic nematodes is a function of their metabolic rate and their initial levels of energy reserves. Availability of energy reserves is essential to support the physiological and behavioural processes accompanying acclimatization to environmental stress. The importance of energy sources for stress tolerance will be discussed further.

8.3. Extreme Temperature

8.3.1. Cold tolerance

Nematodes inhabiting the soil are exposed to sub-zero temperatures in temperate, arctic and sub-arctic regions as well as at high altitudes (Wharton, 1986). Steinernematids have been isolated at many sites in northern Europe (Burman *et al.*, 1986; Husberg *et al.*, 1988) and Canada (Mracek and Webster, 1993). Heterorhabditids have been isolated from northern parts of Europe (Griffin and Downes, 1991). Entomopathogenic nematode isolation from such cold regions indicates that they are capable of withstanding sub-zero conditions. However, little is known about their cold tolerance mechanism.

There are two main strategies by which cold tolerant organisms are able to survive freezing temperatures. They are either freezing-tolerant, surviving ice formation in their tissues, or they are freezing-susceptible, avoiding ice nucleation and maintaining their body fluids as liquid at temperatures well below their melting point by supercooling. Freezing-tolerant animals are usually thought only to survive ice formation in their body cavity and extracellular spaces; intracellular freezing is generally fatal. However, survival of intracellular freezing has been reported in the Antarctic nematode *Panagrolaimus davidi* (Wharton and Brown, 1991). Freeze avoidance occurs in the eggs of *Globodera rostochiensis* and *P. davidi* (Wharton and Block, 1993), and the soil-dwelling stage of the animal-parasitic nematode, *Trichostrongylus colubriformis*, where freezing is prevented by a sheath (Wharton and Block, 1993).

Brown and Gaugler (1995) demonstrated that *S. feltiae, S. anomali* and *H. bacteriophora* were all freezing tolerant with lower lethal temperature of − 22, − 14 and − 19°C respectively. Wharton and Surry (1994) showed that *H. zealandica* is freeze avoiding. The sheath of *H. zealandica* prevents innoculative freezing, allowing extensive supercooling to − 32°C, whereas exsheathed infective juveniles freeze above − 6°C and do not survive. They do so by withstanding extracellular ice formation (‘freezing tolerant’) or by avoiding freezing at sub-zero temperatures by supercooling, but die when the body freezes (‘freezing avoiding’) (Wharton *et al.*, 1984). Supercooling is defined as the ability of an organism to maintain its body fluids in liquid phase at temperatures below the freezing point (Wharton and Block, 1993). Sugars and polyols, such as trehalose, maltose, glucose and dextrose, and polyethylene glycol have roles as cryoprotectants or antifreezes. The production of natural cryoprotectants, such as trehalose, is correlated with enhanced supercooling abilities of arthropods (Wharton *et al.*, 1984).

Some nematodes are able to survive temperatures as low as − 80°C, a temperature at which metabolism is likely to have ceased. Cryopreservation studies (Curran *et al.*, 1992; Popiel and Vasquez, 1989) have demonstrated that entomopathogenic nematodes can be stored indefinately in liquid nitrogen.

8.3.2. Heat tolerance

High temperatures (> 32°C) have an adverse effect on reproduction, growth and survival of many organisms (Wharton, 1986), including nematodes (Grewal *et al.*, 1994; Zervos *et al.*, 1991). Heat-shock proteins (HSP) are known to be involved in survival at elevated temperatures (Schlesinger, 1990). These stress-related proteins and their related genes are highly conserved among organisms (Schlesinger, 1990). Some of the genes involved in HSP production were identified in the free-living nematode *Caenorhabditis elegans* (Jones *et al.*, 1986). Production of *hsp*70 was also detected in *H. bacteriophora* HP88 (Selvan, *et al.*, 1996) even though temperatures above 32°C hamper reproduction, activity and viability of this strain (Grewal *et al.*, 1994). Hashmi *et al.* (1997) analysed the molecular organization of *hsp*70 genes in five species and isolates of heterorhabditids and one steinernematid species using PCR and restriction fragment length polymorphism (RFLP). They found homology with *hsp*70 genes of *C. elegans.* In addition, RFLPs with *hsp*70 probe revealed different banding patterns for *Heterorhabditis* species and isolates.

New heterorhabditid isolates from the arid region of Israel (Glazer *et al.*, 1991) and Egypt (Shamseldean and Abd-Elgawad, 1994) or tropic climate of Sri Lanka (Amarasinghe *et al.*, 1994) were shown to be heat tolerant. Shapiro *et al.* (1995) had shown that the heat tolerance trait in the IS-5 strain of *Heterorhabditis* sp., one of the isolates from Israel, is stable and genetically based. Segal *et al.* (Israel, 2000, personal communication) have isolated two genes that display very high homology to members of the *hsp*70 gene family from *C. elegans.* Their structure was determined and it was demonstrated that the genes are heat inducible, and that their expression is elevated in the IS-5 strain as compared to the HP88 strain.

Improvement of heat tolerance of entomopathogenic nematodes was a prime goal in several research projects conducted in the past few years:

8.3.2.1. Improvement by classical genetics

A trait for heat tolerance was transferred from the IS-5 strain to the HP88 strain of *H. bacteriophora* (Shapiro *et al.*, 1997). The transfer was accomplished by allowing the heat tolerant strain IS-5 to mate with the HP88 strain. The hybrid nature of the progeny was confirmed using a marker mutant of the HP88 strain (*Hp-dpy-2*) and by backcrossing. Virulence, reproduction and storage capacity were compared among the IS-5, hybrid, and HP88 strains and showed no loss of fitness for the hybrid strain (Shapiro *et al.*, 1997). At 32°C, the IS-5 and hybrid strains caused mortality of *G. mellonella* at a faster rate than the HP88 strain. This study demonstrates the potential of using hybridization to genetically improve entomopathogenic nematodes.

8.3.2.2. Genetic engineering

Hashmi *et al.* (1995) developed transformation methods and markers that have resulted in the first genetically engineered insect parasite. They introduced an *hsp*70 gene from *C. elegans* into *H. bacteriophora*, increasing the copy number from one up to ten. The overexpression of HSP resulted in transgenic nematodes much better than unmodified nematodes at surviving high temperatures (Hashmi *et al.*, 1998). The transformation was found to be heritable and stable. No changes in fitness were observed in the transformed strain (Gaugler *et al.*, 1997)

8.4. Desiccation Tolerance

All nematodes are aquatic organisms and need a film of water surrounding their body in order to move (Norton, 1978). Dry conditions adversely affect nematode motility and survival. Some free-living stages of several animal and plant-parasitic nematodes can survive exposure to desiccation for long periods (Cooper and Van Gundy, 1971; Wharton, 1986). These nematodes are capable of anhydrobiosis and can survive in a desiccated state for many years (Cooper and Van Gundy, 1971; Wharton, 1986). Anhydrobiosis is usually reached following a slow rate of water loss (Crowe and Madin, 1975). Some nematodes form tight coils when exposed to desiccation (Wharton, 1986; Womersley, 1987) which slow the rate of water loss by reducing the area of cuticle exposed to air.

The biochemical mechanisms involved in the induction of anhydrobiosis are not fully understood. One biochemical change that has been reported in anhydrobiotic nematodes is the accumulation of polyols and sugars, which are believed to act as protectants of biological membranes and intracellular proteins during dehydration (e.g. Womersley, 1990; Barrett, 1991; Behm, 1997). For example, Crowe and Madin (1975) found that in the mycophagous nematode, *Aphelenchus avenae*, lipid and glycogen levels declined while glycerol and trehalose accumulated during dehydration. The authors showed a strong correlation between survival of the nematode in dry air and the production of these two compounds. Similar observations, in some cases involving different compounds, have been made in other nematode species (Womersley and Smith, 1981; Womersley *et al.*, 1982; Higa and Womersley, 1993). Trehalose has been the main focus of research since it is was found in high concentrations in a number of anhydrobiotic organisms (e.g. bacteria, yeast, nematodes and brine shrimps) and because of this, Crowe *et al.* (1992) suggested that the physical principles governing the stability of certain organisms under dry conditions may be universal.

During dehydration, proteins can be protected by trehalose in two main ways: it can replace 'bound' water and reduce the reaction of dried glucose with amino-acid side chains of proteins (known as the 'browning' or Maillard reaction) (Behm, 1997). There are two mechanisms by which trehalose is thought to stabilize membranes (Hoekstra *et al.*, 1992, 1997). The first involves the direct interaction between the hydroxyl groups of trehalose and the phosphate of phospholipids, which acts to replace water normally associated with the phospholipid bilayer. The end result is a depression in the phase transition temperature (T_m) of the dry phospholipids, maintaining membrane fluidity and keeping the bilayer in the liquid crystalline state. Thus preventing the transition to the gel phase, which would otherwise result in loss of membrane integrity during dehydration (Crowe *et al.*, 1992, 1996). Secondly, trehalose can reduce T_m of dry phospholipids by forming glass (vitrification). This is an indirect interaction, whereby the phospholipids are trapped in a sugar glass, a supersaturated and thermodynamically unstable solid solution (Sun *et al.*, 1996).

On exposed surfaces, steinernematids and heterorhabditids can survive no longer than several hours, depending on species, temperature and relative humidity (Glazer, 1992). In dry soil, entomopathogenic nematodes can persist for 2–3 weeks (Kaya, 1990; Kung and Gaugler, 1990). Most studies that have investigated the desiccation survival ability of steinernematid nematodes concentrated on *S. carpocapsae* (e.g. Simons and Poinar, 1973; Ishibashi *et al.*, 1987; Womersley, 1990; Glazer, 1992). The general

finding has been that under slow drying conditions various strains of *S. carpocapsae* can survive for appreciable lengths of time. In addition, *S. carpocapsae* (All strain) survives desiccation better than *S. glaseri* (Kung *et al.*, 1991) and *S. riobravae* (Baur *et al.*, 1995). Behavioural adaptations, such as loose coiling, have been induced in *S. glaseri* exposed to elevated RHs (97%), and in *S. carpocapsae* on slow drying model substrates (Womersley, 1990), but in general, coiling has not been consistently observed during desiccation and does not correlate with survival. Clumping aids the survival of *S. carpocapsae* infective juveniles when the infective juveniles are desiccated in large aggregates (> 1000 individuals) (Simons and Poinar, 1973). However, it is not known whether such behavioural responses occur naturally, particularly since aggregation and clumping may not be in direct response to dehydration (Womersley *et al.*, 1998).

Womersley (1990) suggested that the sheath of infective juveniles of entomopathogenic nematodes might help to reduce the rate of water loss during desiccation. Campbell and Gaugler (1991a) and Patel *et al.* (1997a), however, did not find evidence for the role of the sheath in desiccation survival in *S. carpocapsae*.

Patel *et al.* (1997a) found that newly emerged *S. carpocapsae* survive better than other nematode species. However, aged infective juveniles (stored at 25°C in distilled water for 75 days) of *S. carpocapsae* were not able to tolerate desiccation as well as newly emerged infective juveniles. The drying profiles for newly emerged infective juveniles desiccated at 80% RH clearly showed that the superior survival ability of *S. carpocapsae* was a result of its lower rate of water loss. Because, the nematodes had been 'fast dried', there was unlikely to have been time for biochemical adjustments and, therefore, a difference in cuticle permeability was the most obvious explanation for the above result.

Solomon *et al.* (1999) studied the desiccation tolerance of the IS-6, IS-15 and SF strains of *S. feltiae*. The IS-6 strain isolated from the desert region of Israel exhibited the highest survival capability (Fig. 8.2), followed by the IS-15 strain isolated from northern Israel. The poorest tolerance was exhibited by the SF strain, which was isolated in Germany (Fig. 8.2).

The desiccation tolerance of the IS-6 and IS-15 strains was associated with a dispersal response of aggregated infective juveniles at the slow dehydration regime, which was followed by entrance into a state of anhydrobiosis as individually coiled infective juveniles (Fig. 8.3). Survival of individual infective juveniles at 85% RH was positively correlated to the initial pre-conditioned clump size which ranged from 70 to 7700 infective juveniles (Solomon *et al.*, 1999).

Solomon *et al.* (1999) recorded physiological changes during the low relative humidity experiment and noted increased trehalose levels, as reported for other anhydrobiotic nematodes during the dehydration process (Womersley 1987, 1990; Crowe and Crowe, 1992). Increased trehalose levels were determined in infective juveniles after 3 days' exposure to 97% RH (from 0.3 to 0.6 g trehalose per g protein), while an opposite trend was observed in glycogen (from 0.09 to 0.02 g glycogen per g protein). Upon rehydration, glycogen returned to its original concentration after 24 h, while trehalose reached only 50% from the original. This result suggests that during the rehydration process, trehalose is used as an energy source or is hydrolysed for the glycogeneogenesis process. Nematode virulence and invasion capability were not hampered by the dehydration and rehydration processes.

Synthesis and accumulation of proteins during the desiccation process were characterized among bacteria, fungi, yeast and plant seeds (Close, 1996; Dure, 1993).

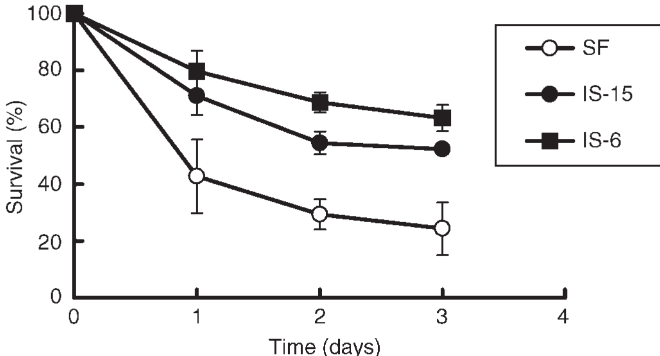

Fig. 8.2. Survival curves of pre-conditioned infective juveniles of three different strains of *Steinernema feltiae* (SF, IS-15, and IS-6) in 85% RH at 23°C ($n = 3$; error bars show ± SE).

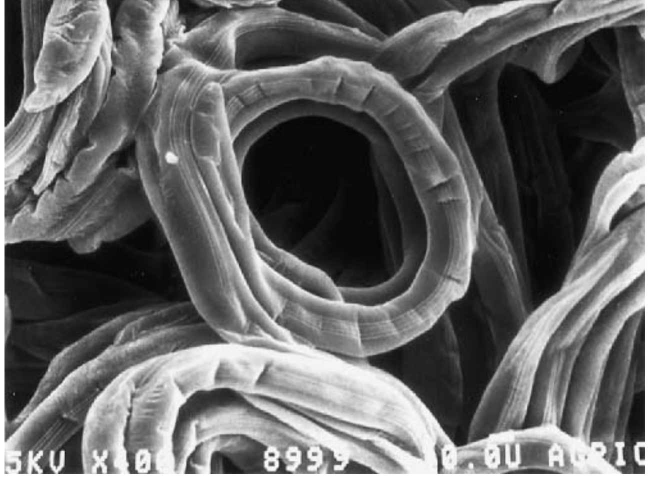

Fig. 8.3. Scanning electron micrograph of desiccated infective juveniles of *Steinernema feltiae* aggregated in a coiled position.

However, little is known about these aspects in regard to nematodes. Solomon *et al.* (2000) identified in *S. feltiae* IS-6 a heat-stable, water stress-related protein with a molecular mass of 47 kDa (designated *desc*47). This protein was accumulated 10-fold in infective juveniles (following exposure to 97% RH for 72 h) which lost 34.4% water content as compared to fresh nematodes (Fig. 8.4). The *desc*47 protein remained at a high level even after the nematodes were rehydrated. No homology to other known proteins was found by the mass spectrometer electrospray-ion-trap system analysis. However, from five obtained sequences of short peptides (ranging from 10 to 21 amino acids), the 21 amino acids peptide – N V A S D A V E T V G N A A G Q A G (D/T) A V – showed a high similarity (79%) and identity (52%) to the *C. elegans* late embryogenesis abundant (LEA) homologue protein (g2353333) and to the LEA group-3-like protein (78.9% similarity and 52.6% identity) from the fern *Onclea sensibilis* (g322390).

The LEA proteins are a diverse group of water-stress related proteins which are expressed in maturing seeds and in water deficit stressed vegetative tissues of higher

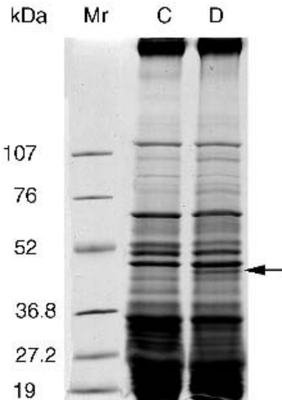

Fig. 8.4. SDS–PAGE (8–20% acrylamide gradient gel) showing a profile of a heat-soluble, uncoagulated proteins. C = control, nematodes kept in distilled water for 3 days at 23°C. D = desiccated clump of infective juveniles that lost 34.4% of its initial water content following exposure to 97% RH for 3 days. R = rehydrated infective juveniles, that were immersed for 3 days in water following desiccation. Molecular markers (Mr) are shown on the left. Each lane was loaded with 40 μg heat-soluble proteins. The arrow indicates *Desc*47.

plants (Chandler *et al.*, 1988; Close, 1996). Xu *et al.* (1996) demonstrated that rice plants transformed with the barley HVA1 gene (group 3 LEA protein) were highly resistant to desiccation and salt stress. These stress proteins were found to be highly hydrophilic and resistant to coagulation by heat. It was suggested, based on the 'proteins' structure-predicted amino acid sequence', that they prevent cellular damage during desiccation by protecting the structure of membranes and proteins, or by renaturating unfolded proteins (Dure, 1993). Ultrastructural localization of plant dehydrins (group 2 LEA proteins) indicated an association with endomembrane-rich areas of the cytoplasm (Egerton-Warburton *et al.*, 1997). The latter suggested that dehydrins protect membrane integrity which is essential for survival of cells during desiccation stress. Nevertheless, the biological function of the LEA protein in the protection mechanisms against desiccation stress remains cryptic.

Current knowledge of the gene function and molecular mechanisms involved in desiccation tolerance of entomopathogenic nematodes is far less than that of physiological aspects. Only recently Zitmann-Gal *et al.* (2001) identified novel genes of *S. feltiae* IS-6 that exhibit changes in transcript levels upon dehydration. These included glycogen synthase (*Sf-gsy-1*), the rate-limiting enzyme in the synthesis of glycogen, that is likely to play a role in desiccation survival. In this study, Zitmann-Gal *et al.* (2001) established changes in the steady-state level of *Sf-gsy-1* transcripts upon dehydration. Our results suggest a shift from glycogen to trehalose synthesis upon dehydration, which is regulated, at least in part, by suppression of glycogen synthase transcription. Currently, new genes and their putative function during desiccation in entomopathogenic nematodes are being explored by this team and others around the world (Ann Burnell, Maynooth, personal communication).

As indicated earlier, fewer studies have been devoted to determining the desiccation tolerance of heterorhabditids. Surrey and Wharton (1995) tested the desiccation

survival of *H. zealandica*. Their experiments indicated that survival was poor once water had been lost from the substrate. They concluded that: 'survival would not be improved by adjusting the treatment temperature, the source of the infective larvae, the method of rehydration or the addition of trehalose'. Menti *et al.* (1997) exposed *S. feltiae* and *H. megidis* from Greece and the UK to different RH levels. They showed that although *H. megidis* survival was superior to that of *S. feltiae*, desiccation tolerance for both species was poor (minutes). Poor survival of *H. megidis* (strain UK211) was also reported by O'Leary *et al.* (1998) who exposed the nematodes directly to 57% RH for 3 h without preconditioning at higher RH levels.

Liu and Glazer (2000) determined the desiccation tolerance of heterorhabditid populations from Israel. First, the most suitable desiccation conditions that lead to induction of a shallow anhydrobiotic state using *H. bacteriophora* HP88 were verified. Substantial differences in survival ability were observed among infective juveniles of 12 new heterorhabditid populations, isolated from different climatic regions in Israel. The nematodes were preconditioned at 97% RH for 72 h followed by exposure for an additional 72 h at 93% RH (Fig. 8.5). Maximum survival was recorded with IS-19 (64%), moderate survival was observed with seven other isolates including HP88, and five isolates displayed poor (< 25% viability) desiccation tolerance.

8.5. Osmotic Tolerance

Nematodes may have to tolerate considerable variation in the concentration of salts in the environment and thus are subject to osmotic stress (Evans and Perry, 1976; Wharton, 1986). The greatest osmotic problem is the influx of water under hyposmotic conditions. Some nematode species possess specific mechanisms for the removal of excess water, a problem reduced in many nematodes by having restricted cuticular permeability. Some nematodes actively remove excess water under hypotonic conditions through the intestine. Influx of water under hypotonic conditions may be reduced by excreting salts and thus decreasing the osmotic gradient (Wharton, 1986).

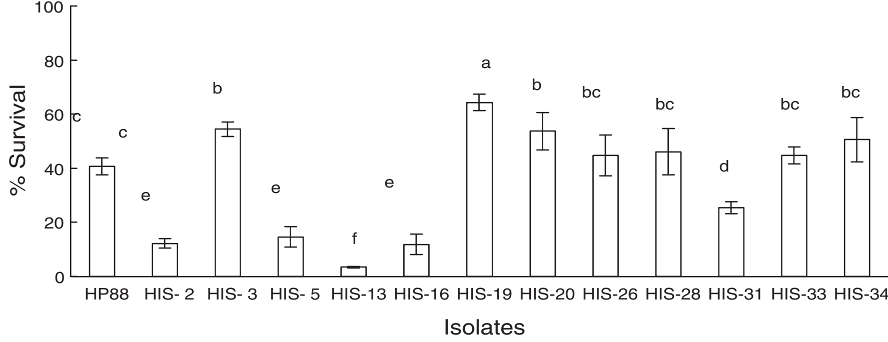

Fig. 8.5. Effect of exposure of different heterorhabditid isolates to 97% RH for 72 h followed by further exposure to 93% for an additional 72 h, at 25°C. Viability was determined after rehydration in distilled water for 24 h at room temperature. Bars indicate standard deviation of mean. Columns within treatments annotated with the same lower case letter are not significantly different ($P = 0.01$), according to SNK multiple range test.

The mechanisms of osmoregulation among steinernematids and heterorhabditids are not known, although studies have demonstrated their capability to withstand osmotic stress (Thurston *et al.*, 1994; Finnegan *et al.*, 1999). Glazer and Salame (2000) evaluated the effect of different osmolytes on the viability of *S. carpocapsae.* Exposing fresh infective juveniles to high temperature (45°C) resulted in a rapid reduction in viability, yet under the same assay condition evaporatively and osmotically desiccated nematodes showed enhanced ability to withstand temperature stress. When infective juveniles of *S. carpocapsae* were exposed to different salt solutions, high mortality was recorded among the nematodes within the first 24 h of exposure. By contrast, non-ionic solutes tested did not reduce viability of the infective juveniles although all nematodes were found to be shrunk after 48 h (Fig. 8.6).

Furthermore, 72 h exposure to solutions of non-ionic solutes resulted in an increase in heat tolerance similar to that of evaporatively dehydrated nematodes, notably in glycerol solutions at concentrations from 2.2 to 3.8 mol. per litre, polyethylene glycol 300 mol. wt. at concentrations of 1.2 to 1.6 mol. per litre, and polyethylene glycol 600 mol. wt at 0.8 mol. per litre. High viability was also recorded among nematodes that were stored for 72 days following a gradual increase in glycerol concentration. Exposure of these nematodes to 45°C resulted in 87.3% and 49.2% survival after 4 and 8 h, respectively (Fig. 8.7).

This study showed that osmotically desiccated *S. carpocapsae* infective juveniles are capable of withstanding heat stress similarly to evaporatively desiccated ones. However, there is no direct evidence showing that removal of water by osmotic pressure induces a dormant state similar to that induced by slow evaporation.

8.6. Pesticides

Steinernematid and heterorhabditid nematodes can survive exposure to many chemical pesticides (Hara and Kaya, 1982; Rovesti and Deseö, 1990; Rovesti *et al.*,

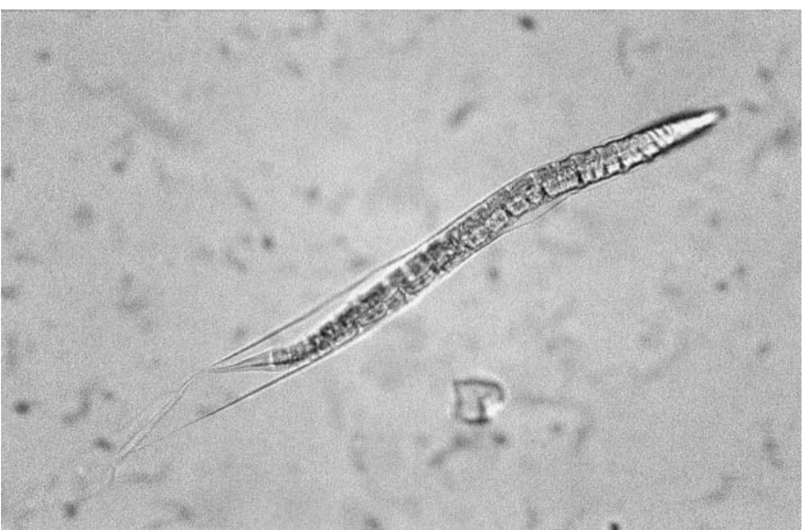

Fig. 8.6. Shrunken infective juvenile of *Steinernema carpocapsae* after 24 h exposure to 2.5 mol per litre glycerol.

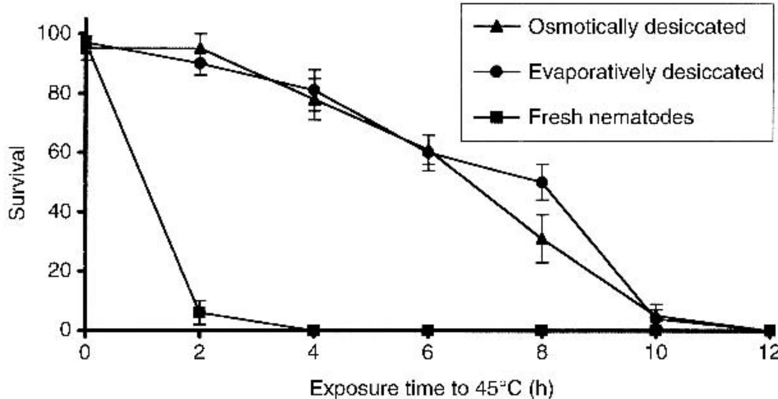

Fig. 8.7. Effect of exposure to high temperature (45°C) on the viability of *Steinernema carpocapsae* 'All' infective juveniles following osmotic dehydration by exposure to 2.5 mol per litre glycerol for 72 h, evaporative dehydration by exposure to 97% RH for 72 h, or fresh infective juveniles suspended in distilled water. Bars are standard error of the means.

1989). However, infective juveniles are highly susceptible to several nematicides likely to be found in the agroecosystem (Rovesti and Deseö, 1990, 1991).

Glazer *et al.* (1997) examined genetic selection as a means of enhancing resistance of *H. bacteriophora* strain HP88 to the nematicides: fenamiphos (organophosphate), oxamyl (carbamate), and avermectin (biologically-derived product). After 11 rounds of selection, resistance to the nematicides as well as several traits relevant to biocontrol efficacy including virulence, heat tolerance, and reproduction potential were examined. Selection resulted in an eight- to ninefold increase in resistance to fenamiphos and avermectin, and 70-fold to oxamyl. No deterioration in traits relevant to biocontrol efficacy was observed in the selected lines as compared to the baseline population.

8.7. Aeration

Since nematodes are aerobic organisms, low oxygen availability can reduce their survival (Evans and Perry, 1976; Wharton, 1986). Oxygen becomes a limiting factor in clay soils, water-saturated soil or soils with high levels of organic matter. In laboratory studies, it has been shown that *S. carpocapsae* survives oxygen tensions as low as 0.5% saturation at 20°C (Burman and Pye, 1980). In sandy soil, survival of *S. carpocapsae* and *S. glaseri* decreased as oxygen concentration decreased from 20% to 1% (Kung *et al.*, 1990).

Reduction of oxygen concentrations induces a dormancy state (anabiosis) in several free-living nematode species (Wharton, 1986). This phenomenon has not been reported with steinernematids and heterorhabditids.

Qiu and Bedding (1999) investigated the effect of anaerobic conditions on survival infectivity and physiological changes in infective juveniles of *S. carpocapsae*. Under aerobic conditions survival rate of infective juveniles decreased slightly to 91% in the first 6 weeks then dropped sharply to about 78% at week 7 and 55% at week 8 of storage. When infective juveniles were incubated in M9 buffer at 23°C under absolute

anaerobic conditions, they were fully inactivated in 16 h but could be revived when returned to aerobic conditions if exposure to anaerobic conditions was for not more than 7 days. Time needed to recover was proportional to the time under anaerobic conditions, varying from several minutes to more than 24 h for those under anaerobic conditions for 7 days. The survival time of the nematodes under anaerobic conditions was significantly affected by temperature (90% survival times at 5, 23, 28°C were 20, 7, 5 days respectively) but not significantly affected by nematode density (from 10^4 to 10^5 per ml) and CO_2 level (from 0% to 6%). Analysis of changes in key energy reserve materials under anaerobic conditions indicate that levels of glycogen and trehalose declined rapidly over time from initial levels of about 5 and 1.5 ng per nematode to 1.33 and 0.3 ng per nematode at day 6, respectively. However, the lactate level increased corresponding and the lipid level was not significantly changed. When anaerobically incubated nematodes were returned to an aerobic environment, both glycogen and trehalose levels increased sharply while the lipid and lactate levels decreased correspondingly. This indicates that, like most other animals, under anaerobic conditions infective juveniles of *S. carpocapsae* depend on carbohydrate reserves to provide energy.

8.8. Other Survival Strategies

When the environment is unfavourable, some nematode populations may be able to avoid stress by moving into protective niches. For example, some plant parasitic nematodes migrate downward in the soil profile to cooler or wetter layers of the soil to escape extreme heat or dryness (Varin, 1986). This behavioural pattern might explain the findings of Glazer *et al.* (1996) who isolated higher numbers of heterorhabditid nematodes in deeper soil layers (35–40 cm) during summer season as compared with upper soil layer (5–10 cm depth). The insect host cadaver can also serve as a protective niche by providing protection from adverse conditions such as extreme temperatures (Brown and Gaugler, 1995) and low humidity (Brown and Gaugler, 1997; Koppenhöfer *et al.*, 1997). In addition, the antibiotic compounds produced by the symbiotic bacterium can provide protection from antagonists (Akhurst, 1995).

8.9. Conclusions

Survival mechanisms play a central role in entomopathogenic nematodes' persistence. As inundative biological control agents, entomopathogenic nematodes are released in massive numbers with the aim of obtaining immediate pest suppression. As such, entomopathogenic nematodes are expected to persist in the treated field at high numbers for at least 2–3 weeks (Georgis and Manweiler, 1994). The use of nematode strains with enhanced survival capability will increase nematode efficacy when applied in the field. If long-term persistence is required for successful application, then the ability of the applied nematode strain to tolerate the specific conditions should be predetermined.

The infective juveniles of steinernematids and heterorhabditids rely on stored food reserves for persistence in the environment until a host is located. Elucidating the metabolic and physiological processes involved in infective juveniles survival will provide the information that may enable preloading infective juveniles, during the production process, with sufficient storage material to support their persistence.

In the present review, I have summarized some of the new research avenues that nematode survival studies are taken. Understanding the fundamental basis of molecular and physiological architecture of survival in entomopathogenic nematodes will enable the improvement of nematode ability to withstand environmental extremes and thus persist longer. New advances in molecular techniques, the acomplishment of *C. elegans* genome sequencing and progress in informatic and genomic technologies provide powerful tools for making significant progress in this area of research. Our findings (Glazer and Salame, 1999) suggest that the ability of entomopathogenic nematodes to survive desiccation, osmotic or heat stresses may have common mechanisms or certain mutual molecular bases. Revealing these fundamental aspects will provide the basis for further comprehension of nematode survival. This requires a joint effort by nematode ecologists, molecular biologists and physiologists.

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9 Natural Enemies and Other Antagonists

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9.1. Introduction

Entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) and their symbiotically associated bacteria (*Xenorhabdus* spp. for steinernematids and *Photorhabdus* spp. for heterorhabditids) are vulnerable to natural enemies (Kaya and Koppenhöfer, 1996; Kaya *et al.*, 1998). The non-feeding, free-living, infective third-stage juveniles occur in the soil environment where they are exposed to microbial and metazoan natural enemies. Although a number of natural enemies attack infective juveniles, we know little about the impact of these enemies on nematode populations under field conditions. Our knowledge of natural enemies on the free-living stage of entomopathogenic nematodes has resulted primarily from empirical studies in the laboratory and field. Moreover, when the surviving infective juveniles infect and kill their insect hosts, organisms within the insect cadavers may antagonize the development of the nematode–bacterium complex. Another source of antagonistic interactions occurs when nematode-killed insects are attacked by scavengers or omnivores that result in the demise of the developing nematodes and the associated bacteria.

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Under natural conditions, entomopathogenic nematodes should be pre-adapted to specific ecological conditions in their environments (Glazer, 1996). Factors that affect nematode survival are intrinsic (i.e. genetic, physiological and behavioural characteristics of the infective juvenile nematodes) and extrinsic (i.e. abiotic and biotic) (Curran, 1993). Abiotic factors including temperature, moisture, soil texture, ultraviolet light, and chemical pesticides affect nematode persistence (Kaya, 1990; Smits, 1996). Biotic factors that are favourable for the nematodes include the presence of hosts and of plants that moderate the physical factors (i.e. soil moisture, temperature, porosity, etc.) creating a suitable environment for survival. Biotic factors can also be antagonistic, affecting the survival of entomopathogenic nematodes and their symbiotic bacteria. These antagonistic factors can be broadly identified as antibiosis, competition, natural enemies (Kaya and Koppenhöfer, 1996; Kaya *et al.*, 1998) and scavengers (Baur *et al.*, 1998). Antibiosis occurs through the release of plant chemicals from the roots into the soil that may adversely affect the host-finding behaviour of the infective stage nematode (Kaya and Koppenhöfer, 1996), or the presence of these chemicals in the host insect may negatively affect nematode reproduction (Barbercheck *et al.*, 1995). Intraspecific (Selvan *et al.*, 1993) and interspecific competition can result in reduced nematode fitness or lead to competitive displacement (Kaya and Koppenhöfer, 1996; Barbercheck and Millar, 2000). The focus of this chapter is on the antagonistic effect of (i) interspecific competition between entomopathogenic nematodes and other biological control agents, (ii) natural enemies, and (iii) omnivores and scavengers on entomopathogenic nematodes.

9.2. Competition with Other Biological Control Agents

Interspecific competition from a microbial control standpoint may enhance efficacy by increasing insect susceptibility to nematode infection, reducing the period of lethal infection, or having an additive or synergistic effect on mortality (Kaya, 1993; Koppenhöfer *et al.*, 1999). Competition, however, often results in an antagonistic relationship that reduces fitness or displaces another species. The most common interspecific competition documented with entomopathogenic nematodes is with other biological control agents because the nematode and the other agent are vying for the same resource – the insect.

The interaction between entomopathogenic nematodes and other biological control agents (i.e. viruses, bacteria and fungi) of insects can be classified as indirect antagonism because the competition is for the host tissues and not directly between the biological control agents. The nucleopolyhedrovirus and granulovirus, for example, infect various host tissues and usually kill their insect hosts within 5–20 days (Tanada and Kaya, 1993). *Steinernema carpocapsae* will infect and develop in insect hosts previously infected with a nucleopolyhedrovirus (Bednarek, 1986; Kaya and Burlando, 1989) or a granulovirus (Kaya and Brayton, 1978). Nucleopolyhedrovirus-infected insects have a fragile integument, especially a day or two before death. When infective *S. carpocapsae* juveniles penetrate a nucleopolyhedrovirus-infected insect just prior to death from the virus, the nematodes hasten its death and develop normally until the insect's integument ruptures. At this time, the developing nematodes are exposed to the external environment, desiccate upon the disintegration of the insect host and die before they can produce infective juveniles. In the case of the granulovirus of the

armyworm, *Pseudaletia unipuncta*, the integument does not disintegrate during the course of the viral infection, but *S. carpocapsae* produces significantly fewer progeny in virus-infected than non-infected hosts. Presumably, the virus-infected hosts have less available nutrients or have catabolic by-products that adversely affect nematode reproduction.

Bacillus thuringiensis-infected hosts can be infected with *S. carpocapsae* or *Heterorhabditis bacteriophora*, but nematode development is dependent upon the timing of *B. thuringiensis* exposure to the host (Bednarek, 1986; Kaya and Burlando, 1989; Poinar *et al.*, 1990). Lepidopterous insect hosts exposed to the nematode first for 24 h and then to *B. thuringiensis* resulted in normal nematode development (Kaya and Burlando, 1989; Poinar *et al.*, 1990). When the insect hosts were exposed to *B. thuringiensis* first for 24 h and then to the nematode, nematode development was poor or non-existent. In the latter case, *B. thuringiensis* prevents the nematode's symbiotic bacterium from developing, whereas in the former case, the nematode's symbiotic bacterium prevents *B. thuringiensis* from developing. When the insect host was exposed to *B. thuringiensis* and nematodes simultaneously, dual infections occurred in a few cadavers. In these dual infections, the host's resources were partitioned with *B. thuringiensis* found in the anterior part of the host and the nematode found in the posterior part of the host (Kaya and Burlando, 1989). Developing nematodes in *B. thuringiensis*-infected insects were smaller, appeared more hyaline, and had less food reserves stored in their intestinal cells than those of the controls (Poinar *et al.*, 1990). In *B. thuringiensis* subspecies *israelensis*-killed mosquito larvae, the nematodes penetrated the cadavers, but the cadavers disintegrated before the nematodes could initiate their development (Poinar *et al.*, 1990).

Barbercheck and Kaya (1990) demonstrated that when the entomopathogenic fungus, *Beauveria bassiana*, and the nematode, *S. carpocapsae* or *H. bacteriophora*, were applied simultaneously to a wax moth (*Galleria mellonella*) host, the nematodes developed normally and produced progeny, almost always excluding the fungus. Lezama-Gutiérrez *et al.* (1996) obtained similar results using the fungus *Nomuraea rileyi* and *H. bacteriophora* against the fall armyworm, *Spodoptera frugiperda*. Fungal exclusion was probably due to the antibiotics produced by either *Xenorhabdus nematophilus* or *Photorhabdus luminescens* that inhibited the growth of *B. bassiana* (Barbercheck and Kaya, 1990). Application of *B. bassiana* to the insect host before the nematodes had an antagonistic effect on *S. carpocapsae* or *H. bacteriophora* (Barbercheck and Kaya, 1990). Although mycotoxins that adversely affected the nematodes may have been produced (Roberts, 1981), the evidence suggests that the fungus simply out-competes the nematodes for available resources. Given a choice between non-infected and fungal-infected hosts in the soil, the nematodes tended to avoid hosts infected with *B. bassiana* (Barbercheck and Kaya, 1991) – a behaviour that may minimize antagonistic interactions.

9.3. Natural Enemies

Entomopathogenic nematodes have their own suite of natural enemies as they are susceptible to infection by microorganisms or predation. Circumstantial evidence suggested that natural enemies adversely affect the nematodes because infective juveniles placed in sterilized or pasteurized soils survived longer than infective juveniles

placed in untreated soils (Ishibashi and Kondo, 1986, 1987). Empirical studies have shown that predation by invertebrate predators and parasitism by microorganisms can reduce the effectiveness of the infective nematodes. The bacterial symbionts also have their own natural enemies that can negatively impact the nematodes.

9.3.1. Phages and bacteria

Phages have been isolated from the bacterial symbionts, *P. luminescens* (Poinar *et al.*, 1989) and *Xenorhabdus* spp. (Boemare *et al.*, 1993). Lytic activity only occurred with the phase I (primary form) *Photorhabdus* but not with the phase II (secondary form) (Poinar *et al.*, 1989). The DNA of the bacteriophage was distinct from the DNA of *P. luminescens*, suggesting that this phage was probably from a non-*Photorhabdus* source. Conversely, Boemare *et al.* (1993) demonstrated that a phage induced by heat or mitomycin C in *Xenorhabdus* cells had DNA that was nearly homologous with DNA of the bacterial symbiont. Phages from foreign bacterial hosts or lysogenic phages that infect the bacterial symbiont may affect nematode production or the virulence of the nematode–bacterium complex. Furthermore, reduction of the bacterial symbiont will lessen the food supply for the developing nematodes and negatively affect the efficacy of the infective juveniles.

No viral or bacterial pathogens have been isolated from entomopathogenic nematodes. Recently, however, Marti and Timper (1999) reported that sporangia of an unidentified *Paenibacillus* sp. was found frequently attached to the sheath of infective juveniles of *Heterorhabditis* sp. that had emerged from wax moth larvae exposed to soil. The sporangia were spindle-shaped, 9–11 μm in length, and contained a central refractive endospore. The sporangia differed from *Paenibacillus popilliae* and their attachment appeared to resemble *Pasteuria* spores. Large numbers of sporangia occurred within the wax moth cadaver, and these sporangia attached to the cuticle of the infective juveniles. There was no evidence of pathogenicity to the infective juveniles, but more tests are needed to resolve the relationship of the sporangia with the insect cadaver and the infective juveniles.

9.3.2. Protozoa

Protozoan parasites (i.e. microsporidia), though rare, have been isolated from different nematode species including entomopathogenic ones (Poinar and Hess, 1988). Veremtchuk and Issi (1970) exposed *S. carpocapsae* to microsporidian-infected insects and found that *Pleistophora* (= *Plistophora*) *schubergi* and *Nosema mesnili* were pathogenic to the nematode. *P. schubergi* infected the intestinal cells of a few nematode individuals, whereas *N. mesnili* caused a more generalized infection in the nematode. However, the impact of either microsporidium on nematode fitness was not evaluated. These interactions where the pathogen infects both the natural enemy (in this case, *S. carpocapsae*) and the host of the natural enemy have been called ‘intraguild predation’ (Rosenheim *et al.*, 1995). Intraguild predation may prove to be a common source of antagonism for entomopathogenic nematodes.

An undescribed microsporidium was found infecting *S. glaseri* from a cerambycid beetle in Sao Paulo, Brazil (Poinar, 1988). This microsporidium produced 10–28 spores per sporophorous vesicle. It infected a number of tissues including hypodermal, intestinal and reproductive cells. The antagonistic effect on the nematode

varied from little apparent damage to mortality depending upon the degree of infection by the microsporidium. The infected infective juveniles were also smaller than uninfected ones and did not survive as long at 22°C. The source of the microsporidium is unknown; it may be from the insect host or specific to *S. glaseri*.

Poinar (1988) warned that nematodes should be checked for presence of parasites before they are introduced into mass culture systems. Their presence could adversely affect the quality of nematodes being produced for laboratory or field tests or for commercial sale.

9.3.3. Nematophagous fungi

Nematophagous fungi, found in a wide range of soil habitats throughout the world, occur in two basic forms (Barron, 1977; Gray, 1988). One form is the predatory or trapping fungi that capture nematode prey by using specialized hyphae (adhesive hyphae, branches, nets, or knobs or non-constricting or constricting rings). The hyphae then penetrate into the body cavity, killing the nematode host (Jaffee *et al.*, 1992). These trapping fungi can also survive as saprophytes. The other form is the endoparasitic fungi that infect hosts by using conidia or zoospores that attach to the nematode's cuticle or are ingested producing germ tubes that penetrate into the body cavity. The endoparasitic fungi are obligate parasites in nature. Both fungal forms have assimilative hyphae that consume the nematode's body contents with sporulation taking place either internally or externally. Some nematophagous fungi show host specificity, and others appear to be generalists. However, a generalist fungus may show greater virulence against one nematode species than another (Gray, 1988; Jaffee and Muldoon, 1995).

9.3.3.1. Trapping fungi

Jaffee *et al.* (1992) studied trap production of nematophagous fungi growing from *S. glaseri* (Fig. 9.1). Two days after exposure of *S. glaseri* to the fungi in 1.5 ml of soil extract, *Arthrobotrys oliogospora, A. dactyloides, Monacrosporium ellipsosporum*, and *M. cionopagum* produced traps from this nematode. Although *A. dactyloides, M. ellipsosporum*, and *M. cionopagum* produced traps when growing from fungal-killed *S. glaseri* on agar, more traps were produced when healthy nematodes were introduced onto the agar plates. For example, *M. cionopagum* produced 14 traps without healthy nematodes, whereas it produced 138 traps in the presence of the nematodes. *A. oliogospora*, in contrast, produced traps only in the presence of the healthy nematodes. Similarly, Van Sloun *et al.* (1990) showed in a sand bioassay that the addition of the free-living nematode, *Panagrellus redivivus*, induced trap production in *Arthrobotrys robusta* and caused a 52% reduction in *S. feltiae* infective juvenile populations.

Infective juveniles of several steinernematid and heterorhabditid species are susceptible to trapping fungi (see Poinar and Jansson, 1986a; Van Sloun *et al.*, 1990; Koppenhöfer *et al.*, 1996). The adhesive traps of *Arthrobotrys* spp., *Monacrosporium eudermatum*, and *Geniculifera paucispora*, the adhesive branches of *M. cionopagum*, the adhesive glandular cells of *Nematoctonus concurrens*, and the adhesive knobs of *M. ellipsosporum* captured and infected infective juveniles. Some infective juveniles managed to detach themselves from *M. ellipsosporum* and escape (Poinar and Jansson, 1986a), but the knobs remained attached to the cuticle and could cause a fungal

infection in the nematodes. Infection occurred only when the infective juveniles were without their sheathed second-stage cuticle. Infective juveniles with a sheathed cuticle escaped infection by exsheathing. In another laboratory study, soil bioassays with *A. oligospora, M. eudermatum, G. paucispora, M. cionopagum* and *N. concurrens* reduced penetration of *Heterorhabditis marelatus* (= *hepialus*) into wax moth larvae by up to 54% (Koppenhöfer *et al.*, 1997).

Jaffee *et al.* (1996) hypothesized that nematophagous fungi were significant mortality factors of the entomopathogenic nematode, *H. marelatus*, in a coastal shrubland and affected the abundance of the lupine bush. The larvae of the ghost moth, *Hepilaus californicus*, feed on the roots of lupine initially and then bore up into the crown of lupine to pupate in the lower shoots (Strong *et al.*, 1995). In some areas where the ghost moth larvae occurred in high numbers, they killed the lupine bush,

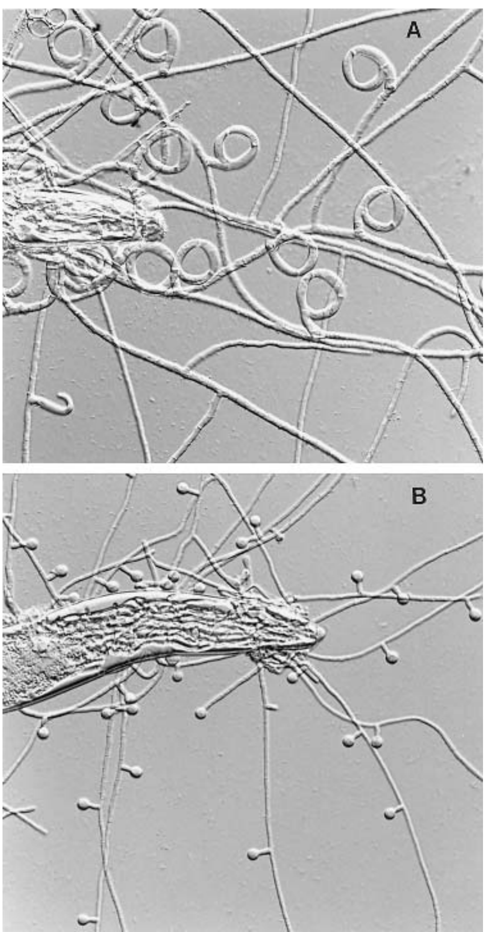

Fig. 9.1. Micrographs of nematode-trapping fungi growing from infective juveniles of *Steinernema glaseri*. (a). Constricting rings produced by *Arthrobotrys dactyloides*. (b) Adhesive knobs produced by *Monacrosporium ellipsosporum*. Magnification 160 ×.

and this mortality was attributed to the low numbers of *H. marelatus* available to control the larvae (Strong *et al.*, 1996). In other locations, the lupine bush flourished and *H. marelatus* numbers were high. Jaffee *et al.* (1996) isolated 12 species of nematophagous fungi with *A. oligospora* being the most abundant. Although some soil samples contained a large number of nematode-trapping fungi, areas with high lupine mortality did not contain larger numbers of fungi than did areas with little lupine mortality. They concluded that the spatial and temporal patterns in fungal abundance and in nematode suppression did not support the hypothesis that nematode-trapping fungi caused the patchy distribution of *H. marelatus* and therefore of the lupine. That is, there was no evidence for a subterranean trophic cascade (Strong *et al.*, 1999; Strong, this volume).

An extraordinary type of antagonism affecting entomopathogenic nematodes was observed with the trapping fungi, *Dactylaria* and *Arthrobotrys*, on the integument of mole crickets in Brazil (Fowler and Garcia, 1989). When field-collected mole crickets (*Scapteriscus borellii*) with and without the nematophagous fungi were exposed to a steinernematid species in the laboratory, those with the fungi had reduced mortality. For example, mole crickets with the fungi had 13% mortality, whereas those without the fungi suffered 63% mortality suggesting that these fungi afforded protection from nematode infection. This type of relationship between the trapping fungus and mole cricket has been referred to as protocooperation.

9.3.3.2. Endoparasitic fungi

Endoparasitic fungi rely on nematodes as hosts, but some may have a restricted host range. Poinar and Jansson (1986b) and Van Sloun *et al.* (1990) showed that *Drechmeria coniospora* did not infect entomopathogenic nematodes on an agar substrate even though the conidia attached to the nematode's cuticle. On the other hand, *Verticillium balanoides* did infect *S. feltiae* on the agar substrate but had no effect in sand (Van Sloun *et al.*, 1990). This observation was substantiated by Galper *et al.* (1995) who demonstrated that fungal activity on agar provided little indication of fungal infectivity in soil.

The endoparasitic fungus, *Hirsutella rhossiliensis*, has received considerable attention because it responds to nematode density (Jaffee, 1992). It has potential for biological control of plant-parasitic nematodes, but its wide host range includes entomopathogenic and free-living nematodes. An anomaly of this fungus is that its conidia must remain on the phialides to be infectious (McInnis and Jaffee, 1989). Detached conidia are non-infectious making the use of this fungus unlikely for inundative control against plant-parasitic nematodes.

Studies with entomopathogenic nematodes showed differential susceptibility to this fungus (Timper *et al.*, 1991). More *H. rhossiliensis* conidia adhered to the cuticle of *S. glaseri* and *H. bacteriophora* than to *S. carpocapsae*, demonstrating differential conidial adhesion and therefore differential fungal susceptibility. This observation was validated in artificially and naturally infested soil as this fungus caused higher mortality in *S. glaseri* than in *H. bacteriophora* or *S. carpocapsae*. The higher mortality of *S. glaseri* to the fungus was attributed to the nematode's inability to retain the second-stage cuticle and to its widely foraging (cruise) behaviour in the soil. This behaviour allowed *S. glaseri* to come into frequent contact with conidia attached to phialides that grew from hyphae emanating from the nematode cadavers. *Steinernema glaseri*'s larger size may

play a role in its susceptibility by having a greater surface area that could come into contact with the conidia in the soil pores. The lower susceptibility of the cruise forager *H. bacteriophora* was attributed to its ability to retain the second-stage cuticle (Timper and Kaya, 1989, 1992). Timper and Kaya (1989) observed that the conidium could attach to the second-stage cuticle and send a germ tube through this cuticle, but the germ tube could not penetrate through the third-stage cuticle. Thus, *H. bacteriophora* should be more effective as a biological control agent in *H. rhossiliensis*-infested soil where insect pests occur deeper in the soil profile than *S. glaseri* (Timper and Kaya, 1992).

9.3.3.3. Nematophagous fungi as antagonists

Nematophagous fungi have potential as biological control agents of plant-parasitic nematodes (Kerry, 2000) but could also adversely affect entomopathogenic nematodes. Linford *et al.* (1938) showed that nematode-trapping fungi appeared to be responsible for the decline of plant-parasitic nematodes. Some encouraging reports indicate that nematophagous fungi can exert a degree of population suppression with some plant-parasitic nematode species, but the overall assessment under field conditions was disappointing (Stirling, 1988). Jaffee *et al.* (1993) demonstrated that the endoparasitic fungus, *H. rhossiliensis*, had the greatest response to plant-parasitic nematode host density, whereas the trapping fungi, *A. oligospora* and *M. cionopagum*, had a low response to host density. Timper and Kaya (1992) concluded that *H. rhossiliensis* would probably have the greatest impact upon inundatively applied entomopathogenic nematodes, but through the proper selection of nematode species for insect suppression, this negative impact may be minimized. For instance, *H. bacteriophora* and *S. carpocapsae* are less susceptible to *H. rhossiliensis* because of the presence of the second-stage cuticle and the ambush foraging behaviour, respectively. Based on Stirling's (1988) finding, the nematode-trapping fungi would probably have less impact upon inundatively applied entomopathogenic nematodes. In fact, representatives of nematophagous fungi (i.e. *Dactylaria, Dactylella* and *Arthrobotrys*) did not show any increase in the number of propagules after application of *S. carpocapsae* and *H. bacteriophora* in the field (Forschler and Gardner, 1991). However, the infected juveniles could serve as foci for trapping healthy infective juveniles (Jaffee *et al.*, 1992). In natural ecosystems, there is no correlative evidence that nematophagous fungal populations suppress nematode populations (Jaffee *et al.*, 1996). Jaffee *et al.* (1996) stated that the correlative approach is not the most satisfactory method of determining the importance of nematophagous fungi as significant mortality factors of entomopathogenic nematodes. They suggest that experimental approaches in which fungal numbers are manipulated by augmentation or inhibition may be more useful.

9.3.4. Invertebrate predators

A number of invertebrate predators including protozoans, turbellarians, nematodes, tardigrades, oligochaetes, mites, and insects have been linked to population reduction of nematodes (Small, 1988). This linkage is not strong because the data are mostly qualitative.

One of the first records of entomopathogenic nematode predation was by Poinar (1979) who observed mites in the genus *Macrocheles* feeding upon *S. carpocapsae*

infective juveniles. Subsequently, Ishibashi *et al.* (1987) reported that the mesostigmatid mite *Eugamasus* sp., the tardigrade *Macrobiotus richtersi*, the mononchid nematode (*Clarkus* sp.), and the dorylaimid nematode (*Actinolaimus* sp.) preyed upon live infective juveniles of *S. carpocapsae*.

A large variety of predatory soil mites, especially the mesostigmatids, will feed on nematodes (Walter, 1987, 1988). Some mesostigmatid species can access small pore spaces in the soil, have high reproductive potential, and prey preferentially on nematodes (Walter, 1988). Studying the mesostigmatid mite, *Gamasellodes vermivorax*, Epsky *et al.* (1988) demonstrated that it reduced *S. carpocapsae* efficacy against wax moth larvae in the laboratory. This mite could complete its development by feeding only on the infective juveniles, but survival was poor. Epsky *et al.* (1988) also showed that the endeostigmatid mite, *Alycus roseus*, and a collembolan, *Hypogastura scotti*, completed their development from late-instar nymphs to adult and produced viable eggs by feeding on infective juveniles. Two other collembolan species, *Folsomia candida* and *Sinella caeca*, readily consumed *S. carpocapsae, S. feltiae* and *S. glaseri* (Gilmore and Potter, 1993). In Petri dish tests, wax moth larval mortality caused by *S. carpocapsae* was inversely related to the length of time that the nematodes were exposed to predation by *F. candida*. On a positive note, mortality of the Japanese beetle larvae by *S. glaseri* was not affected by the presence of *F. candida* in turfgrass plug tests. *Steinernema glaseri*'s effectiveness was attributed to its cruise foraging strategy and the restricted vertical movement of *F. candida* in heavy soil. In one field evaluation, Forschler and Gardner (1991) showed that predatory mite (i.e. Rodacaridae) populations increased 1–4 weeks after application in plots treated with entomopathogenic nematodes. However, there was no relationship between mite populations and insect control.

9.4. Omnivores and Scavengers

Omnivores and scavengers may play a significant role in the population dynamics of entomopathogenic nematodes. After the infective juveniles infect an insect host, the host dies within 48 h from the mutualistic bacteria associated with the nematodes. The nematode-killed host remains in or on the soil surface for 7–15 days or longer (depending on the nematode species and environmental conditions) before the infective juveniles exit from the host. During this period, the nematode-killed host (i.e. cadaver) is at risk of consumption by omnivores or scavengers. Are there mechanisms to protect the cadaver from being consumed? Akhurst and Boemare (1990) speculated that the bioluminescence produced by *Photorhabdus* in heterorhabditid-killed insects may serve to deter scavenger activity in soil. No experiments, however, have been conducted to determine the function of the bioluminescence.

Baur *et al.* (1998) conducted field experiments showing that several ant species fed readily on steinernematid-killed insects but poorly on heterorhabditid-killed insects (Table 9.1). More detailed field research was conducted with the Argentine ant, *Linepithema humile*, by placing nematode-killed insects near ant trials and observing the scavenging activity of the ants. Worker ants fed readily on 4-day-old steinernematid-killed insects but scarcely fed on 4-day-old heterorhabditid-killed insects. Some heterorhabditid-killed insects that were attacked had one or two bite punctures in their integument, resulting in desiccation of the cadavers and subsequent death of the

developing nematodes and their associated bacteria. The ants, whether they consumed nematode-killed insects or just made punctures in the integument, may have significant effects on the recycling of steinernematids and heterorhabditids. The impact of scavenger activity by ants on nematode-killed insects will probably be greatest on steinernematid recycling because heterorhabditid-killed insects appeared to have a 'deterrent' factor present.

Tests showed that the deterrent factor was present in wax worm larvae that were injected with the symbiotic bacterium (*P. luminescens*) associated with *H. bacteriophora* (Baur *et al*., 1998). The deterrent factor was associated with phase I of *P. luminescens* and not phase II (Kaya, unpublished data). In addition, 2-day-old *H. bacteriophora*-killed wax worm larvae were readily consumed by worker ants, whereas 4-day-old *H. bacteriophora*-killed wax worm larvae were not consumed. The data showed that the bacterium had not yet produced sufficient deterrent factor in the 2-day killed larvae to deter scavenging ants. Generally, other scavengers and omnivores were deterred from feeding on *P. luminescens*-killed insects, although the woodlouse (*Armadillidium vulgare*), fed on *H. bacteriophora*-killed termites and birds (scrub jay), removed *H. bacteriophora*-killed wax moth larvae from our ant test sites (Table 9.1). However, we did not observe the bird feeding on the cadavers.

The deterrent factor can also be produced by *P. luminescens* in nutrient broth with high repellency at 120–132 h of fermentation (Fig. 9.2; Zhou and Kaya, unpublished data). When the nutrient broth containing the fermentation product is mixed with sucrose and placed near Argentine ant trials, the ants will feed on the nutrient broth alone plus 5% sucrose (control) or the bacterial fermentation product plus 5% sucrose with equal or slightly higher intensity between 0 and 108 h. After this time, the percentage of ants at the bacterial suspension declines reaching a low point at 132 h. However, if the percentage of sucrose in the broth is at 20 or 25%, the deterrent factor appears to be masked as some feeding by ants will occur. Our observations indicate that broth plus 5% sucrose provides consistent data to demonstrate the presence of the deterrent factor.

9.5. Conclusions

The infective juveniles of entomopathogenic nematodes occur in the soil rhizosphere in which they encounter a wide array of antagonistic microbial and metazoan organisms. Yet, entomopathogenic nematodes have been ecologically successful as exemplified by their wide distribution in various soil habitats throughout the world. Their high reproductive capability (i.e. *r*-selected species) accounts, in part, for their ecological success. Avoiding or escaping from their natural enemies is another means of being successful. The infective juveniles can evade antagonists by their foraging behaviour or have morphological structures (e.g. second-stage cuticle) and physiological factors (e.g. lack of receptor sites on the cuticle) that protect them from nematophagous fungi. The production of antibiotics by the mutualistic bacteria within a host creates a favourable environment for nematode development. Although there is no evidence that bioluminescence associated with *Photorhabdus* protects the cadaver from scavengers, there is strong evidence that a factor(s) deters scavengers from consuming heterorhabditid-killed insects. The faster life cycle of steinernematids allows infective juveniles to exit the host quicker and may minimize the detrimental

Table 9.1. Feeding activity of scavengers and omnivores to *Photorhabdus* species associated with *Heterorhabditis* species offered in various venues.

Scavenger/omnivore scientific or common name + Family	Source of *Photorhabdus*	Response of scavenger/omnivore	Reference
Veromessor andrei Formicidae	4-day-old *H. bacteriophora*-killed wax moth larvae	Deterred from feeding on cadaver	Baur *et al.* (1998)
Pheidole vistana Formicidae	4-day-old *H. bacteriophora*-killed wax moth larvae	Deterred from feeding on cadaver	Baur *et al.* (1998)
Formica pacifica Formicidae	4-day-old *H. bacteriophora*-killed wax moth larvae	Deterred from feeding on cadaver	Baur *et al.* (1998)
Monomorium ergatogyna Formicidae	4-day-old *H. bacteriophora*-killed wax moth larvae	Deterred from feeding on cadaver	Baur *et al.* (1998)
Linepithema humile Formicidae	4-day-old *H. bacteriophora*-killed wax moth larvae	Deterred from feeding on cadaver	Baur *et al.* (1998)
L. humile Formicidae	4-day-old *H. bacteriophora*-killed *Tenebrio* larvae	Deterred from feeding on cadaver	Zhou and Kaya, unpublished
L. humile Formicidae	4-day-old *H. megidis*-killed wax moth larvae	Deterred from feeding on cadaver	Baur *et al.* (1998)
Pheidole megacephala Formicidae	4-day-old *H. bacteriophora*-killed wax moth larvae	Deterred from feeding on cadaver	Kaya, unpublished
P. megacephala Formicidae	2-day-old *H. bacteriophora*-killed wax moth larvae	Feeding on cadaver	Kaya, unpublished
L. humile Formicidae	*Photorhabdus* in nutrient broth plus 5% sucrose	Little feeding on sucrose compared with control	Zhou and Kaya, unpublished
Armadillidium vulgare Armadillidiidae	4-day-old *H. bacteriophora*-killed *Zootermopsis*	Feeding on cadaver	Nguyen and Kaya, unpublished
Periplaneta americana Blattidae	4-day-old *H. bacteriophora*-killed *Zootermopsis*	Deterred from feeding on cadaver	Nguyen and Kaya, unpublished
Scrub jay Corvidae	4-day-old *H. bacteriophora*-killed wax moth larvae	Removed cadavers in ant tests	Zhou and Kaya, unpublished

effects of scavenging activity. By understanding how natural enemies and antagonists affect entomopathogenic nematodes, use of these nematodes in inundative or augmentative biological control programmes can be enhanced.

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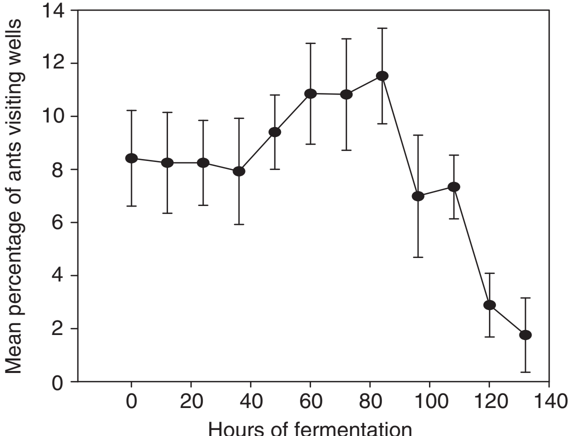

Fig. 9.2. Mean percentage (± SEM) of ants visiting *Photorhabdus luminescens* suspension fermented over time in nutrient broth. Bacterial samples were taken at 12-h intervals and frozen until all samples were taken. Bacterial suspension was thawed, sucrose added to give a 5% concentration, and 0.21 ml of the sucrose/bacterial suspension was placed into a well of a 96-microtitre well plate. The treatments were randomized and only wells along the edge of the plates were used. The total number of Argentine ants at the wells was counted at 10-min intervals for 1 h after placement into the field. The grand total number of ants was divided into the grand total number of ants at each well. This experiment was conducted at four field sites and repeated twice.

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10 Behavioural Ecology

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10.1. Introduction

Many studies of entomopathogenic nematode behaviour and ecology over the past 10 years were originally undertaken to discover why these lethal insect parasites provided unpredictable field efficacy. Laboratory tests found a single species of entomopathogenic nematode, *Steinernema carpocapsae*, able to infect hundreds of species of insects (Poinar, 1990). However, field tests against many of these pests proved

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disappointing. Poorer-than-expected field results have been attributed to several aspects of ecology and behaviour which were not understood at the time of the application (Georgis and Gaugler, 1991; Gaugler *et al.*, 1997). Here I discuss entomopathogenic nematode behaviours that impact biological control success either directly or indirectly. In the first section, I discuss behaviours that may help predict the outcome of field releases. The rest of the chapter addresses more specific aspects of entomopathogenic nematode behavioural ecology. I have grouped behaviour into four major categories: infective juvenile dispersal and location in the soil, foraging strategies, host discrimination, and infection dynamics. These distinctions are artificial and constructed for convenience, so when reading about one of these categories of behaviours, one must remember that they are not independent.

Most research has focused on the infective-stage juvenile because this stage is applied by users to kill insects. The infective-juvenile stage is an ideal subject for the study of basic foraging ecology. Important decisions confront all animals as they search for and assess resources. The outcomes of the decisions made are strong agents of selection on life history strategies (Koops and Abrahams, 1998). For entomopathogenic nematodes, the infective juvenile is the only free-living stage, and so it is faced with a single, vital decision: whether or not to infect a potential host. Infective-stage juveniles do not feed, mate, or go through development outside the host. Once the infective juvenile commits to infection it resumes development, thus removing the possibility of exiting one host and finding another, should the present one prove unsuitable. This single decision potentially has more impact on future success than any other decision by that individual because it is intractable, and impacts all aspects of habitat quality for the future of that individual and the following generation. Indeed, this single decision could represent one of the most pivotal of any decisions made by any animal. For that reason, we would expect infective stage parasites to have extremely well-developed capabilities of finding hosts and assessing their quality. Much of the following chapter is devoted to analysing the work that has been aimed at understanding the infection process from the standpoint of the infective stage juvenile.

One general challenge facing entomopathogenic nematology is to broaden the taxonomic base of our research on behavioural ecology. Most knowledge is based upon studies conducted on a narrow representation of entomopathogenic nematode species. Indeed, when searching for refereed journal articles on *Steinernema* behaviour, one finds that more than 90% of the literature deals with only four species (Fig. 10.1). Fewer than half of *Steinernema* species have any citations about their behaviour. Not surprisingly, most studies focus on those species that are available currently for purchase as biological control agents. While reading this chapter, keep in mind that the diversity of entomopathogenic nematode behaviour may be poorly represented in most of the sections.

10.2. What Behaviours Predict Field Results?

Lack of consistency and predictability are arguably the two most formidable impediments to wider adoption of biological control in mainstream pest management (Georgis and Gaugler, 1991). There are certain aspects of behaviour and ecology that indicate biological control potential for entomopathogenic nematodes.

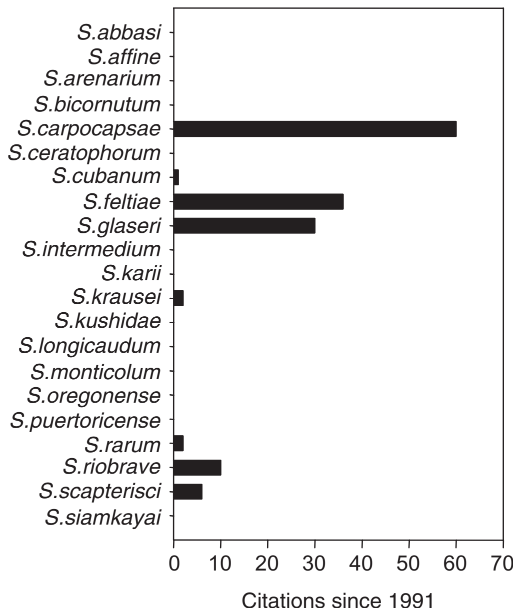

Fig. 10.1. Number of citations since 1991 of entomopathogenic nematode behaviour and ecology for each species of *Steinernema*. Source: The Insect-Parasitic Nematode Home Page (Smith, 2000).

Perhaps the most basic prediction we can make for any biological control agent is its location after application. Entomopathogenic nematodes forage for hosts in several ways, but all of them involve some kind of activity, so they do not necessarily stay where they are applied. Location may pertain to nematode vertical distribution within the soil profile or spatial distribution within an area. Vertical distribution has been studied in the laboratory and the field, and the results have yielded fairly consistent results: field populations of *S. carpocapsae* most often are found in the upper 1–2 cm of the soil whereas *H. bacteriophora* is distributed more-or-less evenly in the 8 cm of soil tested (Campbell *et al.*, 1996). Similar results were reported by Ferguson *et al.* (1995), who compared the relative locations of *H. bacteriophora* (NC and Oswego strains), *S. carpocapsae* (NY001), and *Steinernema* sp. (NY008) that were applied to fields. These authors found *H. bacteriophora* strains to be more evenly distributed vertically than either of the *Steinernema* species tested. In fact, *H. bacteriophora* was isolated from as deep in the soil as 35 cm. Laboratory studies have shown that *S. carpocapsae* infective juveniles tend to move upwards (Georgis and Poinar, 1983; Schroeder and Beavers, 1987), whereas *S. glaseri* and *H. bacteriophora* move primarily upwards, but also move throughout the soil column. Relative position in the soil profile has also been suggested as a contributor to spatial distribution, in that *S. glaseri* and *S. carpocapsae* may not compete for the same host species and therefore are able to maintain syntopy (Koppenhofer and Kaya, 1996). Position in the soil profile also constrains host affiliations.

Spatial distribution has received attention in natural and applied populations of entomopathogenic nematodes. Studies show that entomopathogenic nematodes are

patchily distributed, but that the degree of patchiness varies among species (Stuart and Gaugler, 1994; Campbell *et al.*, 1995, 1996; Glazer *et al.*, 1996; Strong *et al.*, 1996). These studies are not wide-area surveys such as that conducted by Gaugler *et al.* (1992), but studies of distribution on a within-field scale. In general, *H. bacteriophora* populations are more patchy than either *S. carpocapsae* or *S. feltiae* populations (Campbell *et al.*, 1998). These authors also showed that within 2 months of application of *H. bacteriophora* in a uniform distribution, their populations resembled natural patchy populations. Further, these authors showed that Japanese beetle grub populations were less dense within patches of *H. bacteriophora* than in areas where the nematodes did not exist, which suggests that these nematodes decrease host populations at natural densities. The driving forces behind these types of distributions are unclear. Edaphic factors and host distribution are potential causes, however no conclusive studies have shown their relative importance. A greater understanding of population distribution is essential to conserving nematodes at high densities in target habitats (Lewis *et al.*, 1998).

Determining entomopathogenic nematode host relationships is essential to predicting efficacy. Because new strains are generally collected using the *Galleria*-bait technique (Bedding and Akhurst, 1975), their natural host associations are usually unknown. Laboratory-based tests of host recognition may prove helpful in this area, and will be covered later in the chapter. Search tactics also govern host relationships, and will be covered in the next section. Host behaviour plays a key role as well.

Entomopathogenic nematode longevity is well-documented in the laboratory, but remains poorly understood in the field. Shelf-life is also affected by nematode behaviour. Lewis *et al.* (1995a) showed that *S. carpocapsae* has a relatively low metabolic rate compared with *H. bacteriophora* and that there were significant differences in behaviour that corresponded to differences in metabolic rate and survivorship. Corresponding differences are seen in the shelf-life of products that are composed of these species; *S. carpocapsae* products last longer on average than do *H. bacteriophora*-based products. Several accounts of field persistence have been published. Parkman *et al.* (1993a,b) have described the establishment and dispersal of *Steinernema scapterisci*, and have shown that these nematodes have had a suppressive effect on mole crickets of the genus *Scapteriscus* for up to several years. Klein and Georgis (1992) have also documented long-term establishment of entomopathogenic nematodes. These authors documented that the NC strain of *H. bacteriophora* was suppressing Japanese beetle populations 8 months after application. Despite a number of anecdotal reports in the literature, the reasons behind why some entomopathogenic nematode applications become established and others don't remains unknown.

10.3. Infective Juvenile Dispersal and Location within the Soil Profile

According to nearly all entomopathogenic nematode literature, when infective stage juveniles emerge from their host cadaver, their sole purpose is to parasitize a new host. This statement, while basically correct, implies that all individuals from a cadaver do the same thing, and that the only influence on the emerging infective juveniles comes from prospective hosts for which the infective juveniles are searching. This is an over-simplification, as there are differences between nematodes that emerge first versus later from a single cadaver (Lewis and Gaugler, 1994; Stuart *et al.*, 1996). The presence

of the cadaver from which the infective juveniles exit can also have a profound effect on how the nematodes disperse and respond to new prospective hosts (Shapiro and Glazer, 1996; Shapiro and Lewis, 1999). This section is dedicated to nematode movement and location in the soil that is not in response to host cues.

10.3.1. Not all nematodes are equal

All nematodes that emerge from a single cadaver are not necessarily the same with respect to behaviour. Considering that infective juveniles emerge from a cadaver over a period up to 3 weeks in some species, differences are likely to occur among individuals. Lewis and Gaugler (1994) demonstrated that male *S. glaseri* infective juveniles were the first to emerge from hosts (a condition termed 'protandry'), and that males were more responsive to volatile cues from an uninfected host than were females. However, *S. carpocapsae* males did not emerge before females, and in *S. feltiae*, females emerged first (Lewis, 2000, unpublished observation). These authors went on to show that female *S. glaseri* infective juveniles responded more strongly to volatile cues from infected than uninfected hosts. Stuart *et al.* (1996) found through selection experiments with *S. glaseri*, that the pattern of emergence (how many infective juveniles emerged when) and size both had genetic underpinnings. O'Leary *et al.* (1998) found that not only were *Heterorhabditis megidis* infective juveniles that emerged from their cadaver early better host-finders than were those that emerged later, but the early-emerging infective juveniles were more tolerant to warm temperatures and less tolerant to desiccation than their late-emerging counterparts. These phenomena taken separately add complexity to what we understand about entomopathogenic nematode infection dynamics. Together these results suggest that infection strategies are likely to be much more complicated than previously suspected, and that each species may have a strategy that is unique.

After emergence, as infective stages age their behaviour may change. Lewis *et al.* (1995a) compared three species of infective stages through time. The ability to move and infect a host generally decreased with time for *S. carpocapsae, S. glaseri* and *H. bacteriophora*. Again, it is difficult to make general conclusions for all species, other than the observation that all species became be less effective foragers with time. *Steinernema carpocapsae*, which forage by standing on their tails waiting to attach to passing hosts, began to do so with lower frequency, which made them less efficient foragers. The other two species, which both forage by moving through the soil, became less mobile with age. *Heterorhabditis bacteriophora* infective juveniles had the highest metabolic rate of the group, and the shortest lifespan. *Steinernema carpocapsae* infective juveniles had the slowest metabolism, and *S. glaseri*'s metabolic rate was intermediate. *Steinernema glaseri* lived the longest of the three species, probably because they are much larger and carry more energy reserves.

10.3.2. The role of the cadaver

Differences in infective juvenile movement and infection rate also have been related to the presence or absence of the host cadaver. Standard laboratory rearing collects emerging infective juveniles in a White trap (White, 1927) and stores them in water for days to weeks before use. Essentially all research on entomopathogenic nematodes is conducted with nematodes that are collected and stored in this manner. The dispersal

of *H. bacteriophora* and *S. carpocapsae* was measured when nematodes were applied to sand either in *G. mellonella* cadavers or in aqueous suspension collected in White traps. Both species moved farther when tested on agar and in sand columns when they were allowed to exit naturally from their host compared with those added in water (Shapiro and Glazer, 1996). Shapiro and Lewis (1999) showed that infectivity of *H. bacteriophora* was ten times greater when infective juveniles emerged naturally, as opposed to being collected in water and applied artificially. Interestingly, when a water extraction of homogenized host cadaver infected with conspecific infective juveniles 10 days previously was applied with the infective juveniles harvested from water, high levels of infectivity were recovered. These studies strongly suggest that chemicals associated with the host cadaver influence infective juvenile behaviour as they exit the host. What they also suggest is that predictions of natural population dynamics based upon experiments conducted with nematodes collected from White traps are unlikely to be accurate. However, nematodes harvested from White traps may be close in behaviour to nematodes that are applied as biological insecticides.

10.3.3. Abiotic factors

Other factors affecting entomopathogenic nematode movement are associated with abiotic, biotic and anthropogenic conditions in the soil. Soil type has been found to influence movement, survival and infectivity (Kung *et al.*, 1990), in that soils with small particles inhibit nematode movement, whereas sandy loam and sand appeared to provide a medium through which nematodes move easily. Cultural practices in agriculture, including addition of fertilizers and pesticides may also affect nematode behaviour.

10.4. Foraging Strategies

Parasites exhibit a range of strategies to increase the probability of encountering and recognizing a suitable host. Foraging strategies of entomopathogenic nematodes, and the many biological phenomena that are linked to them, have been studied extensively over the past 10 years. Several aspects of life history characters are either directly or indirectly linked to foraging strategy. The relevance of contact (Lewis *et al.*, 1992) and volatile (Lewis *et al.*, 1993) host cues, sex ratio (Lewis and Gaugler, 1994), mode of movement (Campbell and Kaya, 1999a,b), metabolic rate (Lewis *et al.*, 1995a), and longevity (Lewis *et al.*, 1995a; Lewis *et al.*, 1997) are some examples that have been studied. Within the genera *Heterorhabditis* and *Steinernema*, there is considerable variation in foraging strategies. The foraging strategies used by infective juveniles to find a host vary along a continuum between ambush and cruise foraging (Lewis *et al.*, 1992, 1993; Campbell and Gaugler, 1993, 1997; Grewal *et al.*, 1994). Nematode infective juveniles are motile, and their search behaviour can be divided into two broad categories: crawling and standing on their tails (i.e. nictation) (Campbell and Gaugler, 1997).

Most studies of foraging ecology follow the theoretical framework of optimal foraging (Pyke *et al.*, 1977). The utility of optimal foraging theory as it applies to entomopathogenic nematodes is not clear. As the theoretical framework is commonly used, optimal foraging theory is based upon the costs (measured in risk of predation or starvation) and benefits (measured in calories or host quality) for various kinds

of foraging patterns and decision making. This includes an enormous variety of situations, however all of them have in common that the subject of the study forages for more than a single resource. For example, parasitoid wasps lay eggs in several different hosts and lacewings eat more than one aphid. Entomopathogenic nematode infective juveniles find a single host. There is no opportunity for repeated foraging bouts, or assessment by the forager of the quality of the patch in which they are foraging, based on repeated samples of resources. There is also no obvious opportunity for the forager to learn about the quality of the resource through repeated foraging bouts. A separate foraging paradigm may be necessary for entomopathogenic nematodes and for all foraging of infective stage parasites that enter a single host and begin development.

10.4.1. Phylogenetic origins

Campbell (2000, unpublished results) has explored the phylogenetic origins of foraging strategies within the *Steinernema*. A proposed phylogeny, based on molecular (28S ribosomal DNA) and morphological data (P. Stock, 2000, unpublished results) suggests the evolutionary relationships among the species and serves as the framework for the evolution of foraging strategies. Some behavioural data for most *Steinernema* species have been collected, but not yet published. The species have been categorized into behavioural classes, 'ambusher', 'cruiser' or 'intermediate', based on their efficiency at attaching to mobile versus sedentary hosts (Table 10.1). In general, species

Table 10.1. Summary of behavioural tests for most species of *Steinernema* (Campbell, unpublished data).

Species	Foraging strategy based on attachment to mobile versus immobile	Nictate?	Jump?	Dispersal decreased by sand?	Ranging to localized search by host contact?	Attraction increased by host contact?
S. abbassi	ND	No	Yes	No	No	Yes
S. carpocapsae	Ambusher	Yes	Yes	Yes	No	Yes
S. ceratophorum	Intermediate	Yes	Yes	No	Yes	No
S. cubanum	Cruiser	No	No	No	Yes	No
S. feltiae	Intermediate	No	No	No	No	No
S. glaseri	Cruiser	No	No	No	Yes	No
S. karii	Cruiser	No	No	Yes	Yes	No
S. kraussi	ND	No	No	Yes	No	Yes
S. longicaudum	Cruiser	No	No	No	No	Yes
S. monticolum	Intermediate	No	Yes	No	Yes	No
S. oregonenses	Cruiser	No	No	No	Yes	No
S. puertoricenses	Cruiser	No	No	No	No	No
S. riobrave	Intermediate	No	Yes	No	No	No
S. siamkayai	Ambusher	Yes	Yes	Yes	Yes	No

ND = No data available.

that nictate (a behaviour explained in detail below) are classified as ambushers, species that do not nictate are classified as cruisers, and species that lift part of their bodies from the substrate, but do not do so for periods of more than a few seconds are termed intermediate. Movement on substrates that allow nictation or standing behaviour is slowed compared with movement on smooth agar for ambushers and sometimes for intermediate species, but not for cruisers. Responses to host cues are less consistent. The tests represented in Table 10.1 were all performed with *G. mellonella* serving as the source of host materials, and this insect species is not likely to be an optimal host for all nematode species tested. Differential responses to host cues are covered in the host recognition section in detail. This information has enabled probing of the evolution of foraging strategies in the form of the following questions: (i) Is ambushing or cruising the primitive state? (ii) How many times have the various foraging strategies arisen? (iii) Can foraging strategy be predicted based on entomopathogenic nematode phylogeny?

10.4.2. Movement during foraging

Understanding the mechanics of foraging behaviours is key to constructing predictions of how foraging strategy influences nematode biology. Ambushing nematode species nictate during foraging. By raising nearly all of their bodies off the substrate, nictating nematodes reduce the surface-tension forces holding them to the substrate, which enables them to attach to passing insects (Campbell and Gaugler, 1993). Nictation takes many forms, and ranges from straight motionless behaviour to partial lifting from the substrate and waving back and forth. Infective juveniles of some species, *S. carpocapsae* and *S. scapterisci* for example, spend most of their time in prolonged bouts of motionless nictation which may last several hours (Campbell and Gaugler, 1993), which is typical of ambushing species. For other species, *S. feltiae* or *S. riobrave*, for example, standing is rare in occurrence and short in duration. Standing behaviour for an ambush forager can be considered an immobile scanning or ambushing bout separated by repositioning moves that can take the form of crawling or jumping. One way to measure the propensity of a particular group of nematodes to nictate is to compare movement rates on smooth agar versus agar sprinkled with sand. The sand allows nematodes able to nictate to do so, while nematodes on plain agar will crawl unimpeded. If the nematodes nictate, the net movement rate is slower on sand-sprinkled agar (Table 10.1).

Many *Steinernema* species exhibit jumping behaviour, which is initiated from a standing posture (Campbell and Gaugler, 1993). To jump, nematodes form a loop with their bodies, with the head held to the side of the body by the surface tension. By contracting the loop until the body ‘kinks’, the nematode generates enough stored energy that when the loop is released, they are propelled through the air (Campbell and Kaya, 1999a,b). *Steinernema carpocapsae*, which is approximately 0.56 mm in length, can jump an average of 4.8 mm (nearly 10 body lengths) in distance and 3.9 mm in height (Campbell and Kaya, 1999a). The frequency of jumping, like standing behaviour, varies among species, and is increased by mechanical contact, air movement, and volatile host cues (Campbell and Kaya, 2000). These authors also found that *S. carpocapsae* and *Steinernema scapterisci* jump toward the source of host volatile cues, which suggests that jumping plays a role in host finding.

10.4.3. Response to host cues

When are host cues relevant to parasites? Entomopathogenic nematode species respond differently to cues associated with hosts, depending primarily on their foraging strategy. Lewis *et al.* (1992, 1993) tested the hypothesis that ambushing nematodes, represented by *S. carpocapsae*, would not respond as strongly to host cues as would cruise-foraging nematodes, represented by *S. glaseri.* The logic behind this hypothesis is that ambushers wait for potential hosts to come to them, whereas cruisers move in search of sedentary hosts. When responses to host volatiles were compared on an agar substrate, *S. carpocapsae* infective juveniles were weakly attracted to volatile cues from *G. mellonella* whereas *S. glaseri* were strongly attracted (Lewis *et al.*, 1993). In another study of nematode responses to host cues, Campbell and Kaya (1999a) have shown that *S. carpocapsae* infective juveniles do respond to host volatiles by jumping toward the source. In this case, substrate contributes to behaviour; Lewis *et al.* (1993) tested *S. carpocapsae* infective juveniles on smooth agar where they were unable to nictate or jump. The relevance of contact cues is often tested by determining whether or not exposure to a particular cue stimulates that forager to switch from ranging (more-or-less linear, high speed movement in the absence of cues) to localized search (a slower, more area-concentrated movement with an increased turning rate) (Bell, 1991). Lewis *et al.* (1992) found that when *S. glaseri* infective juveniles were exposed to host cuticle, their searching behaviour changed by becoming localized, whereas *S. carpocapsae* infective juveniles did not change their search patterns after contact with host cuticle. Grewal *et al.* (1994) went on to test responses to various types of host cues by several species of entomopathogenic nematodes, and found that the nematodes' responses to host cues were reliable predictors of foraging strategies for these species. In other words, if the infective juveniles of a species did not respond to volatile cues from a host, that species was likely to be an ambusher.

Considering that nematodes are limited to mostly chemical information to assess their surroundings, it stands to reason that *S. carpocapsae* and other ambushing species use some kind of chemical cue to find and assess hosts. Lewis *et al.* (1995b) proposed that for ambushing nematodes, cues must be presented in a particular order. A cascade of events leading to host recognition and penetration must be started by one specific cue. The order of events during host finding is predictable for ambushing nematodes (Fig. 10.2). For ambushing nematodes, a likely first host cue would be contact with the cuticle when the infective juvenile encounters a passing host. The portal of entry for *S. carpocapsae* into hosts is commonly the spiracles, from which CO_2 would escape. Indeed, *S. carpocapsae* responded strongly to volatile cues from *G. mellonella* only after contact with *G. mellonella* cuticle (Lewis *et al.*, 1995b). In the same study, these authors showed that exposure to host cuticle also resulted in infective juveniles penetrating into the host haemocoel at a faster rate than infective juveniles not previously exposed to cuticle. This work suggested that *S. carpocapsae* infective juveniles responded to host cues in a strict hierarchical order; host volatiles were attractive only after infective juvenile nematodes were in contact with the host.

The order of events for cruise-foraging nematodes is probably not as predictable as it is for ambushers (Fig. 10.2). A proposed order of events of cruise-foraging nematode species is easily understood. The nematodes move through the soil matrix, respond first to remote (volatile) cues from a host and follow the gradient that the

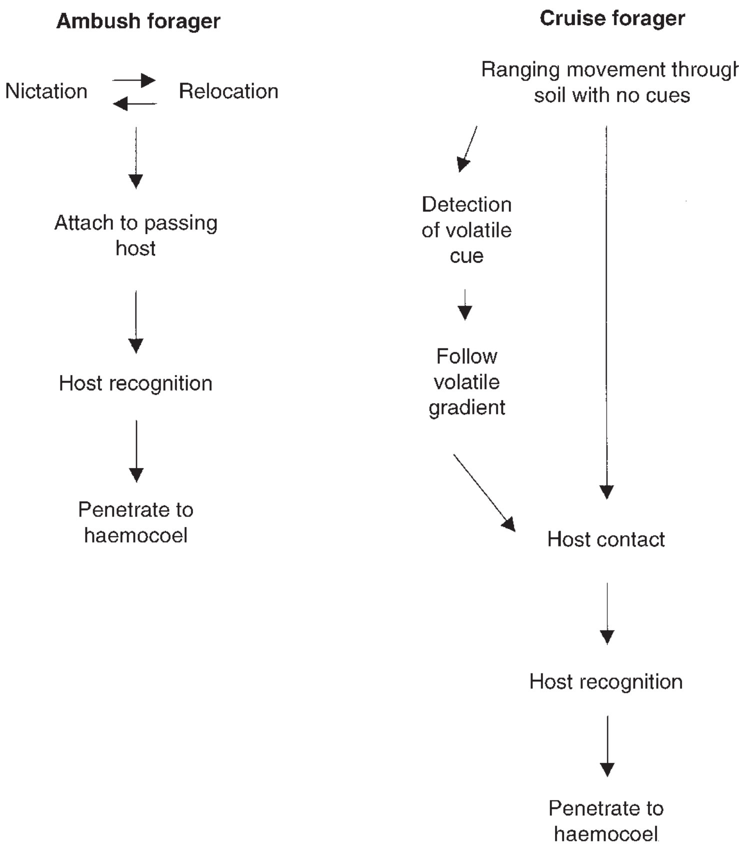

Fig. 10.2. The order of events that occur during a bout of host finding for ambushing nematodes and cruising nematodes.

volatiles form. Upon contacting a potential host, if the infective juveniles receive information via contact cues associated with the insect confirming its acceptability, they penetrate the host haemocoel. However, the presence of the volatile cues is not necessary to stimulate a response to contact cues, and vice versa. In other words, the order of cues need not be consistent. For example, a nematode could be foraging and approach a host from a direction other than where a volatile gradient has formed, or the forager could come into contact with materials (such as faeces) that are not part of the host but nevertheless indicate the host's proximity. Thus, cruising foragers are more flexible in their response to cues. *Steinernema glaseri* infective juveniles are strongly attracted to volatiles and switch their movement from ranging search to localized search after contact with host cuticle.

10.5. Host Discrimination

Location and foraging strategy only partially govern a nematode species' range of hosts. Host recognition behaviours, acceptance behaviours, and infection behaviours all play significant roles in the infection process. Host recognition has been measured

by logging changes in several types of behaviours in response to host-related materials. Grewal *et al.* (1993a) tested the responses of infective juvenile *H. bacteriophora, S. glaseri, S. carpocapsae* and *S. scapterisci* to gut contents of four candidate host species: *Acheta domesticus, Popillia japonica, Spodoptera exigua* and *Blattella germanica.* By cataloguing several movement parameters (duration of crawling, body waving, head waving, etc.) and a behaviour termed 'head thrusting', these authors related changes in these behaviours due to contact with gut contents with the nematodes' effectiveness killing these hosts. Relating these behavioural shifts to the nematodes' pathogenicity toward the hosts was an essential part of these studies. It is necessary to show that the behaviours are strongest in response to the host species that are most suitable in terms of parasite fitness, which can be measured by infectivity or fecundity.

10.5.1. Host recognition by *S. carpocapsae*

Lewis *et al.* (1996) studied the behavioural response of *S. carpocapsae* infective juveniles to host cues presented in the hierarchical order explained by Lewis *et al.* (1995b). Exposure to host cuticle was followed by testing their response to a volatile gradient. Nine species of candidate arthropod hosts (*Musca domestica, Agrotis ipsilon, Diabrotica virgifera, Leptinotarsa decimlineata, P. japonica, Acheta domestica, B. germanica*, an isopod and a chilopod) and two control surfaces (agar and wax) were the contact cues. The response of the exposed nematodes to volatiles from *G. mellonella* larvae was the parameter used to indicate the recognition response. Like studies on host gut contents and faecal material, infectivity to each host was compared with the recognition response. At a dose of 200 infective juveniles per host, there was a positive relationship between recognition and host mortality. However, at 500 infective juveniles per host, this relationship degraded. This finding suggests that natural host relationships can be obfuscated by exposing hosts to very high doses of nematodes. Further, this study showed that the strongest behavioural responses by *S. carpocapsae* were elicited by those candidate hosts that supported the highest levels of infective juvenile production. These studies support the idea that measures of host recognition might be useful in the characterization of new isolates from the field. However, the various host recognition assays must be designed specifically to match the foraging strategy of the nematodes being tested. The assay described is likely to work exclusively with nematode species that ambush. Ambushing nematodes can be recognized using movement on sand-covered versus smooth agar as described above.

10.5.2. Host recognition by *S. glaseri*

A one-step bioassay for host recognition has been suggested by Glazer and Lewis (2000). This assay would resemble the work on contact cues by Lewis *et al.* (1992) in that test nematodes would be exposed to host cuticle for 5 min and then placed on plain agar. The variable, 'giving-up-time', is measured by placing a nematode in the centre of a 1-cm diameter ring drawn on the agar dish and recording the amount of time the nematode takes to leave the ring. This test is based on the nematode switching from ranging to localized search, which is described above. Typical values of giving-up-time for *S. glaseri* are 1600 s after exposure to *P. japonica* larvae, and 700 s after exposure to *A. domestica* cuticle. Control values (no exposure to cuticle) are in the region of 100 s (Glazer and Lewis, 2000). A meaningful range of hosts remains to be tested.

10.5.3. Standardization

Excluding Petri dish assays, there have been no serious attempts to test host affiliations for most species other than *S. carpocapsae, S. feltiae, S. glaseri* and *H. bacteriophora*. Using the assays described above for ambush and cruise foraging nematode species, this could be accomplished. The first step would be to determine which of the tests to use against a particular species. One way to choose could be to categorize the species as either nictating or non-nictating. The two-step test would be used for nictating species and the one-step test for non-nictating species. A group of insect species to test should be chosen for all species, and this group could be used as a standard. The group should include easily attainable members of the major insect orders. Nematode response to each of the candidate hosts would be recorded. To validate the association, infectivity and the level of infective juvenile production per gram of host tissue would also be tested for all host species with the idea that infectivity and reproduction should be highest in the hosts that elicit the strongest behavioural response. If this test was conducted for new species and strains as they were collected, we would have a much better concept of natural host affiliations than we do at present. This would also enable more informed predictions about what species of insect pests a particular nematode species might be effective in controlling.

10.6. Infection Dynamics

Despite a reasonably complete understanding of the events that lead to and culminate in infection, what happens among the nematodes within the host, and how those inside the host affect approaching infective juveniles is still largely unexplored. These relationships have a great impact on field biology, population ecology, and population genetics. There are also more proximate effects of these interactions such as how they impact infection decisions by infective juveniles outside an infected host.

10.6.1. The infection as a resource

The quality of a host is dynamic and can be measured in a number of ways, but the ultimate currency of value for the parasite is the level of fecundity that is supported by a particular host. The quality of a non-infected host can be measured in these terms and is relatively constant. However, only one individual infective juvenile infects a host that was not infected previously. Because entomopathogenic nematodes can have several to well over 100 individuals infecting a single host, infective juveniles must assess the quality of the host in terms of the on-going infection. Once a host has been entered by a single infective juvenile, the quality of the host as a resource is dynamic, and there are costs and benefits associated with entering a host that has been previously infected (Grewal *et al.*, 1993b, 1996). Potential costs include competition among conspecific or heterospecific parasitic stages for limited resources. Selvan *et al.* (1993) demonstrated that a low number of founding nematodes causes the *per capita* reproductive rate to be low (the Allee effect). This indicates that for a given host, there is an optimal number of founders. Low numbers of founders could be detrimental because the mortality risk from the host's immune system might not be surmounted by too few individuals, or (for steinernematids only) the likelihood of finding a mate is low if too few conspecific individuals are present. The costs of over-infecting a host

include rapid depletion of resources and an increased risk of secondary invaders. Thus, each parasite's success depends on its ability to assess the condition of a potential host. Inside the infected insect, rapid changes take place as the infection progresses (Fig. 10.3). The symbiotic bacteria begin log-phase growth several hours after the infective juvenile enters the host haemocoel (Wang *et al.*, 1995). Nematodes feed on the bacteria and develop into fourth-stage juveniles and then to adults. The insect host dies usually within 3 days of infection, so any immune response has been mounted and overcome within this period. The speed at which these events occur increases with warmer temperatures and is faster with greater numbers of nematodes, so Fig. 10.3 is a generalized representation of events.

10.6.2. How infective juveniles respond to infected insects

Given the conditions described above, it is not surprising that infective juveniles respond differently to infected hosts than they do to uninfected hosts. Lewis and Gaugler (1994) found that *S. glaseri* female infective juveniles were more strongly attracted to *G. mellonella* larvae after they had been infected by conspecific nematodes. In fact, several species of entomopathogenic nematode respond differently to infected versus non-infected hosts (Grewal *et al.*, 1993b, 1996). All of these studies showed a heightened attraction to hosts that were infected recently (within 24 h) by conspecific nematodes.

Effects of infections are not limited to increasing attractiveness to conspecific nematodes. A follow-up study to the ones mentioned above demonstrated that not only did infective juveniles respond more strongly to infected hosts, but they responded differently to hosts infected with conspecific versus heterospecific nematodes (Grewal *et al.*, 1996). *Steinernema carpocapsae* infective juveniles were actually repelled from hosts infected with *S. glaseri*. In contrast, *S. glaseri* infective juveniles responded no differently to a host infected with *S. carpocapsae* than they did to a non-infected host. These authors also tested infective juvenile response to hosts injected with *Xenorhabdus* spp. bacteria and found that bacteria alone did not cause

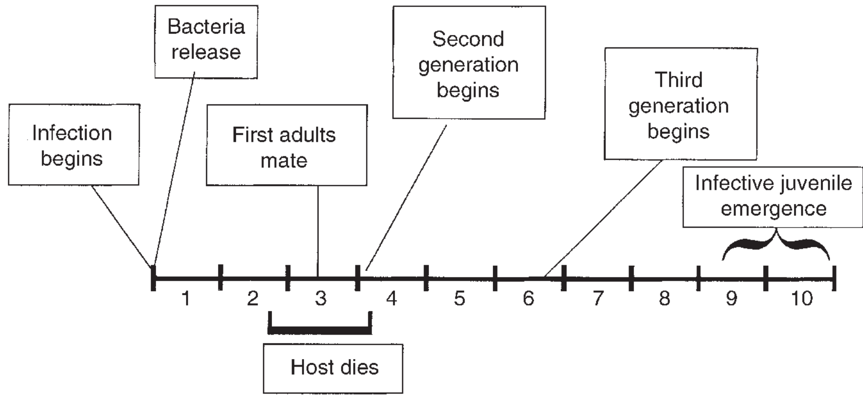

Fig. 10.3. Generalized time line representing the progress of an infection by *Steinernema carpocapsae* at 25°C.

these changes in infective juvenile behaviour. Why are some species of infective juveniles repelled from heterospecific infections while others seem unaffected? One potential reason is that nematode species that are strong competitors within the cadaver may not be selected against by entering a pre-existing infection initiated by another species because they will 'win', whereas weak competitors will be selected against because they will produce no progeny in such a circumstance. Typically, when an insect is co-infected with two species of entomopathogenic nematodes, only one of them will produce infective juveniles (Alatorre-Rosas and Kaya, 1991).

Another type of interaction, found by Glazer (1997), occurred when infective juveniles were exposed to exudates from hosts infected by conspecific nematodes. Penetration rate was decreased by this exposure. This finding may represent a mechanism that enforces an optimum number of founding nematodes in an infection. A comparable task is decision making by female parasitoid wasps. When searching for insect hosts, females assess potential hosts for previous parasitization by conspecific and heterospecific parasitoids before oviposition (Godfray, 1994 and references therein). There are significant differences between insect parasitoids and entomopathogenic nematodes, the most important probably being that the parasitoid making the assessment does not actually enter the host, whereas the infective juvenile does, although the cues used by the two decision makers may be similar. In some ways the entomopathogenic nematode infection is potentially more complicated than that of most parasitoids. The complication of how many nematodes penetrate over what period of time is still unresolved. The number of founders and the age of the infection are both likely to affect host quality, but their respective contributions have not been elucidated. The infection dynamics of entomopathogenic nematodes are complicated, with many influences that work in synchrony and opposition.

10.6.3. Descriptions of infection dynamics

Several studies have been attempted to describe these interactions mathematically with varying degrees of success. Fan and Hominick (1991) suggested that only a proportion (less than 40%) of *S. feltiae* infective juveniles that emerged from a host cadaver were infectious at any time, regardless of host availability. The so-called 'phased infectivity hypothesis' has been tested and discussed at great length since this initial observation. Mathematical descriptions of infection dynamics have been published for *S. feltiae* (Bohan and Hominick, 1996, 1997) that describe the short- and long-term interactions between a cohort of infective juveniles and potential hosts, and concur with the phased infectivity hypothesis: that a portion of infective juveniles that emerge from a host is not infective. These studies were the first to describe infection dynamics of entomopathogenic nematodes mathematically, and were useful in that respect. These studies assumed, for the sake of simplification of their models, that infective juvenile nematodes found hosts randomly.

Infective juveniles do not find hosts via random movement, and they make infection decisions based on characteristics of the hosts. Campbell *et al.* (1999) found that when provided enough hosts, nearly all infective juveniles that result from a given infection will infect a host immediately. The poorly-understood interactions between infective juvenile parasites and potential hosts have resulted in difficulties in interpreting the infection dynamics of entomopathogenic nematodes. Changes in host

suitability during infection may help to explain why not all infective stages will infect a host even when placed in close contact (Campbell *et al.*, 1999). Preliminary studies (J.F. Campbell, Kansas, 2000, personal communication) indicate that infective juvenile nematodes will penetrate a previously-infected insect up to 72 h after the initial infection. At this point in the infection, the founding nematodes have reached the adult stage and the host has died. The fitness consequences of infection at different times are not known, nor are the cues that the nematodes use to assess host status. The idea of phased infectivity remains in dispute.

10.7. Future Prospects and Conclusions

Despite years of intensive study of entomopathogenic nematodes, a great deal remains to be learned about their behaviour and ecology. Of the four basic areas of behaviour and ecology named early in this chapter, infective juvenile dispersal and location in soil, foraging strategies, host discrimination, and infection dynamics, our knowledge of infective juvenile foraging strategies is the most complete. The level of understanding of foraging ecology has made predictions of field success more accurate, and this work has been helpful in explaining why some early control failures may have occurred. A shortcoming here is that few species have been studied in this context. A primary challenge in the coming years is to expand this information to include more of the species and strains that have been isolated recently.

The location of nematodes in the soil has received some attention, usually in the context of where nematodes that have different foraging strategies might be located in the field. These laboratory studies have been limited for the most part to tests where nematodes were applied to sand and their movement measured. Most of this work focused on vertical movement through tubes filled with sand. The artificial conditions of these studies and many of the studies on entomopathogenic nematode behaviour in general has caused some scepticism as to the utility of the results. Some field studies have been conducted, and they have for the most part, but not always, upheld predictions based on laboratory results.

Spatial dynamics of entomopathogenic nematode populations in the field has been studied to some extent with endemic populations and applied nematodes. Entomopathogenic nematode populations are patchily distributed, and are best described as meta-populations (Lewis *et al.*, 1998), which is a group of separate populations that have limited genetic exchange. The underlying reasons for this type of population structure remains unknown. Another fruitful area for research will be to elucidate what drives the spatial and temporal population dynamics and structure of these parasites in the field. For example, do entomopathogenic nematode populations mirror the spatial distribution of their hosts, or are other abiotic factors more influential in their distribution? Answers to questions like this will help to predict the fate of applied populations and hopefully enhance our ability to conserve populations that exist and to alter the habitat to encourage populations.

The area of host recognition and discrimination has received relatively little attention, considering the potential value of the information to be gained. The host ranges of some species have been shown to be much narrower than originally thought based on Petri dish assays of infectivity. Mechanisms for host recognition have been proposed for few species of entomopathogenic nematode. A standardized method of

determining host range would add much to the existing body of knowledge in this area. In addition, by linking host range with phylogeny we may develop a powerful predictive tool for new isolates.

The area of entomopathogenic nematode behavioural ecology that remains least understood is their infection dynamics. Although infection dynamics are central to understanding many of the other aspects of behavioural ecology addressed in this chapter, they are still unpredictable based upon what is known. This unpredictability stems in part from an incomplete understanding of how infections change with time, and what those changes mean to infective juveniles searching for hosts. Three important questions that remain to be asked are: (i) What happens to the quality of a host during infection, and how do changes in the host impact parasite decision making? (ii) How does the endogenous condition of the infective juvenile (age and experience) influence host assessment? (iii) What are the sources and identities of chemical cues from hosts of varying quality, and what role do they play in host assessment?

The significance of the relationship between behavioural ecology and biological control potential has been recognized for many years. So far, only the most obvious and direct relationships have been studied extensively for entomopathogenic nematodes. Other aspects of behavioural ecology that are less directly related to biological control are beginning to come under investigation. These aspects, such as infective juvenile decision making and the dynamic value of infected hosts as resources, are two such areas. Over the next 10 years, these areas of behavioural ecology will see more attention, and they may well be the key to predicting entomopathogenic nematode efficacy in the field.

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11 Populations of Entomopathogenic Nematodes in Foodwebs

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11.1. Introduction

The focus of this review is upon the foodweb roles of entomopathogenic nematodes. These worms are enemies of insects in soil, leaf litter, and other moist habitats in close contact with soil. They are widespread on all continents except Antarctica (see Hominick, Chapter 6 this volume). More than 90% of insect species have life history stages in the soil (Klein, 1990), thus exposure to entomopathogenic nematodes should be common. The few reviews indicate that the potential hosts of entomopathogenic nematodes include most of the orders of insects found above ground, but especially in non-agricultural habitats many litter-dwelling and underground insect species are probably still undetected (Brown and Gagne, 1990). The dearth of information means

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that our understanding of the ecology of the ecological roles of entomopathogenic nematodes is tentative.

The major idea of foodwebs is that indirect interactions follow from direct, pair-wise interactions between species (Menge, 1995). I will advance the argument that the natural enemies of entomopathogenic nematodes make for interesting and novel foodwebs that should be more branched and complex than the linear indirect chains of predator, herbivore and plant species: these linear trophic cascades gain most attention of ecologists (Polis *et al.*, 2000). My apostate claim is in spite of our experimental demonstration in nature of a trophic cascade in which the entomopathogenic nematode *Heterorhabditis marelatus* suppresses larvae of the ghost moth, *Hepialus californicus*, which, unchecked, feed heavily upon the roots of lupine bushes. This heavy feeding causes severe harm and even death to lupine. Evidence from larger scales of time and space than our experiment does not square with a trophic cascade. The high rate of mortality of *H. marelatus* infective juveniles in the soil appears to be partly the product of natural enemies, and nematophagous fungi are the most prominent suspects as predators; the dual nutrition of these predators make for reticulate interactions. As well, in recent months we have confirmed long-held suspicions of multiple insect host species for *H. marelatus*. This raises the possibility of apparent competition in the foodweb, which is not a linear trophic interaction.

11.2. Survival of Infective Juveniles in the Soil

Infective juveniles are both the dispersive and the resistant life history stage of entomopathogenic nematodes, roughly equivalent in an ecological sense to plant seeds and microbial cysts. While high short-term mortality of infective juveniles is a bane to inundative biological control (Smits, 1996), it is balanced by high fecundity. Tens of thousands to hundreds of thousands of infective juveniles can emerge from a single large, host cadaver (Mason and Hominick, 1995; Strong *et al.*, 1996).

11.2.1. Constant rates, short-term experiments and half-lives

Constant rates of survival can be expressed in terms of half-lives per chosen period of time. The length of the period is chosen relative to the rate of survival and to the purposes that the statistic is put. An advantage of expressing survival in terms of half-life is ease of extrapolation to the time of virtual extinction of a cohort. This indicates how frequently a lineage of entomopathogenic nematodes must recycle through hosts in order to persist. Low rates of survival of infective juveniles in soil amount to half-lives of days or weeks, while the highest measured rates of survival amount to half-lives of several months (Baur and Kaya, 2001). Half-lives of weeks extrapolate cohort survival to no more than a few months, while half-lives of several months extrapolate a large fraction of a cohort surviving for over a year. There are no published data that show definitively that infective juveniles survive in the soil for more than one annual cycle, although there is no reason to rule this out on principle. Good evidence of super annual survival would have to concern single cohorts of infective juveniles and rule out population turnover, 'recycling' through hosts.

For a constant rate of survival, half of a cohort is alive after one time period, a quarter of the cohort is alive after two periods, and an eighth of the original cohort is

alive after three periods. This progression yields about 1/100 of the cohort alive ($1/2^7 = 1/128$) after seven periods. Using the mnemonic that $2^{10} \cong 10^3$, then, *c.* 1/1000 of the original cohort will be alive after about 10 periods. After 16 or 17 periods, about one of 100,000 remain. Thus, from 10 to 15 half-lives should completely exhaust the potential for a cohort of entomopathogenic nematodes to persist. As I will argue below, even though many data on field survival rates of infective juveniles have been reported, we have very few data pertinent to the question of constancy or inconstancy of these rates. There are no experimental data on the minimum number of survivors required for a cohort of entomopathogenic nematodes to have one successful reproduction.

Soil survival rates of infective juveniles of both *Steinernema* and *Heterorhabditis* species have been measured many times in both the laboratory and the field. The most complete compilation is by Baur and Kaya (2001), who give data for tests done in the absence of hosts, for which no reproductive 'recycling' of infective juveniles occurred. I have plotted these data for survival in sand or soil as a histogram, with the shortest half-lives plotted per week and progressively longer half-lives plotted by increasing numbers of months up to 5 months (Fig. 11.1). About 18% of the estimated half-lives are of less than 1 week (thus, fewer than 1/1000 infective juveniles in these circumstances would live for 10 weeks). The mode of the data is a half-life of about 1 month. About 30% of the data yielded estimates of half-lives of 2 months or greater, with the extrapolation that a fraction of a cohort of thousands will persist in the soil for years, with constant rates of survival. Laboratory tests had a mean half-life of *c.* 62 days, while field tests yielded about half of this statistic (34 days). The mean half-life for the 47 tests done on *Steinernema* species was almost 60 days and all of the half-lives exceeding 1 month are for *Steinernema*. Thus, modal survival rates for this family are amply high to carry tens or even hundreds of infective juveniles from typically sized cohorts through the 6–10 month period of winter or other season inimical to growth in the mid and high latitudes.

11.2.2. Heterorhabditids have shorter half-lives than *Steinernema*

Lower survival rates for heterorhabditids than for steinernematids have been noted previously (Gaugler, 1981). Only about 18% (9 of 57) of the data in Baur and Kaya (2001) are for *Heterorhabditis* species. Heterorhabditids had a mean half-life of *c.* 34 days, about a month (Fig. 11.2). Recalling the generalization from above about half-lives yielding about one survivor in 1000 after ten periods, this mean rate makes for at least ten survivors of a cohort of 10,000 infective juveniles for the 8 to 10 months between the end of one annual growing season and the beginning of the next. However, most of the data for heterorhabditids are for half-lives shorter than 1 month. The data are skewed by two long half-lives (40 and 23 days), and seven of the nine data are less than the mean: 15, 12, 11, 10, 8, 5 and 3 days. For the maximum of these, 15 days, after ten periods (5 months) 10 of a cohort of 10,000 would remain, and after 15 periods (7.5 months), one would remain. So, for cohorts of 100,000 and more it is conceivable that the 15-day half-life would be sufficient to tide the cohort over an inhospitable season of 8 months. However, about two-thirds of the measured rates for heterorhabditids fall below 15 days, into a range where, as I point out below,

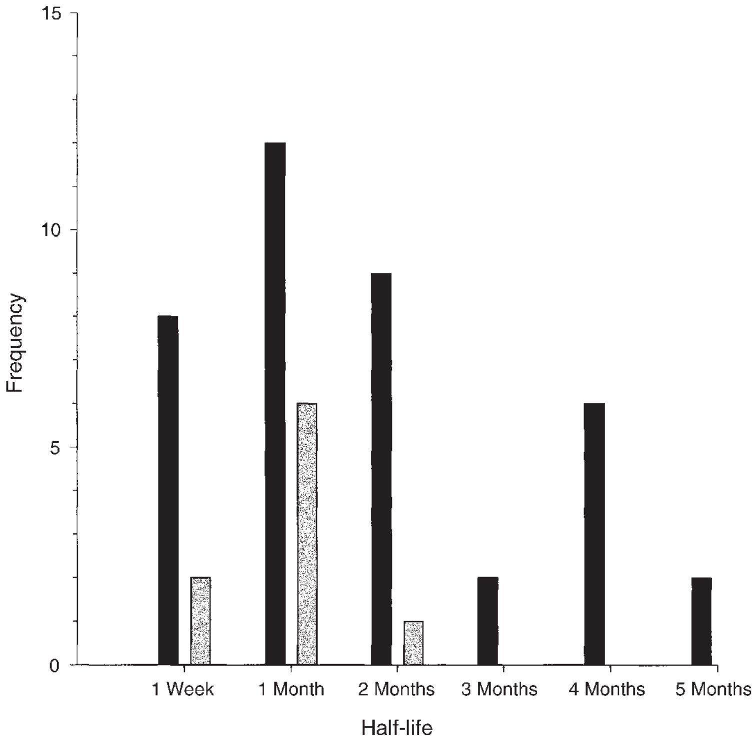

Fig. 11.1. Frequency distribution of half-lives of entomopathogenic nematodes plotted by months. Steinernematidae shown in black and Heterorhabditidae in grey. Data from Baur and Kaya, 2001.

half-lives of *H. marelatus* from Bodega Bay fall. Thus, for many heterorhabditids, either periodical recycling through new hosts or increased survival after an initial period in the soil is likely to be the way that cohorts persist through the annual cycle.

Environmental conditions can have tremendous influence upon survival rates. A dramatic example is shown in the difference of survival of *Steinernema riobravis* over a gradient of a mere 15 cm in soil (Fig. 4 in Duncan and McCoy, 1996). The data comprised survivors of nematodes that were placed in field soil. After 7 days, the *S. riobravis* surviving at the surface of the soil had decreased to 1% of those at day zero, at 3 cm in soil depth survivors had decreased by 69%. However, at 15 cm there was little or no decrease in survivors! Another example of extreme differences in survival will suffice to show the sensitivity of infective juveniles to a normal range of environmental conditions. *Steinernema feltiae* at 10°C had an estimated half-life of 330 days, from a 32-week laboratory experiment of Molyneux (1985). At 15, 23 and 28°C, the corresponding half-lives for these species were 11, 4 and 5 days, respectively (Baur and Kaya, 2001).

11.2.3. Changing rates and heterogeneous cohorts

The estimates of half-lives for infective juveniles from short-term experiments is only a first step in understanding the ecology of entomopathogenic nematodes. The next

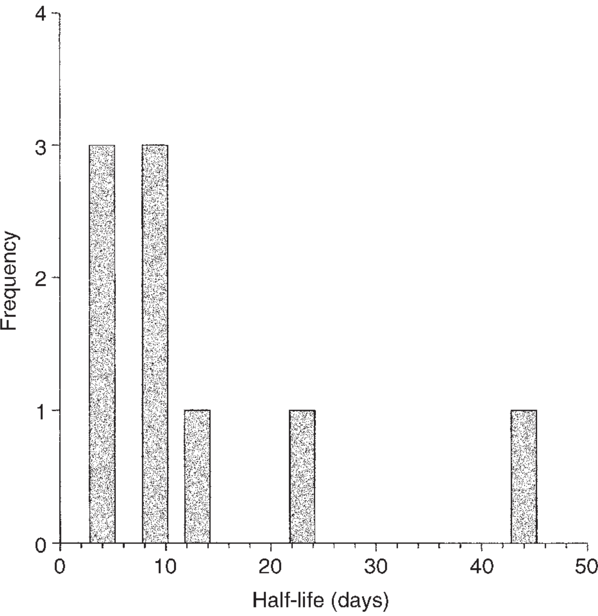

Fig. 11.2. Frequency distribution of half-lives of Heterorhabditidae plotted by days. Data from Baur and Kaya, 2001.

step is to consider what might lead to changing or heterogeneous survival rates within cohorts. Survival over the long term can increase relative to the rates observed during the first few days or weeks in the soil (Griffin, 1996; see Glazer, Chapter 8 this volume). The diversity of behaviours of entomopathogenic nematodes lead to the expectation that cohort survival can be affected by the response of infective juveniles to changed environmental conditions (see Lewis, Chapter 10 this volume).

Inhospitable conditions are weathered by inactive infective juveniles, and induced inactivity during stressful times can conserve resources for later more salubrious times. Low soil moisture (Kung *et al.*, 1991) and temperature (Fan and Hominick, 1991; Brown and Gaugler, 1997; Griffin, 1996) can temporarily reduce activity and lead to increased survival. Low soil moisture that induces a quiescent dehydration-survival state of *Steinernema carpocapse* is the most deeply understood from a physiological perspective (see Glazer, Chapter 8 this volume). Non-dispersing infective juveniles within cadavers have a higher survival rate in dry soil than those outside of cadavers (Koppenhöfer *et al.*, 1997a). In this category is the remarkably great increase in infectivity of a species of *Heterorhabditis* with increasing storage time in tap water (Griffin, 1996).

Finally, density affects the survival of infective juveniles. Positive density dependence means that per capita survival is higher at higher densities, and negative density dependence means that per capita survival decreases at higher densities (Stiling, 1987). Host cadavers represent an *en masse* refuge within which survival can be higher, per capita, than in temporarily inhospitable soil (Koppenhöfer *et al.*, 1997a); this is positive density dependence. Natural enemies that respond numerically to infective juveniles can create negatively density dependent survival (Jaffee 1996). The soil is rife

with natural enemies of entomopathogenic nematodes (see Kaya, Chapter 9, this volume).

Phased infectivity describes the observation that only a fraction of infective juveniles present and viable in the soil actually infect host insects presented to them, even under favourable conditions. This could mean that a fraction of the cohort follows a risk-spreading strategy, '. . . if nematodes are all infectious at the same time and no hosts are available, the nematodes may become locally extinct' (Kaya and Gaugler, 1993). As well, it could be that a kind of endogenous periodicity of infectiousness exists within cohorts (Bohan and Hominick, 1997).

Recent experiments showed that high densities of hosts (30 in a 90-mm Petri dish with 50 g of sand) quickly exhausted the competent fractions of infective juveniles of three species of *Steinernema*; few hosts placed subsequently in these arenas became infected (Campbell *et al.*, 1999). The pattern was different for *Heterorhabditis bactiophora*, however. Even this large number of hosts failed to exhaust the pool of infective juveniles in the arenas, and subsequent rounds of testing yielded substantial numbers of infected hosts. Developmental and environmental heterogeneity can form within cohorts of infective juveniles to produce the appearance of phased infectivity in the absence of innate heterogeneity. Differential movement of the worms (i.e. dispersal and emigration) can result in only a fraction of the cohort being close enough to a host to infect it at any time. Selective entry owing to the state of a host could also create heterogeneity in the cohort and a pattern of phased infectivity. For the dioecious *Steinernema* species, infective juveniles were clumped within hosts, consistent with this idea. As well, infective juveniles that emerge early from hosts were found to be more vigorous than those emerging later, creating another sort of heterogeneity in the cohort. In most situations in nature, hosts will be far less dense than in these experiments, leaving fractions of viable cohorts in the soil for subsequent infections. Thus, while a pattern consistent with phased infectivity should not be surprising in field data, especially for heterorhabditids, the physiological and genetic bases remain unclear.

11.3. *Heterorhabditis marelatus* at Bodega Bay, California

The first focus of our research programme was a three-species trophic cascade in which the entomopathogenic nematode *Heterorhabditis marelatus* indirectly protects bush lupine, *Lupinus arboreus*, by killing root-feeding ghost moth larvae (Lepidoptera, Hepialidae, Strong *et al.*, 1995). Lupines can suffer heavy root damage from these larvae, and mortality of lupine was correlated among sites at Bodega Bay with the number of larvae inside the root (Fig. 11.3). Experimental exclusion of ghost moth larvae by means of systemic insecticides demonstrated that even low numbers of larvae caused increased mortality and reduced seed set of bush lupine (Maron, 1998).

H. marelatus can cause high mortality to its natural hosts on roots of bush lupine: 65, 75 and 80% of large ghost moth larvae were killed in three sites respectively (Strong *et al.*, 1996; Table 1). The spatial distribution of *H. marelatus* was correlated with the long-term fluctuation in coverage of bush lupine. Where *H. marelatus* had the greatest incidence, at Mussel Point, lupine cover fluctuated less; where the nematode had the lowest incidence, lupine cover fluctuated most (Fig. 11.4). These correlations are consistent with the three-species model of a trophic cascade.

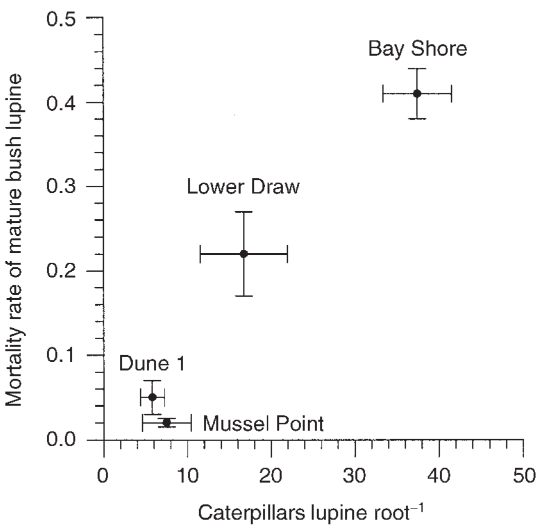

Fig. 11.3. Mortality of *Lupinus arboreus* as a function of numbers of ghost moth caterpillars, *Hepialus californicus* in the roots, at four sites at Bodega Bay. Redrawn from Figure 5; Strong *et al.*, 1995.

11.3.1. Survival of infective juveniles in the laboratory

The natural population of *H. marelatus* from Bodega Bay, California had short half-lives in laboratory experiments with raw soil. For example, in a 2-day experiment in containers with 15 cc of soil, infective juvenile survival ranged from 71 to 91% (Koppenhöfer *et al.*, 1996; Table 3), yielding half-lives between 4 and 15 days (Fig. 11.5). In other laboratory experiments, we have found half-lives as short as 2 days (survival of eight or nine from a cohort of 100, for 1 week) to as long as 1 month (85% survival after 1 week). There was no correlation between duration of the experiment and estimated half-life.

A few infective juveniles lingered in the soil of these experiments for as long as 8 weeks. These last few infections did not change the picture of half-lives for heterorhabditids. So few worms lived that the half-lives were almost identical to those calculated from the first week of the experiments. For example, the half-life of one of 100 infective juvenile recovered after 8 weeks yields a half-life of about 8 days [ln (0.5)/(ln (0.01))/56) = 8.4]. Thus, no evidence of survivorship rates increasing with time is found in the few survivors in these experiments, and *H. marelatus* half-lives are quite similar to those of other members of this family (Fig. 11.2). We must do long-term studies under field conditions in arenas without hosts to understand more about the survivorship of entomopathogenic nematodes.

11.3.2. A field experiment of plant protection by *Heterorhabditis marelatus*

Large bush lupine can withstand the feeding of a few ghost moth larvae each year, but seedlings are more vulnerable. Tiny larvae can sever young tap roots within the first few months of germination, and entomopathogenic nematodes kill small ghost moth

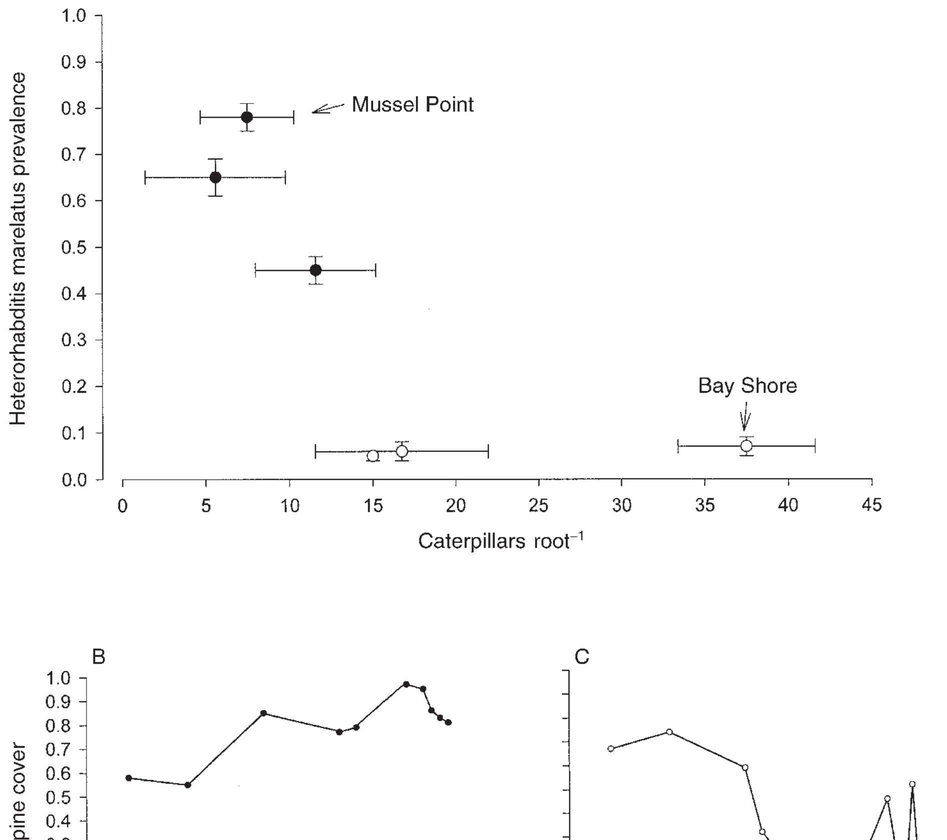

Fig. 11.4. (A) Prevalence of *Heterorhabditis marelatus* among six sites at Bodega Bay, 1994–1995, as a function of the numbers of larvae per root of *Lupinus arboreus*. The sites are Mussel Pt., Cove, Dune 1, Lower Draw, Upper Draw, and Bayshore, ranked in descending order on the ordinate. Data from Strong *et al.* (1996), Table 2. (B). Cover of *L. arboreus* for Mussel Pt. (C). Cover of *L. arboreus* for Bayshore. Data for (B) and (C) from Strong *et al.* (1995).

larvae in the soil in spring. We placed hatchling ghost moth larvae near the stems of lupine seedlings growing in the field (Strong *et al.*, 1999). The experiment was a random, factorial design with four levels of neonate ghost moth larvae (0, 8, 16 and 32) crossed with the entomopathogenic nematode (present or absent). Each treatment combination had 15 replicate seedlings. The results demonstrated a powerful trophic cascade (Fig. 11.6). Two months after the beginning of the experiment, 46% (21 of 45) of seedlings had died in treatments lacking entomopathogenic nematodes, compared with 11% (5 of 45) mortality of seedlings in treatments with the entomopathogenic nematode.

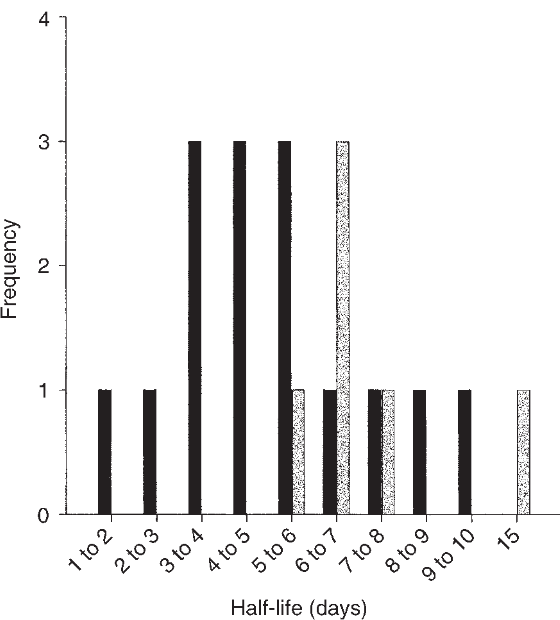

Fig. 11.5. Half-lives of *Heterorhabditis marelatus* in laboratory experiments in raw soil, without pasteurization. Grey, in 15 cc of soil for a 2- day experiment (Koppenhöfer *et al.*, 1996, Table 3, raw soil, no fungi). Black, in 100 cc of soil, 20% moisture, one infective juvenile per cc of soil, at room temperature, ten replications per test. Methods as in Strong *et al.* (1996).

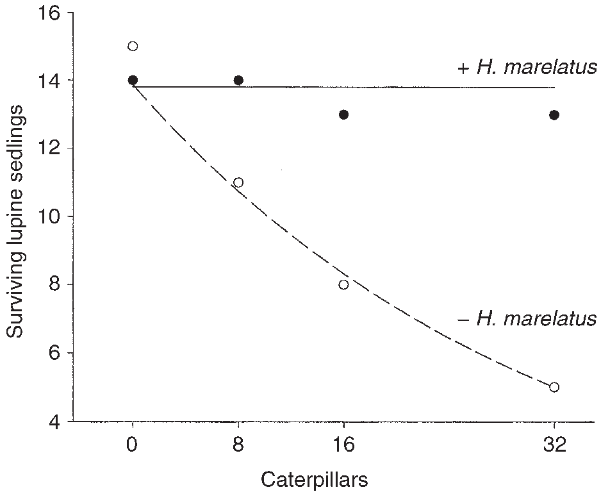

Fig. 11.6. Survival of *Lupinus arboreus* seedlings as a function of increasing numbers of ghost moth caterpillars (*Hepialus californicus*) in a field experiment. Open circles, without application of *Heterorhabditis marelatus* to soil around the stem. Closed circles, with *H. marelatus* applied to soil. Redrawn from Figure 2, Strong *et al.* (1999).

11.3.3. Survival in the field

Most field-released *H. marelatus* disappeared (Strong *et al.*, 1999). Of 60 rhizospheres inoculated with *H. marelatus* in April, the nematode was recovered from only six when soil was assayed in September with *G. mellonella* larvae. We recovered one *H. marelatus* from five of these rhizospheres, and four *H. marelatus* from the sixth. Rough calculations are that *c.* 120,000 infective juveniles were placed out, 2000 into each of 60 rhizospheres. Approximately 120 days passed between inoculation and recovery efforts. This gives a half-life of about 9 days, which is in the range of the half life estimates of the short-term laboratory studies with *H. marelatus* (Fig. 11.5). The small fraction of nematodes that we did not recover would not affect the half-life estimates (see 11.3.1, above). Of course, some nematodes were likely to have reproduced in the experimental ghost moth larvae used in the experiment. Alternatively, the experimental larvae were small and would have produced few infective juveniles.

11.3.4. Natural enemies

Many nematophagous fungi are found in the soils at Bodega Bay where *H. marelatus* lives (Table 11.1; Jaffee *et al.*, 1996). These fungi are polyphagous nematode enemies that can cause high mortality to entomopathogenic nematodes (see Kaya, Chapter 9 this volume). Other enemies of entomopathogenic nematodes (nematophagous insects, mites, Collembola, tardigrades and carnivorous nematodes) were sparse in our study site. We have identified 15 species of nematophagous fungi in the soils of our study site. The species most abundant in our samples was *A. oligospora*. It was found at all sites and in 92% (44/48) of the samples, with as many as 695 propagules of these fungi per gram of soil from the study area. Two other species, *Monacrosporium eudermatum* and *Geniculifera paucispora*, were distant second and third in the Most Probable Number sampling. In laboratory studies, five species of these nematophagous fungi from the study site caused 38–98% mortality to *H. marelatus* after 36 h on agar (Koppenhöfer *et al.*, 1996). In soil, pasteurized or not, mortality that could be

Table 11.1. Nematophagous fungi observed and isolated from soil in stands of *Lupinus arboreus* at the Bodega Marine Reserve, California (from Jaffee *et al.*, 1996).

Fungus	Trophic structures or mode
Arthrobotrys brochopaga	Constricting rings
Arthrobotrys musciformis	Adhesive networks
Arthrobotrys oligospora	Adhesive networks
Arthrobotrys superba	Adhesive networks
Geniculifera paucispora	Adhesive networks
Hirsutella rhossiliensis	Adhesive conidia
Monacrosporium cionopagum	Adhesive branches
Monacrosporium deodycoides	Constricting rings
Monacrosporium eudermatum	Adhesive networks
Monacrosporium parvicollis	Adhesive knobs
Nematoctonus concurrens	Adhesive, glandular cells
Stylopage sp.	Adhesive hyphae
Pleurotis ostreatus	Nematoxin from secretory cells

attributed to these fungi was much less than that observed on agar. While four of the five fungal species killed more than 80% of the nematodes on agar, none caused more than 30% mortality in soil. This suggests that physical mycostatic properties of soil can mediate fine-scale spatial interactions between these fungi and infective juveniles (Stirling, 1991); even small volumes of soil provide refuge from these fungi. Interspecific competition between the nematophagous fungi does not appear to be intense (Koppenhöfer *et al.*, 1997b).

11.3.5. Local density and survival

Mortality to *H. marelatus* caused by *A. oligospora* can be very high in cadavers of ghost moth larvae, *H. californicus*, which are major hosts of *H. marelatus* in the rhizospheres of lupine at Bodega Bay. Many field-collected cadavers of the ghost moth have a coating of fungi, which proliferates upon White traps in the laboratory. Trapping structures of *A. oligospora* can be seen on these cadavers, intertwined among dead infective juveniles of *H. marelatus* that have issued from the cadaver. The hyphae do not extend into the cadaver, within which reproductive *H. marelatus* thrive and continue to produce infective juveniles. These observations suggest a mechanism for the density-dependent mortality observed in our laboratory experiments; predatory activity of the fungi could increase with higher densities of infective juveniles. This is a hot spot of high mortality; away from the cadaver where density of infective juveniles is much lower, mortality caused by *A. oligospora* would be much lower.

11.3.6. Dual nutrition of the nematophagous fungi

While some species of nematophagous fungi are completely carnivorous or 'endoparasitic' (Jaffee and Muldoon, 1995), others practice dual nutrition and are cellulolytic as well as carnivorous. Species with dual nutrition can flourish upon dead plant material as well as upon live nematodes (Cooke, 1977). One idea is that nematodes provide N that is in short supply in the plant detritus upon which species practising dual nutrition proliferate (Barron, 1992). A nutritional spectrum exists among species of these fungi, from more detritivorous to more carnivorous (Stirling, 1991). We found that a large fraction of wet soil samples from our site produced rapid, abundant growths of *A. oligospora* upon lupine detritus that was added to the sample.

11.3.7. A foodweb for entomopathogenic nematodes

The basic element of a foodweb for entomopathogenic nematodes that attack herbivorous insects is a three-species trophic cascade; the nematodes suppress populations of herbivorous insects and thereby protect the plant. The simplest way to include the nematophagous fungi known to be so common and injurious to *H. marelatus* at Bodega Bay is to extend the chain by adding a link. Thus, the fungi could reverse the plant-protective effects of the trophic cascade (Fig. 11.7a). However, this linear four-species trophic cascade is probably not correct. Nematophagous fungi are not confined in diet to entomopathogenic nematodes but consume a variety of nematodes. In soil rich with fresh detritus, microbivorus nematodes are the main food for these fungi (Fig. 11.7b; Van Den Boogert *et al.*, 1994; Jaffee, 1996). Finally, the dual

nutrition means that the populations of these fungi could be sustained directly by detritus as well as by nematodes (Fig. 11.7c). In the models of Fig. 11.7b and 11.7c the protection of the host plant would be independent of the populations of herbivorous insects because of the independent sustenance of nematophagous fungi.

11.4. Conclusions and Future Prospects

Half-life is a useful summary statistic for survivorship of cohorts of entomopathogenic nematodes. With the assumption of a constant rate of mortality, this statistic provides a baseline for study of mortality rates of infective juveniles. Steinernematids range in half-life upwards to 5 months, which is a mortality rate so

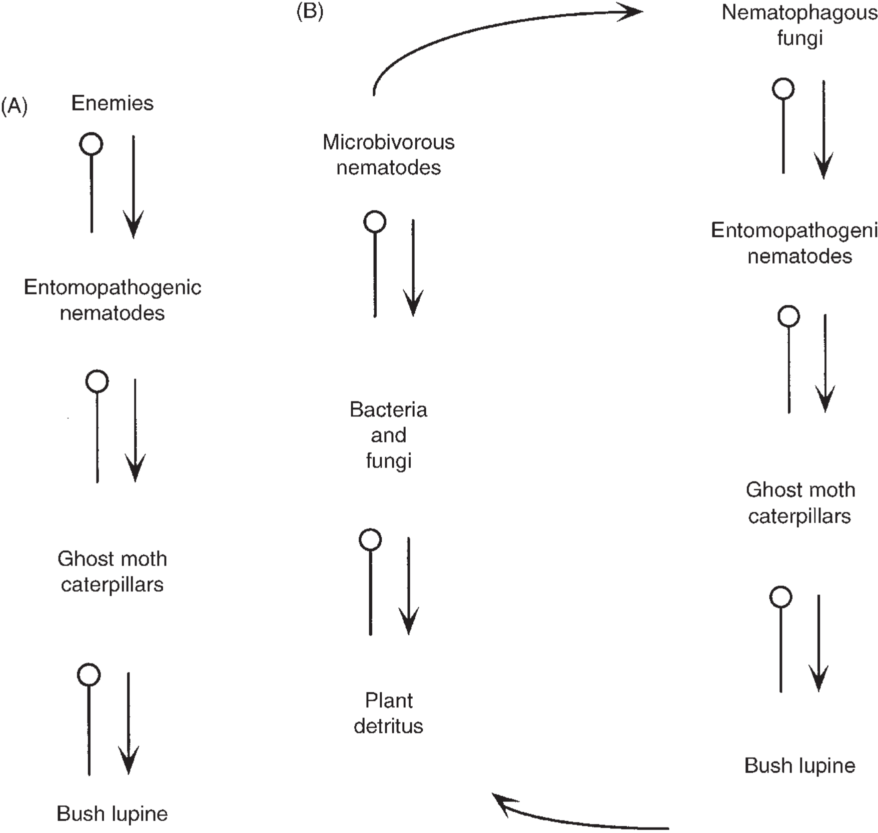

Fig. 11.7. Hypothetical component foodwebs for entomopathogenic nematodes inspired by the webs of *Heterorhabditis marelatus* at Bodega Bay. (A) Four-species, linear trophic cascade with an enemy specific to the entomopathogenic nematode. (B) A web with a polyphagous enemy (nematophagous fungi) which is sustained by microbivorous nematodes, sustained in turn by bacteria produced in plant decomposition. (C) A web based upon dual nutrition, in which nematophagous fungi are sustained by decomposition of plant material as well as by entomopathogenic nematodes.

low that a substantial fraction of the cohort will live through the inactive period between growing seasons. Heterorhabditids have shorter half-lives. About two-thirds of the measured half-lives for this family are less than 15 days, a range in which only one in 100,000 of a cohort would remain after a long winter or dry season of 7.5 months. Thus, either survivorship rate increases during the inactive period in the soil or recycling through hosts between growing seasons is the norm for populations with half-lives in this low range.

Half-lives of *H. marelatus* measured in the laboratory range from a few days to a week and are roughly comparable to those of other species of *Heterorhabditis*. Thus, mortality is probably too high for cohorts to persist through the 8 months or so of dry summer weather in the Mediterranean climate of Bodega Bay. Either recycling of successive cohorts through different hosts between growing seasons or increased rates of survival in a small fraction of the cohort that persists long enough to enter the dry summer soil are possibilities. Host-free, long-term experiments in nature are necessary to resolve the question.

Infective juveniles of entomopathogenic nematodes are killed in the soil by diverse natural enemies. At Bodega Bay, *H. marelatus* lives in the soil with a

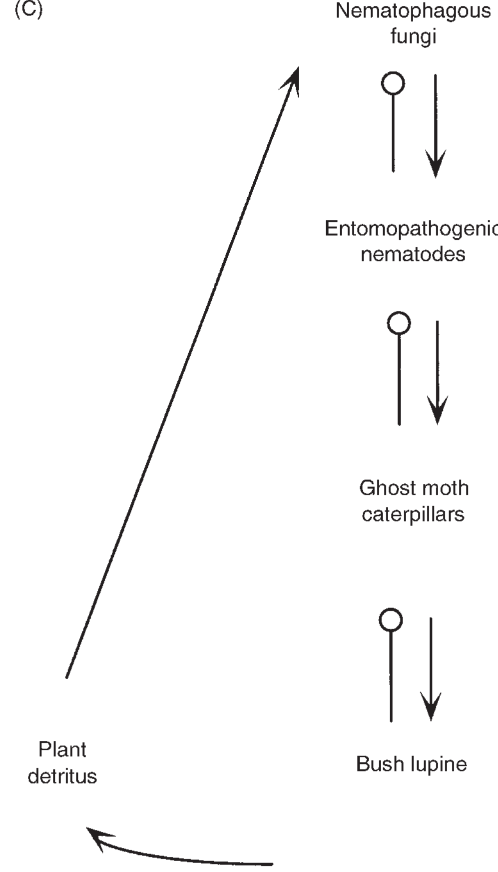

Fig. 11.7. *Continued.*

community of nematophagous fungi. We have observed that the most common species of these, *A. oligospora*, proliferates upon cadavers of larvae of the ghost moth, *H. californicus*, which is a prime host of *H. marelatus* at Bodega Bay. Trapping structures intertwine with dead infective juveniles of *H. marelatus* that have issued from the cadaver.

These hot spots of mortality contrast with lower death rates away from cadavers; even small volumes of soil provided a physical refuge from the fungi, (*viz.* the greater mortality on agar than in soil in our laboratory experiment). This is consistent with density-dependent mortality. Similarly, Fowler and Garcia (1989) observed density-dependent attack of *S. feltiae* by *A. oligospora* as infective juveniles attacked subterranean crickets.

The foodweb of *H. marelatus* provides a model for webs involving other entomopathogenic nematodes. *H. marelatus* kills the larvae of the root-feeding Lepidopteran, *H. californicus*, and thereby protects the lupine plants abundant on the site. The populations of *H. marelatus* are patchy, and lupine populations in areas that support higher incidences of this nematode have had more constant populations of this plant. I speculate that the food web of *H. marelatus* is more complex than a linear trophic cascade. First, the polyphagous nature of nematophages means that microbivorous nematodes in the soil, rather than infective juveniles, will often be the main food source of the enemies that are most dangerous to entomopathogenic nematodes. Second, dual nutrition of nematophagous fungi further increase the reticulation of these foodwebs. These factors mean that the dynamics of foodwebs involving entomopathogenic nematodes will be complex. I will speculate, additionally, that entomopathogenic nematodes exist in metapopulations, with high local heterogeneity, low overall densities, and high patchiness (Stuart and Gaugler, 1994).

Finally, we have recently learned that small soil-dwelling insects such as Tenebrionidae are also hosts of *H. marelatus* at Bodega Bay. The polyphagous nature of *H. marelatus* in other places is well known; this nematode attacks several species of agricultural insect pests in Oregon (Berry *et al.*, 1997a,b). Multiple host species raise the possibility of apparent competition in the food web, between two or more insect victim species that share *H. marelatus* as an enemy (Holt and Lawton, 1993). The host insect species tolerating the highest levels of attack by *H. marelatus* should win in apparent competition, with extinction for the other insect species. Theory suggests that one possible explanation for the coexistence of different hosts species of *H. marelatus* is different spatial and or temporal refuges from the infective juveniles produced by the most tolerant host species. If the different host insect species prove to have different host plant species, a ready avenue for understanding the mechanisms of coexistence will be available. Entomopathogenic nematodes are important natural enemies in soil and litter. Wide geographical distribution, heavy mortality imposed upon host insects, and involvement in complex trophic interactions qualify entomopathogenic nematodes as prime subjects for both applied and basic ecological research.

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12 Genetics and Genetic Improvement

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12.1. Introduction

The impetus for research in entomopathogenic nematodes and their bacterial symbionts has come about because of their commercial importance as biological control agents. Consequently much of the focus in entomopathogenic nematode genetics is on applied aspects relating to strain improvements in areas such as environmental tolerances, infectivity to target pests, formulation and shelf-life (Burnell and Dowds, 1996). Techniques of classical genetics – mutagenesis, hybridization and artificial selection have been successfully used in such strain improvement programmes. By contrast, the techniques of molecular genetics have not been widely applied to entomopathogenic nematodes, except in the area of molecular diagnostics and in studies of molecular phylogeny.

Entomopathogenic nematodes belong to the same family as *Caenorhabditis elegans* whose genome has been fully sequenced and annotated. In principle, the molecular

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tools that have been developed for *C. elegans* could be developed and applied to studies on entomopathogenic nematodes but, in practice, such technology transfer has been rare. In this chapter I describe the current state of knowledge of entomopathogenic nematode genetics, both classical and molecular, outline the research methodologies which are currently available, and consider how the tools of molecular genetics and the *C. elegans* genome sequence can be utilized by the entomopathogenic nematode research community. Finally, I briefly review the potential use of these nematodes and their symbionts as excellent model systems in which to study a range of fundamental biological problems.

12.2. Progress to Date

Significant progress has been made since the publication of *Entomopathogenic Nematodes in Biological Control* (Gaugler and Kaya, 1990) in the classical genetics of entomopathogenic nematodes and many research tools and protocols have been developed and refined. In order to fully appreciate and understand the organismal and molecular complexity of entomopathogenic nematodes, however, research approaches using molecular genetics will be necessary.

12.2.1. General biology and life cycle

The non-feeding infective juvenile of *Heterorhabditis* and *Steinernema* is a modified third-stage juvenile which is adapted for dispersal, survival, host finding and infection. It is analogous to the dauer juvenile stage of *C. elegans. C. elegans* is a free-living, bacterial feeding nematode found in soil and in compost. It is an 'r-selected', voracious feeder with a high reproductive capacity and rapid growth potential, developing from egg to adult in 36 h at 20°C. This reproductive strategy rapidly leads to overcrowding and depletion of food resources in the nematode's natural environment. Under these conditions and modulated by high temperatures, and by the production of a density dependent pheromone, *C. elegans* reproduction ceases and dauer juveniles are produced at the second juvenile moult (Golden and Riddle, 1984). *C. elegans* dauer juveniles can survive for months in the absence of food and when they encounter food they rapidly resume development to become self-fertile hermaphrodite females. When infective juveniles of entomopathogenic nematodes resume development in an insect host, *Heterorhabditis* juveniles, like *C. elegans*, mature to hermaphrodite females. These first generation *Heterorhabditis* females give rise to a second generation of amphimictic males and females (the early second generation progeny) and also to self-fertile hermaphrodite females and infective juveniles, which occur later in the second generation (Dix *et al.*, 1992; Strauch *et al.*, 1994; Wang and Bedding, 1996). By contrast, a steinernematid infective juvenile typically matures to become either a male or an amphimictic female, however Griffin *et al.* (2001) have recently identified a hermaphrodite strain of *Steinernema*. The life cycles of *Heterorhabditis, Steinernema* and *C. elegans* are illustrated in Fig. 12.1.

Most rhabditid nematodes are small soil-dwelling bacterial feeders. Some rhabditids have evolved a necromenic life cycle with their soil-dwelling invertebrate hosts. Dauer juveniles of these nematodes enter their host and remain quiescent until it dies and is invaded by saprophytic bacteria. Then the dauer juveniles resume feeding, development and reproduction. Sudhaus (1993) has postulated that *Heterorhabditis*

and *Steinernema* evolved from necromenic nematodes which developed a symbiotic association with an entomopathogenic bacterium. Figure 12.2 is a portion of the nematode phylogram of Blaxter *et al.* (1998) which shows that *H. bacteriophora* belongs to the same clade as *C. elegans* and was the most closely related parasitic nematode to

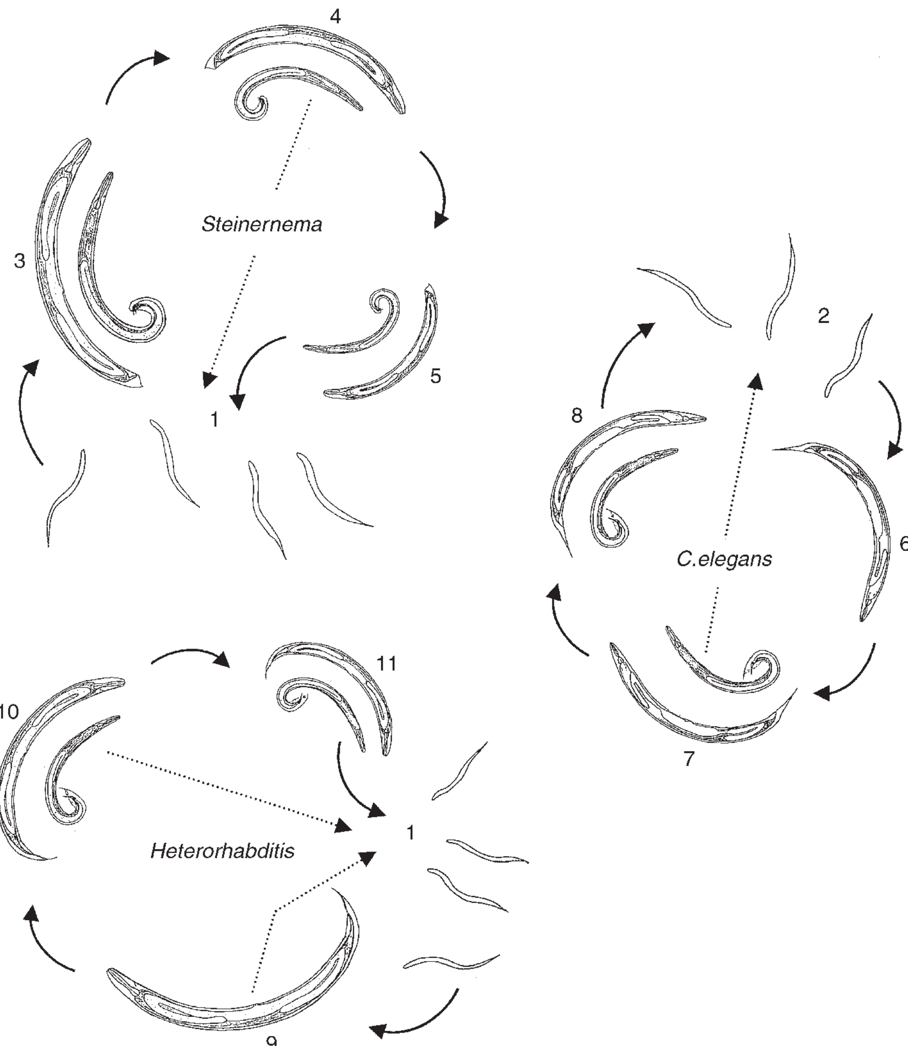


Fig. 12.1. The life cycles of *Heterorhabditis, Steinernema* and *Caenorhabditis elegans*. 1, Infective juveniles (*Steinernema, Heterorhabditis*); 2, dauer juveniles (*C. elegans*); 3, first generation amphimictic females and males; 4, second generation amphimictic females and males; 5, third generation amphimictic females and males; 6, first generation hermaphrodite females; 7, second generation hermaphrodite females and rare males; 8, third generation hermaphrodite females and rare males; 9, first generation hermaphrodite females; 10, second generation females (can be either amphimictic or hermaphrodite) and rare males; 11, third generation hermaphrodite females and rare males (⇢ implies some recruitment from an adult stage to the infective juvenile or dauer juvenile stage).

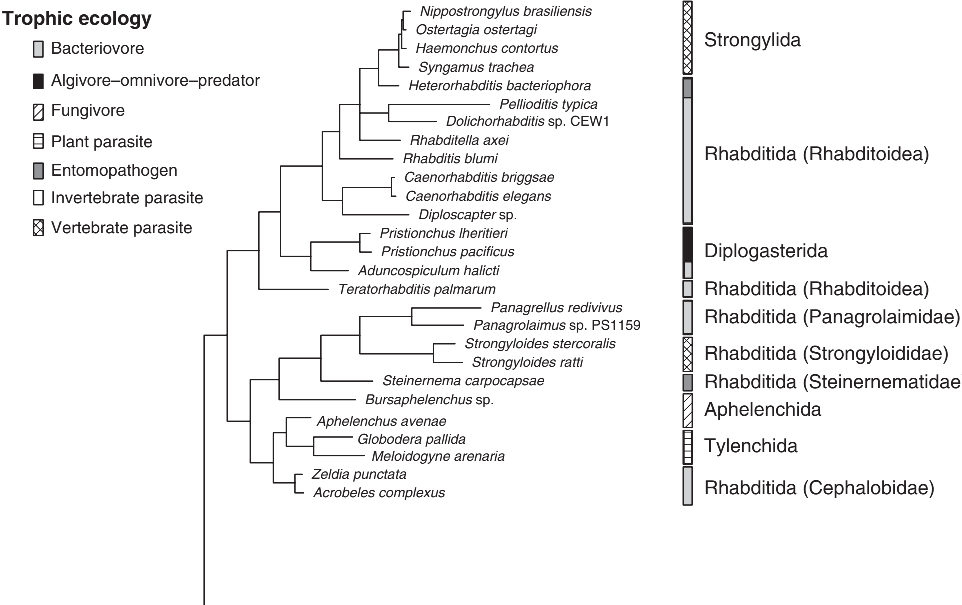


Fig. 12.2. A portion of the nematode phylogram obtained by Blaxter *et al.*, (1998) using small subunit ribosomal DNA sequences and showing the relationship between *Heterorhabditis bacteriophora, Steinernema carpocapsae* and *Caenorhabditis elegans*. Reprinted by permission from Nature (392, 71–75) copyright (1998), Macmillan Magazines Ltd.

C. elegans in this study. *S. carpocapsae* was placed in a neighbouring clade and is more distantly related to *C. elegans*. Thus entomopathogenic nematodes, particularly *Heterorhabditis*, are closely related phylogenetically to *C. elegans* and share many similarities in life cycle and development. This strongly suggests that at the genomic level, too, many genes, genetic processes and pathways will also be conserved between these genera.

12.2.2. Classical genetics

Entomopathogenic nematodes represent one of the most tractable groups of parasitic nematodes on which to carry out genetic research: their life cycle is short; they can be cultured *in vitro* or *in vivo* and they are extremely prolific. Kaya and Stock (1997) provide a useful compendium of general techniques and methodologies for these nematodes. Cross-breeding protocols are well established and routinely used. For *Steinernema*, these tests can be carried out in hanging drops of *Galleria mellonella* haemolymph (Poinar, 1967) or by injection of infective juveniles into living *G. mellonella* (Akhurst and Bedding, 1978). For *Heterorhabditis*, second generation amphimictic virgin females must be carefully selected and they are transferred together with second generation males into prepared *G. mellonella* cadavers or onto lipid agar plates (Dix *et al.*, 1992). Mutagenesis protocols based on those developed by Brenner (1974) for *C. elegans* – using the mutagen ethylmethane sulphonate (EMS) – are routinely applied to entomopathogenic nematodes (Zioni *et al.*, 1992; Rahimi *et al.*, 1993; Tomalak, 1994a). Such mutant isolation programmes have yielded morphological

mutants which are useful as markers in genetic analyses (Shapiro *et al.*, 1997) and in life cycle studies (Dix *et al.*, 1994). Entomopathogenic nematodes can be successfully cryopreserved and stored using liquid nitrogen (Popiel and Vasquez, 1991; Curran *et al.*, 1992; Nugent *et al.*, 1996). This means that nematode strains, together with their symbiotic bacteria, can be stored indefinitely without subculturing, thereby safeguarding their genetic identity and saving them from contamination or loss.

12.2.2.1. Selective breeding

Prior to the advent of genetic engineering, mutagenesis and selective breeding were the main means by which improvements in beneficial traits were achieved in economically important plants and animals. Many beneficial traits are inherited polygenically, being encoded by a large number of genes, each with only a small effect on the phenotype. For these traits, selective breeding is the most appropriate means of genetic improvement. An alternative strategy is to let nature carry out the selection programme. For example, nematode strains endemic to cold and warm climatic regions will be adapted by natural selection to function at low and high temperatures, respectively. Such cold active and warm active strains have been isolated in targeted strain collection programmes (Glazer *et al.*, 1996; Mracek *et al.*, 1998).

Quantitative phenotypes are the outcome of genotype × environment interactions. Some quantitative traits are strongly influenced by environmental conditions and thus have low heritabilities. Phenotypes with high heritability values are more responsive to selective breeding. Figure 12.3 (derived from Gaugler *et al.*, 1989) presents a systematic approach which might be used in a selective breeding programme for entomopathogenic nematodes. The rationale for most of the steps in this figure should be apparent, but for further discussion see Gaugler *et al.* (1989), Hastings (1994) and Falconer and Mackay (1996). Heritability estimates allow the experimenter to predict the selection response in the trait under selection. These estimates are usually obtained from the degree of resemblance between relatives or from inbred lines (Glazer *et al.*, 1991), using analysis of variance tests. Gaugler *et al.* (1989) suggested that an indication of potential heritability of a beneficial trait could be also obtained by collecting a number of ecologically distinct strains and assessing their phenotypic variability for the desired trait. It should also be possible to analyse heritabilities by isolating individual gravid first generation entomopathogenic nematode females containing developing infective juveniles *in utero* and collecting their progeny to yield a series of full sib families, each derived from a single infective juvenile.

Several selective breeding programmes for increased host finding and increased host pathogenicity have been carried out (Gaugler *et al.*, 1989; Grewal *et al.*, 1993; Tomalak, 1994b; Peters and Ehlers, 1998). In some (Tomalak, 1994b), but not all cases, a positive selection outcome in the laboratory did translate into improved performance of the selected strain in the field. Gaugler and Campbell (1991) achieved a 72-fold improvement in the host-finding ability of *S. carpocapsae* towards scarab larvae, however the selected strain failed to provide enhanced parasitism in laboratory or field trials (Gaugler *et al.*, 1994). The selected infective juveniles were using carbon dioxide as a cue to orient towards the host spiracles, but the spiracles of scarab larvae are protected with sieve plates which prevent entry. Thus in selecting for enhanced field efficacy it is important, as Gaugler *et al.* (1994) point out, to choose a nematode species already known to parasitise the target insect, and to select for ability to penetrate, in

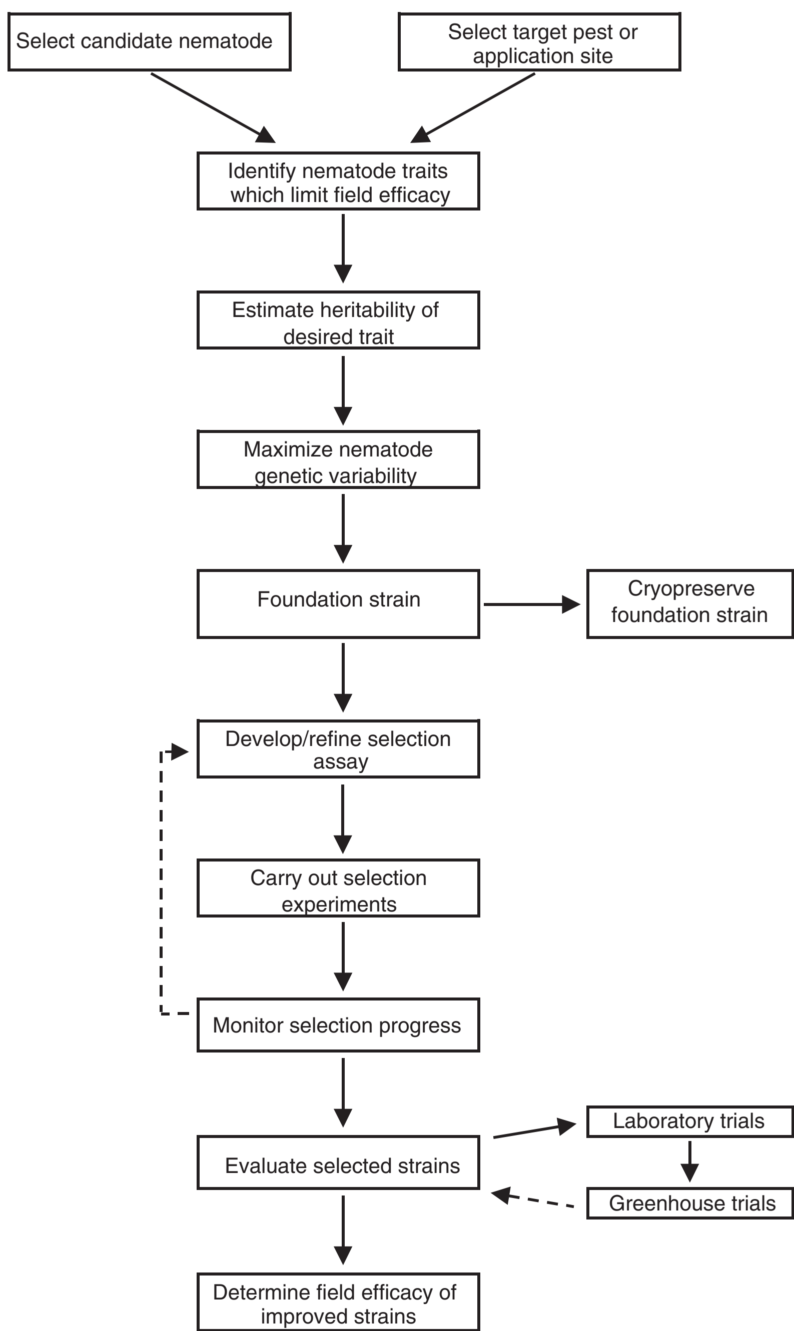


Fig. 12.3. A systematic plan for the design of a genetic selection programme for entomopathogenic nematodes (derived from Gaugler *et al.*, 1989).

addition to selecting for host finding ability. Artificial selection has been successful in enhancing infectivity at low temperatures (Griffin and Downes, 1994; Grewal *et al.*, 1996) and in increasing tolerance towards nematicides (Glazer *et al.*, 1997). A potential problem which can arise in the use of artificial selection for strain improvement is the occurrence of correlated responses in other non-selected characters, and this may affect the fitness of the selected strain. Once the desired phenotype has been obtained and the selection pressure is relaxed, the phenotypes of the selected strains sometimes tend to revert gradually towards the unselected state (Hastings, 1994). This problem can be circumvented by cryopreserving the selected lines and by reapplying selection pressure at regular intervals on the selected strain.

12.2.2.2. Mutagenesis

The high reproductive rate and short generation time of entomopathogenic nematodes suggests that where suitable screens are available, mutagenesis may provide an alternative means for strain improvement. Screening thousands of infective juveniles for a desirable phenotype can be very time consuming unless a suitable means of mass screening is devised. Lethal screens provide an easy method of mutant selection. This method of screening is particularly appropriate for isolating strains with increased stress tolerances. If a dose mortality curve is obtained for the parental strain for an environmental stress such as heat, cold, UV tolerance, nematicides etc., then those environmental conditions which result in 100% mortality of the parental strain can be used to isolate resistant mutants. Behavioural screens may also be devised which facilitate mass screening for mutant selection, as in the isolation of cryophilic or thermophilic mutants or of chemosensory mutants, or strains with alterations in their host range.

Several nematode species including *C. elegans* (Hedgecock and Russell, 1975) and *Steinernema carpocapsae* (Burman and Pye, 1980) show a sensitive thermotactic behaviour. This behaviour can be observed by placing the nematodes on a thermal gradient whereupon they will accumulate at the temperature to which they have been cultured. Temperature shift experiments show that nematode thermal preferences can be reset within 4–12 h by laboratory acclimation to higher or lower temperatures (Hedgecock and Russell, 1975; Burman and Pye, 1980; Nugent, 1996). Hedgecock and Russell (1975) utilized this thermotactic behaviour of *C. elegans* to isolate cryophilic, thermophilic and athermotactic mutants. The cryophilic mutants were selected on the basis of their migration on a thermal gradient to colder temperatures than their culture temperature; thermophilic mutants migrated along the gradient to warmer temperatures than their culture temperature while the athermotactic mutants migrated randomly over the thermal gradient. Using a similar approach to that used for *C. elegans*, Nugent (1996) isolated cryophilic mutant strains of the Irish isolate *Heterorhabditis* sp. K122 following EMS mutagenesis. All the cryophilic *Heterorhabditis* mutants displayed an improved infection potential at 9°C for *Tenebrio molitor* larvae, whereas at 20°C no improvement was observed. Desiccation tolerant mutants of *H. megidis* have been isolated following EMS mutagenesis by O'Leary and Burnell (1997). In general the potential of EMS mutagenesis as a tool in entomopathogenic nematode strain improvement programmes is underexploited, particularly for strain improvements in environmental tolerances.

12.2.2.3. Molecular genetics

With the exception of studies in molecular diagnostics and phylogenetics, studies on molecular genetics of EPN have lagged behind those of their bacterial symbionts. The genome sizes of *S. carpocapsae* and *H. bacteriophora* have been determined (Grenier *et al.*, 1997). The genome of *H. bacteriophora* at 4×10^7 bp is less than half as large as that of *C. elegans* (1×10^8 bp), while that of *S. carpocapsae*, at 2.3×10^8 bp, is twice as large as the *C. elegans* genome. Male and female karyotypes have been determined for several species of *Steinernema*: males are $2n = 9$ and females are $2n = 10$ (Poinar, 1967; Guohan *et al.*, 1989). The diploid karyotypes of *Heterorhabditis* have not yet been determined, but a karyotype of $n = 7$ has been observed in first generation *Heterorhabditis* hermaphrodites from a range of species and isolates (Curran, 1989).

Genetic transformation systems have been developed for both *H. bacteriophora* (Hashmi *et al.*, 1995) and *S. feltiae* (Vellai *et al.*, 1999) using microinjection protocols developed for *C. elegans* (section 12.3.5).

12.3. Technology Transfer of Research Methodology from *C. elegans*

Entomopathogenic nematodes belong to the same family of nematodes as *C. elegans*, thus one might expect that the genetic methodologies which have been developed for *C. elegans* research can be readily applied to these nematodes. When tested this expectation will, in general, prove true. Certain protocols, for example transposon mutagenesis, may however require substantial research and development to obtain a working system for entomopathogenic nematodes. The main problem is one of manpower, resources and research focus. The *C. elegans* community work on a single strain (Bristol N2) of *C. elegans,* whereas entomopathogenic nematode researchers work on a large number of species and strains in two nematode genera, and have a more applied focus. Twenty-five years of intense research effort in *C. elegans* have yielded an unequalled set of genetic resources which are freely accessible to all researchers. These resources include: a gene-rich genetic map, a physical map of overlapping cosmid and YAC (yeast artificial chromosome) clones, a full genome sequence containing 19,000 predicted protein coding genes (The *C. elegans* Sequencing Consortium, 1998), and resource centres from which mutant strains and genomic and cDNA clones can be obtained. Two key works: *The Nematode* Caenorhabditis elegans (Wood, 1998) and C. elegans *II* (Riddle *et al.*, 1997) provide detailed information on all aspects of *C. elegans* genetics and molecular biology. Two excellent methodology books are also available: Epstein and Shakes (1995) and Hope (1999). The *C. elegans* web server at http://elegans.swmed.edu/ provides access to a wide number of sites with resources pertaining to *C. elegans* research.

Genes identified by their mutant phenotypes are cloned by *C. elegans* workers using two approaches: (i) genetic mapping followed by YAC and/or cosmid rescue of the mutant phenotype, or (ii) transposon mutagenesis. The first approach is not available for entomopathogenic nematodes because of the lack of genetic and physical maps for *Heterorhabditis* or *Steinernema*. The second approach cannot be used at present because actively transposing entomopathogenic nematode strains are not available. Thus for the moment, cloning of genes in *Heterorhabditis* and *Steinernema* is restricted to the cDNA-based methods described below or to methods using degenerate PCR primers or heterologous probes.

12.3.1. Transposon mutagenesis

Transposons are molecular parasites which are able to replicate their DNA sequences and insert them into new sites in their host's genome. When a transposon inserts into the coding region of a gene, a gene mutation usually results. Transposons exist in active and inactive forms. Active transposons encode the genes and flanking sequences necessary for transposon mobility. Active elements are relatively rare, since they destabilize the genomes of their hosts by insertional mutagenesis and therefore generate a strong selection pressure in the host to suppress them.

Transposons provide powerful tools for molecular genetics. Transposon tagging can be used to isolate any gene whose mutant phenotype can be readily identified. Mutations caused by transposon insertion are isolated from a strain with active transposons. The mutated gene is identified by transposon insertion display on a polyacrylamide gel. Genomic sequences flanking the transposon insertion site will contain sequences from the mutated gene that can then be used to isolate the wild-type gene from a genomic or cDNA library (Fig. 12.4). Active transposons have been identified in *C. briggsae* and *C. elegans* and 'mutator' strains, in which active transposition occurs in the germline, have been isolated (Collins *et al.*, 1987). The availability of these strains resulted in the first wave of gene cloning in *C. elegans*.

Greiner *et al.* (1999) have cloned and sequenced a range of *mariner*-like transposons from *Heterorhabditis*, but all clones contained stop codons and other mutations in their sequences and do not represent active elements. An active transposon system for entomopathogenic nematodes would provide a huge impetus to research in the molecular genetics of these nematodes. Active transposons could be developed using two strategies: via (i) heterologous or (ii) homologous transposition systems. Several *mariner*-like elements have been identified which are capable of tranposition in heterologous hosts (Schouten *et al.*, 1998). Such transposons could be transformed into entomopathogenic nematodes and tested for their ability to undergo transposition. Even if lines transformed with transposons did not display active transposition in their germlines, it is possible, by analogy with *C. elegans* (Collins *et al.*, 1987) that such strains could be transformed into 'mutator' strains by mutagenesis (section 12.3.4.2). Active (homologous) transposable elements could also be sought in entomopathogenic nematodes by searching for naturally occurring mutator strains. Strains of *C. elegans* (and other organisms) which contain active transposons have high rates of spontaneous mutation and reduced fertility. A large number of species and strains of entomopathogenic nematodes are in culture in various laboratories around the world. A co-ordinated effort by interested laboratories to identify poorly-performing 'sickly' strains in their collections could well yield mutator strains containing active transposons. Transposon mutagenesis could then be used to identify and clone *Heterorhabditis* or *Steinernema* genes for which a suitable lethal, morphological or behavioural mutant screen can be devised.

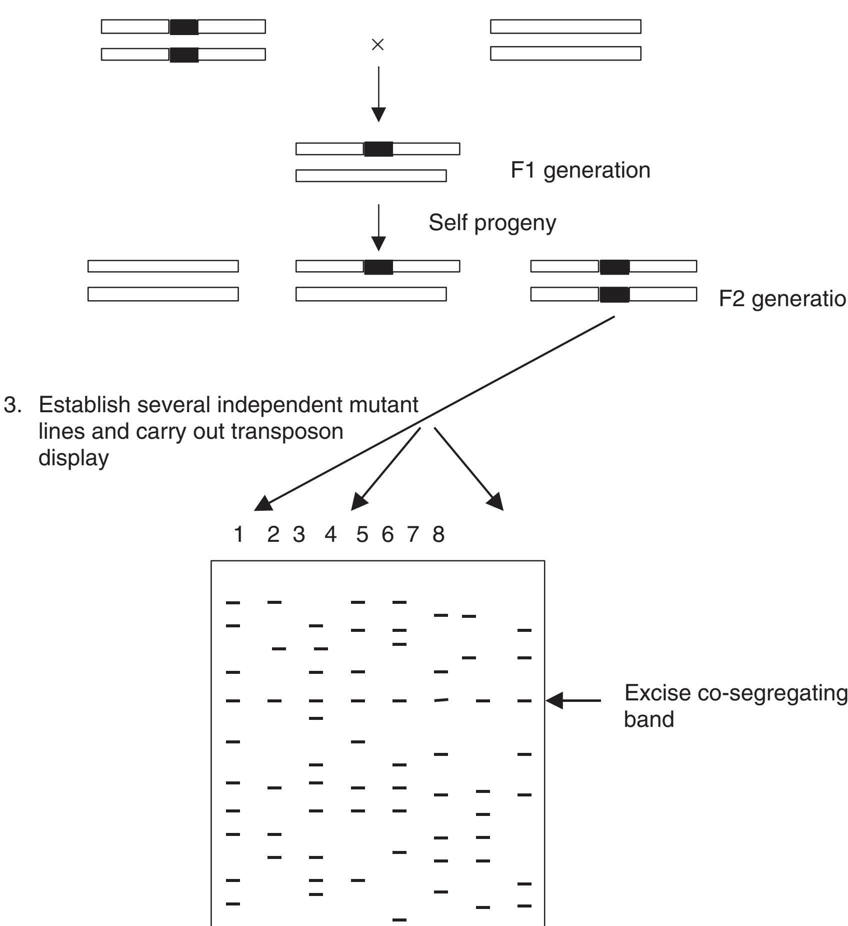
12.3.2. Expressed sequence tags (EST)

All the genes present in an organism can be identified by sequencing, aligning and annotating its genomic DNA. Despite recent technical advances, this is still a major task which is both expensive and slow and is feasible only for a small subset of model organisms. When manpower and resources are limiting, researchers are increasingly

Transposon Tagging

1. Screen mutator strain for phenotype of interest

2. Cross transposon-induced mutant with wild-type strain (= transposon)



4. The band which consistently co-segregates with the mutant phenotype is excised from the gel, reamplified and used as a probe to screen a cDNA or genomic library.

Fig. 12.4. Gene isolation by transposon tagging and transposon display. A mutator strain is screened for the phenotype of interest. A series of sub-lines are established from a single mutant line. Their genomic DNA is digested with two restriction enzymes and PCR adapters are ligated on. PCR is performed using a primer pair corresponding to the transposon and adapter. The products from these reactions are displayed on a denaturing polyacrylamide gel. The band which consistently co-segregates with the mutant phenotype is excised from the gel, reamplified as used as a probe to screen a genomic library (see Frey *et al.*, 1998 and Wicks *et al.*, 2000 for further details).

turning to an expressed sequence tag (EST) strategy for gene discovery. ESTs are short DNA sequence reads, usually 250–400 bp long, which are taken at random from the 5′ or the 3′ ends of individual cDNA clones. An EST programme includes the following steps: (i) the mRNA which is being transcribed from active genes is isolated from the appropriate tissue or developmental stage; (ii) this mRNA is then converted into cDNA using the enzyme RNA reverse transcriptase; (iii) the cDNA is cloned into a plasmid or phage vector to make a cDNA library; (iv) clones are selected at random from the library for sequencing; (v) the resulting DNA sequences are used for database homology searches against either DNA or protein sequences in an attempt to identify the genes from which they were derived (Adams *et al.*, 1991). A public EST database, dbEST, is maintained at GenBank (Boguski *et al.*, 1993). Most EST projects deposit their EST sequences in dbEST and this database is growing rapidly. (The dbEST release of 9 February, 2001 contains 7.35 million entries.)

Many new genes which would be difficult to isolate using any other strategy have been identified in EST projects. *C. elegans* was the first nematode for which an EST project was established (McCombie *et al.*, 1992; Waterson *et al.*, 1992). Several EST projects are currently in progress in other nematodes, with the general objective of utilizing and building on the information gained in the *C. elegans* genome project. The filarial nematode *Brugia malayi* EST project commenced in 1994 and the current dataset contains 20,000 ESTs which define 7000 genes (Williams *et al.*, 2000). The dbEST database (http://www.ncbi.nlm.nih.gov/dbEST/index.html) now contains EST datasets from the animal parasitic nematodes *Ascaris, Haemonchus, Necator, Nipostrongylus, Onchocerca, Strongyloides, Toxocara*, and *Trichuris*; the plant parasitic nematode *Globodera* and the free-living nematodes *C. elegans, C. briggsae, Pristionchus* and *Zeldia*. The genes that have been identified in these initiatives provide information of direct relevance to specific nematode species, but they also contain valuable information for comparative nematode genomics. Among the genes identified in these EST screens are genes which have homologues in non-nematode species, stage-specific genes which may or may not have homologues in *C. elegans*, and novel genes which have not been identified in *C. elegans* or any other organism. Many of these novel gene transcripts are extremely abundant in certain parasitic species, which suggests that they may be carrying out important functions. Should novel entomopathogenic nematode gene transcripts be identified in an EST screen, these transcripts may be important pointers to adaptive changes associated with parasitism or symbiosis.

As the costs of DNA sequencing are no longer prohibitive, EST sequencing strategies are now feasible for entomopathogenic nematodes. Laboratories with little experience in cDNA library construction are not restricted from carrying out EST projects, as several commercial companies custom-make cDNA libraries from total RNA supplied by the experimenter. Even a small scale (~300 clones) EST sequencing effort will identify many functionally important genes, providing the resources and impetus for further studies (Tetteh *et al.*, 1999; Hoekstra *et al.*, 2000). For this reason my laboratory has initiated an EST project on recovering *Heterorhabditis bacteriophora* infective juveniles in the expectation that this project will identify many genes involved in pathogenicity and morphogenesis. Recovering infective juveniles are collected after being placed for 3 h on *Galleria mellonella* cadavers which had been killed by injecting cells of *Photorhabdus luminescens*.

12.3.3. Differentially expressed genes

Differentially expressed genes are those whose expression is restricted to a particular tissue or developmental stage, or whose expression is induced following an environmental stimulus or signal. Two PCR based techniques can be used to isolate differentially expressed genes. These are mRNA differential display (Liang and Pardee, 1992) and cDNA AFLP (Bachem *et al.*, 1996), (AFLP = amplified fragment length polymorphism). See Tawe *et al.* (1998) for the use of differential display to identify stress responsive genes in *C. elegans*. Jones and Harrower (1998) have compared the efficiency of both techniques for the isolation of differentially expressed genes in potato cyst nematodes and they concluded that cDNA AFLP was the superior technique. In this technique cDNAs deriving from two different developmental stages or treatments are each cut by two restriction enzymes and double stranded adapters are ligated onto the ends of the restricted DNA. These adapters can then be used as primer sites for stringent PCR. Reaction conditions need to be controlled so that an optimum number of fragments can be amplified for visualization on a silver stained polyacrylamide sequencing gel (typically 50–100 fragments per lane, Fig. 12.5). cDNA fragments that are differentially expressed can be excised from the gel, PCR amplified, cloned and sequenced.

Suppression subtractive hybridization is a method that generates cDNA libraries that are highly enriched for differentially expressed genes (Diatchenko *et al.*, 1996). This method efficiently amplifies rare transcripts and the reagents for this protocol are available in kit form (PCR-Select cDNA Subtraction Kit, Clontech, Palo Alto, California). Although this technique has been widely applied in mammalian cell biology, there are no reports yet of its application in nematodes, but see Evans and Wheeler (1999) for its application in the study of differential gene expression in honey bees.

12.3.4. RNAi

Fire *et al.* (1998) reported that the microinjection of double stranded RNA (dsRNA) for a portion of the *unc-22* gene into the gonad of *C. elegans* resulted in a potent and specific inhibition of the *unc-22* gene. This resulted in a strong 'twitcher' phenotype in the F1 progeny of the injected animals and a weaker twitcher phenotype in the injected animals themselves. This effect was more powerful than that achieved by injecting single stranded antisense or sense RNA and it could be also achieved by injecting the dsRNA directly into the body cavity. Fire *et al.* (1998) named this effect RNA interference (RNAi). The Fire group and other investigators have studied the RNAi effect using several other well characterized *C. elegans* genes. Typically it is found that the mutant phenotypes obtained with RNAi are indistinguishable from those that eliminate the target gene's function. dsRNA corresponding to non-coding regions such as introns or promotor sequences do not have an RNAi effect (though there may be gene-specific exceptions to this rule). The homology-dependent gene silencing is associated with the complete loss of the endogenous mRNA transcripts, but RNAi leaves the primary DNA sequence of the target gene unchanged and the F2 progeny revert to the wild-type phenotype (Montgomery *et al.*, 1998). The RNAi effect is achieved with very few dsRNA molecules per affected cell, indicating that it is non-stochiometric and may involve a catalytic or amplification mechanism. Subsequently it was shown that disruption of gene activity by RNAi can be achieved

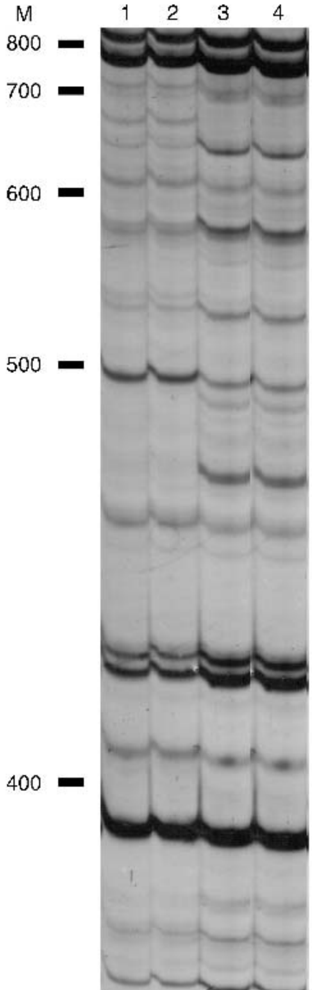


Fig. 12.5. A portion of a cDNA AFLP gel showing differential gene expression during the development of *Caenorhabditis elegans*. The cDNA AFLP profiles are those obtained for *C. elegans* fourth stage juvenile larvae grown at 15°C for 117 h (Lanes 1,2) or 123 h (lanes 3,4) post hatching (Browne and Burnell, 2000 unpublished results).

by soaking worms in dsRNA preparations (Tabara *et al.*, 1998), by feeding worms *Escherichia coli* expressing *C. elegans* dsRNA (Timmons and Fire, 1998), or by transcription from a transgene that synthesises hairpin dsRNA (Tavernarkis *et al.*, 2000) (see Fig. 12.6 for details). RNAi was subsequently shown to occur in organisms as diverse as protozoans, insects, plants, fungi and mice and has been the subject of several reviews (Fire, 1999; Bosher and Labousesse, 2000; Plasterk and Ketting, 2000).

12.3.4.1. RNAi and functional genomics

The discovery of RNAi provides a powerful tool for functional genomics. The *C. elegans* genome project has been completed but the function of 12,000 of the

predicted 19,000 genes is unknown. It is now possible to find out the role of these putative genes using RNAi and several genome-wide RNAi projects have been initiated (Frazer *et al.*, 2000). In addition to its role in genetically well characterized organisms like *C. elegans, Drosophila* and *Arabidopsis*, RNAi has also been used in organisms in which detailed genomic data are lacking such as the cnidarian *Hydra* (Lohmann *et al.*, 1999) and the planarian *Schmidtea mediterranea* (Alvarado and Newmark, 1999). Thus RNAi seems to be one research technology which could be readily transferred from *C. elegans* to entomopathogenic nematodes. Kuwabara and Coulson (2000) recommend the following experimental approach for RNAi: (i) begin with a gene that is likely to display a readily visible knockout phenotype; (ii) the dsRNA should be 0.6–2.0 kb in size; (iii) cDNA sequences are the dsRNA templates of choice; (iv) the dsRNA should be highly homologous to the target sequence. Thus if RNAi were to be used in the functional genomics of entomopathogenic nematodes, the dsRNA would probably have to be synthesized from cloned cDNA cloned from the species of entomopathogenic nematode under investigation. RNAi could be used in conjunction with an EST approach or gene cloning by differential expression to identify genes involved in important pathways in entomopathogenic nematodes. For example, genes with roles in phenotypes such as dauer formation and recovery, sex determination,

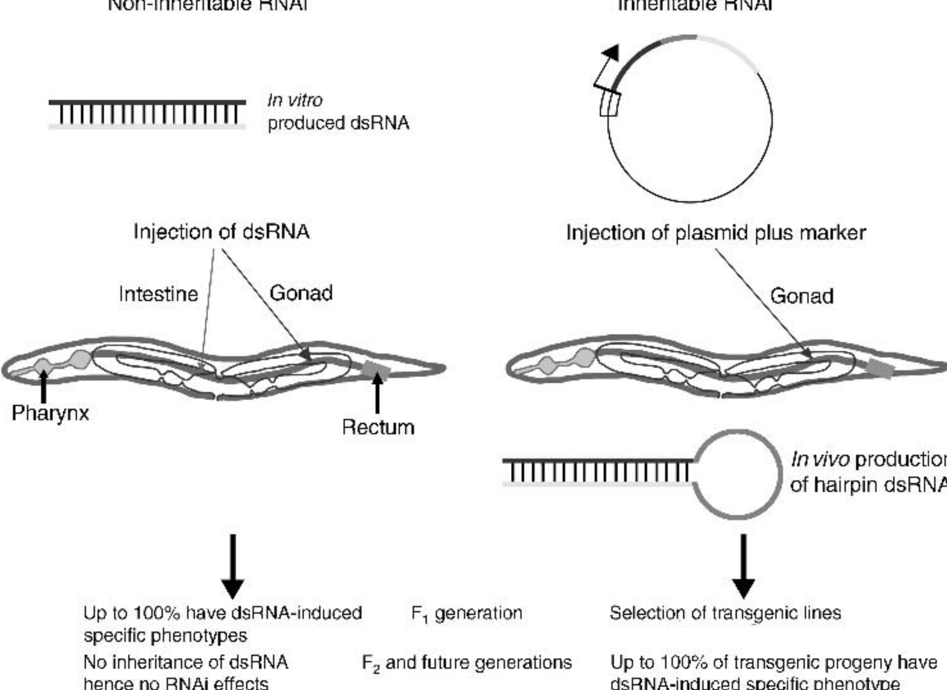


Fig. 12.6. Strategies for dsRNA delivery in *Caenorhabditis elegans*. Non-inheritable RNAi results when dsRNA is injected into the gonad or pseudocoelom; when worms are fed bacteria that express the dsRNA of interest or when worms are soaked in a solution of dsRNA. Transformation of *C. elegans* with a plasmid that expresses dsRNA when transcribed *in vivo* results in inheritable RNAi. (From Boscher and Labouesse, 2000). Reprinted by permission from Nature Cell Biology (2, E31–E36) copyright (2000), Macmillan Magazines Ltd.

symbiosis, host finding, pathogenicity etc. could be identified using RNAi. RNAi could also be used to investigate whether gene function is conserved between homologous genes in *C. elegans* and entomopathogenic nematodes.

12.3.4.2. RNAi and transposon silencing

Transposon mutagenesis requires the transposon to be active in the germline, otherwise the insertional mutagenesis is not passed on to the progeny. Several wild-type strains of *C. elegans*, e.g. the Bergerac strain, have active transposition in the germline and crosses between the standard *C. elegans* N2 lab strain and the Bergerac strain yielded a strain with an N2 genetic background which has active germline transposition. *C. elegans* strains with increased rates of transposon motility in the germline have also been isolated using chemical mutagenesis, yielding 'mutator' strains (Collins *et al.*, 1987). Ketting *et al.* (1999) found that many other transposons of unrelated sequence had also become activated in the germline of these mutator strains, and that in addition, most of these mutator strains were resistant to RNAi. Tabara *et al.* (1999) isolated RNAi deficient mutants in *C. elegans*. These mutants which failed to show gene silencing when treated with dsRNA, were tested for germline transposition and in two out of the four genes isolated, endogenous transposons had become active in the germline. These studies show that the phenomenon of transposon silencing and RNAi are mechanistically linked.

Homology dependent gene silencing was first described in plants where it appears to act as a defence system against RNA viruses, which form dsRNA during their replicative phase (Wassenegger and Pelissier, 1999). Although nematodes are important vectors of plant viruses, nematode viral infections are rare. By contrast, active transposons seem to be common in nematodes, but an RNAi based genetic system ensures that transposase mRNA (the sole gene product of *mariner*-like transposons) is inactivated in the nematode germline. Thus RNAi seems to be an ancient surveillance system designed to protect organisms against viruses and transposons (Boscher and Labouesse, 2000).

If an efficient system for RNAi can be established in entomopathogenic nematodes, which yields a lethal phenotype when an essential gene is inactivated by RNAi, then by analogy with *C. elegans,* an RNAi resistant mutant screen should yield strains in which the transposon surveillance system is inactivated. Such mutator strains could then be used for gene cloning by transposon tagging, as described in section 12.3.1.

12.3.5. Genetic transformation

Genetic transformation provides the technology to transfer genes from any prokaryotic or eukaryotic species into a target organism. Transgenic methods are routinely used to precisely alter single phenotypes of both prokaryotic and eukaryotic organisms for basic research. The power and precision of transgenic technology has also been utilized in several strain improvement programmes, most notably in crop plants and microorganisms.

Hashmi *et al.* (1995) successfully transformed *H. bacteriophora* by microinjection using a β-galactosidase (β-gal) reporter construct under the control of a promoter from the *C. elegans hsp-16* heat shock gene. *H. bacteriophora* was subsequently transformed with a green fluorescent protein (GFP) reporter gene (Hashmi *et al.*, 1997) and with a

heat-inducible heat shock gene (*hsp70A*) from *C. elegans* (Hashmi *et al.*, 1998). More than 90% of the *hsp70A* transgenic nematodes survived a severe heat shock treatment (40°C for 1 h) as compared to 2–3% of wild-type *H. bacteriophora* (Hashmi *et al.*, 1998). Vellai *et al.* (1999) transformed *S. feltiae* with a trehalose phosphate synthase (TPS) gene under the control of the *C. elegans hsp-16* heat-shock promoter. TPS is the key enzyme in the biosynthesis of trehalose, an important cyroprotectant and osmoprotectant molecule. Overexpression of TPS in *S. feltiae* resulted in increased osmotolerance and desiccation tolerance in the transgenic adult nematodes.

The studies described above clearly demonstrate the potential of transgenic methods for strain improvement of *H. bacteriophora* and *S. feltiae*, particularly for enhancements in environmental tolerances. Other traits that could be improved in entomopathogenic nematodes by transformation with single gene constructs might include phenotypes such as cold tolerance, UV tolerance, nematicide resistance. The potency and specificity of biocontrol nematodes might also be improved by expressing novel insecticidal proteins (e.g. toxins, lytic enzymes or immunosuppressants) in their salivary gland secretions. Genetic transformation could also be used for basic research in these nematodes to study developmental and tissue dependent gene expression using GFP or β-gal reporter constructs, or in the cloning of mutationally defined genes by mutant rescue.

Genetic transformation of *Heterorhabditis* and *Steinernema* was achieved using the microinjection methods developed for *C. elegans* (Mello *et al.*, 1991). These transformation techniques are technically demanding and require specialized equipment. Microprojectile bombardment is widely used in plant transformation and this method has been successfully applied to *C. elegans* by Wilm *et al.* (1999). It should be possible to adapt their protocol for use with entomopathogenic species. A morphological marker for identifying entomopathogenic nematode transformants has yet to be developed. [The *rol* marker which is widely used to identify transformants in *C. elegans* is not expressed in *Heterorhabditis* (Hashmi *et al.*, 1995)]. The development of such a construct for use in transformation experiments would be a major advance.

Genetically modified organisms (GMOs) typically have incorporated into their genomes a single foreign gene affecting an individual trait, nevertheless the environmental release of GMOs is a very emotive issue. Concerns about the release of GMOs centre around questions such as: (i) what are the possibilities that the transgenes from the GMO will be transferred (horizontally) to other organisms, especially to deleterious organisms such as pathogens and pests? (ii) What are the possibilities that the GMO will survive better than the parent strain in some environments, with unknown consequences? (reviewed by Hoy, 2000). Gaugler *et al.* (1997) document the regulatory procedures which they were required to fulfil before receiving permission to field test transgenic *H. bacteriophora* containing a heat-inducible heat shock gene (*hsp70A*) from *C. elegans*. These authors also describe the safeguards that they incorporated into their field studies in order to minimize the possibility of escape of the transgenic nematodes. Gaugler *et al.* (1997) report that the US Environmental Protection Agency concluded that registration of transgenic *H. bacteriophora* was not required but that the nematode's bacterial symbiont must be that which occurs in nature with the nematode and that the bacterial symbiont must not be genetically engineered. These authors also report that the US Department of Agriculture's Animal and Plant Health Inspection Service (APHIS) concluded that production of additional heat-shock protein by

transgenic *H. bacteriophora* could be expected to have little effect on nematode survival or ecological relationships because heat-shock events are very rare and are buffered by the soil environment. Thus permission for contained field release of transgenic *H. bacteriophora* with the *hsp70A* gene from *C. elegans* was granted by regulatory authorities.

There have been no field releases of transgenic nematodes in Europe to date. In Europe, such releases are controlled by the council directive 90/220/EEC on the deliberate release into the environment of genetically modified organisms (see Handbook for implementation of EU and environmental legislation, at http://europa.eu/int/comm/environmental/enlarg/handbook). This council directive adopts a preventative approach, rather like that which pertains in the USA. The European system requires the prior notification of the competent national authority that will then evaluate the risks to human health or the environment from the proposed release. If permission is granted by a national authority within the EU, this authority then has the responsibility to establish inspection and monitoring procedures to ensure that the necessary safeguards as required by the EU directive are implemented (see Richardson, 1996 for further details). Gaugler *et al.* (1997) conclude that permission for the release of transgenic nematodes is more likely to be obtained from regulatory authorities when (i) the transgenic traits confer commercial (e.g. improved shelf-life or production efficiency) rather than ecological advantages; (ii) donor and recipient organisms already exist in the ecosystem; (iii) when the genetic manipulation results in the over-expression of an existing nematode gene product; and (iv) risk reduction (e.g. containment) protocols are stringent.

12.4. Conclusions and Future Prospects

The strain improvements obtained to date with *Heterorhabditis* and *Steinernema* have been mainly achieved using conventional genetics. However the strains developed by Hashmi *et al.* (1998) and Vellai *et al.* (1999) clearly demonstrate the potential of transgenic methods for strain improvement, particularly for improved environmental tolerances. Genetic improvements of entomopathogenic nematodes via conventional means will, however, remain important in the future because of public concerns about the use of transgenic organisms. Nevertheless, we are now entering a new phase in entomopathogenic nematode research in which the tools of molecular genetics will be increasingly used to address a range of biological questions, both fundamental and applied.

One strategy that will help the *Heterorhabditis* and *Steinernema* research community in their endeavours to make progress in nematode genetics and biology is to emphasize the importance and utility of these nematodes and their symbionts as exciting subjects for basic research. In this way new researchers with skills in molecular biology might be encouraged to work on entomopathogenic nematodes. These nematodes and their bacteria could be excellent model systems in which to study a range of fundamental biological problems including:

- the pathogenic interactions between the nematodes and their insect hosts;
- the development and maintenance of the symbiotic relationship between the nematodes and their symbiont bacteria;
- the parallels between the developmental pathways of *C. elegans* and entomopathogenic nematodes, especially the dauer formation and recovery pathways;

- the environmental signals and genetic processes involved in sex determination, especially for *Heterorhabditis*;
- the role between chemoreception and host detection.

Entomopathogenic nematodes have many advantages as model animal parasites. They (particularly *Heterorhabditis)* are closely related phylogenetically to *C. elegans* and share many similarities with it in their life cycle and development. This strongly suggests that at the genomic level too, many genes and genetic processes and pathways will be conserved between these genera. Where significant differences in gene identity, organization and expression profiles are detected between entomopathogenic nematodes and *C. elegans* (in an EST survey, for example) these differences may be important pointers to adaptive changes associated with parasitism or symbiosis. The knowledge gained from these endeavours should ensure that entomopathogenic nematodes will become more effective biopesticides and should also ensure that these nematodes and their symbionts gain prominence as unique and intrinsically interesting biological systems.

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13 Formulation and Application Technology

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13.1. Introduction

Poor storage and post-application survival are major obstacles to the expanded use of entomopathogenic nematodes as bioinsecticides. No nematode formulation meets the 2-year shelf-life requirement of standard chemical pesticides. This chapter explores the concepts of nematode formulations and application technology, and critically evaluates the factors affecting nematode survival in formulations developed for storage, transport, and application. Quality control, standardization, handling, and transport issues are briefly described. Practicalities of applying nematodes using conventional pesticide, fertilizer and irrigation equipment are explored and factors affecting nematode survival during application are discussed. Recent research on the use of adjuvants to enhance nematode retention and survival on the foliage is briefly reviewed. Emphasis has been placed on identifying gaps in knowledge and on research needs to enhance nematode storage stability and post-application survival.

13.2. Formulation

Formulation refers to the preparation of a product from an active ingredient by the addition of certain active (functional) and non-active (inert) substances. Formulation is intended to improve activity, absorption, delivery, ease-of-use or storage stability of an active ingredient. Typical examples of pesticide formulation ingredients (additives) include absorbents, adsorbents, anticaking agents, antimicrobial agents, antioxidants, binders, carriers, dispersants, humectants, preservatives, solvents, surfactants, thickeners and UV absorbers. Although the overall concept of nematode formulations is similar to traditional pesticide formulations, nematodes present unique challenges. High oxygen and moisture requirements, sensitivity to temperature extremes, and behaviour of infective juveniles limit the choice of the formulation method and ingredients. Major goals of developing nematode formulations include maintenance of quality, enhancement of storage stability, improvement in ease-of-transport and use, reduction of transport costs, and enhancement of nematode survival during and after application. Expected shelf-life of steinernematid and heterorhabditid nematodes in major formulations is given in Table 13.1.

13.3. Formulations for Storage and Transport

Although infective juveniles can be stored up to several months in water in refrigerated bubbled tanks, high cost and difficulties of maintaining quality preclude the deployment of this method. High oxygen demand, sensitivity of some nematode species to low temperature, susceptibility to microbial contamination, and toxicity of antimicrobial agents are factors influencing nematode quality during storage in water. Therefore, nematodes are usually formulated into non- or semi-liquid substrates soon after they are produced.

13.3.1. Formulations with actively moving nematodes

Placement of nematodes on or in inert carriers has provided a convenient means to store and ship small quantities of nematodes. In these formulations, nematodes are fully active and move freely in or on the substrate. The inert carrier formulations are

Table 13.1. Expected shelf-life of *Steinernema* and *Heterorhabditis* spp. in formulations.

			Shelf-life (months)	
Formulation	Nematode species	Strain	22–25°C	2–10°C
Actively moving nematodes				
Sponge[a]	*S. carpocapsae*	All	0.03–0.1	2.0–3.0
	H. bacteriophora	HP88	0	1.0–2.0
Vermiculite[a]	*S. carpocapsae*	All	0.1–0.2	5.0–6.0
	S. feltiae	UK	0.03–0.1	4.0–5.0
	H. megidis	UK	0	2.0–3.0
Reduced mobility nematodes				
Alginate gels	*S. carpocapsae*	All	3.0–4.0	6.0–9.0
	S. feltiae	SN	0.5–1.0	4.0–5.0
Flowable gels	*S. carpocapsae*	All	1.0–1.5	3.0–5.0
	S. glaseri	NJ43	0.03–0.06	1.0–1.5
	S. scapterisci	Colon	1.0–1.5	3.0–4.0
Liquid concentrate[a]	*S. carpocapsae*	All	0.16–0.2	0.4–0.5
	S. riobrave	RGV	0.1–0.13	0.23–0.3
Anhydrobiotic nematodes				
Wettable powder	*S. carpocapsae*	All	2.0–3.5	6.0–8.0
	S. feltiae	UK	2.5–3.0	5.0–6.0
	H. megidis	UK	2.0–3.0	4.0–5.0
	H. zealandica	NZ	1.0–2.0	3.0–4.0
Water dispersible granules[a]	*S. carpocapsae*	All	4.0–5.0	9.0–12.0
	S. feltiae	SN	1.5–2.0	5.0–7.0
	S. riobrave	RGV	2.0–3.0	4.0–5.0

[a]Commercially available formulation.

easy and less expensive to make, but all products require refrigeration during storage and transport, and are thus expensive.

13.3.1.1. Sponge

Polyether-polyurethane sponge-based formulations are widely used to store and ship small quantities of nematodes in the USA cottage industry that caters mainly to the home lawn and garden markets. The sponge formulation is made by applying an aqueous nematode suspension to a sheet of sponge, usually at 500–1000 infective juveniles per cm^2 of surface area. Nematodes on sponges can be stored for 1–3 months at 5–10°C. Normally $5–25 \times 10^6$ infective juveniles are placed on a sheet of sponge that is then placed in a plastic bag. The bags are placed on ice packs for shipping, and the nematodes are removed from the sponges by soaking and hand squeezing in water before application. This formulation is not suitable for large acreage applications owing to this cumbersome removal method and the large quantity of sponges needed.

13.3.1.2. Vermiculite

Vermiculite formulation is a significant improvement over sponge. Advantages include a more concentrated nematode product, longer storage stability, and more convenient application. Normally, an aqueous nematode suspension is mixed

homogeneously with vermiculite, and the mixture is placed in thin polyethylene bags. The vermiculite–nematode mixture is added to the spray tank directly, mixed in water, and applied either as spray or drench.

13.3.2. Formulations with reduced mobility nematodes

Owing to the high activity of nematodes in the inert carriers, the stored energy reserves of infective juveniles are rapidly depleted and sometimes they even escape from the formulations. Therefore, formulations have been developed in which the mobility of nematodes is minimized either through physical trapping or by using metabolic inhibitors. Examples of such formulations include alginate and flowable gels and a liquid concentrate containing a proprietary metabolic inhibitor.

13.3.2.1. Physical trapping

Thin sheets of calcium alginate spread over plastic screens have been used to trap nematodes (Georgis, 1990). For application, the nematodes had to be released from the alginate gel matrix by dissolving it in water with the aid of sodium citrate (Georgis, 1990). Alginate-based *S. carpocapsae* products were the first to possess room temperature shelf-life, and led to increased acceptability of nematodes in high value niche markets (Grewal, 1998). However, the time-consuming extraction steps and the problematic disposal of large numbers of plastic screens and containers, rendered this formulation unsuitable for large-scale application.

A formulation in which nematodes are mixed in a viscous flowable gel or paste to reduce activity has been reported (Georgis, 1990). Although the flowable gel is easy to apply, nematode shelf-life was shorter than in the alginate gels and therefore, the use of this product was suspended. Chang and Gehert (1995) described a paste formulation in which nematodes are mixed in a hydrogenated oil and acrylamide. Over 80% survival of *S. carpocapsae* after 35 days at room temperature was reported in this formulation, which is commercially unacceptable.

13.3.2.2. Metabolic arrest

Grewal (1998) reported that the addition of a proprietary metabolic inhibitor reduces oxygen demand and enables storage of concentrated *S. carpocapsae*, *S. feltiae* and *S. riobrave* without bubbling air for extended periods at room temperature. Over 7×10^9 infective juveniles of *S. carpocapsae* could be stored for 6 days at room temperature in a 10-litre container, without significant loss in viability. This liquid concentrate formulation is used for shipping *S. riobrave* for application in citrus against *Diaprepes* root weevil. Yukawa and Pitt (1985) described a formulation wherein nematodes were trapped in powdered activated charcoal that served as an adsorbent. The charcoal and nematode mixture was stored in sealed containers to minimize oxygen availability. High cost, lack of room temperature stability and difficulties of application rendered this formulation unsuitable for commercial use.

13.3.3. Formulations with anhydrobiotic nematodes

Nematodes require a film of water around their body for optimum metabolism, activity, and movement. However, the energy stores of infective juveniles are constantly depleted during activity. Therefore, one formulation goal has been to reduce the rate

of consumption of stored energy reserves by infective juveniles. This has been accomplished in some formulations by desiccating infective juveniles. Entomopathogenic nematodes are capable of only partial anhydrobiosis, and are therefore referred to as quiescent anhydrobiotes (Womersely, 1990). Although desiccation of nematodes at controlled relative humidities has been demonstrated (Simmons and Poinar, 1973; Popiel *et al.*, 1993), this method had little success commercially. Induction of partial anhydrobiosis has been achieved successfully by controlling water activity (A_w) of the formulations (Bedding, 1988; Silver *et al.*, 1995; Grewal, 2000a). Water activity is a measure of how tightly water is bound, structurally or chemically, to the nematodes. As opposed to water content, A_w is influenced by bonding of water molecules to the surfaces as well as osmosis. A_w equals the relative humidity of air, in equilibration with a nematode sample in a sealed container. A_w is defined as the ratio of water vapour pressure over a sample (P) divided by the water pressure over pure water (P_0). Thus multiplication of water activity by 100 yields the relative humidity of the atmosphere in equilibrium with a sample. Water activity can be easily measured using a water activity meter, such as those routinely used in the food industry (AquaLab Model CX-2, Decagon Devices, Inc., Pullman, Washington). The formulations containing partially anhydrobiotic nematodes include gels, powders, and granules.

13.3.3.1 Gels

Bedding and Butler (1994) developed a formulation in which nematode slurry is mixed in anhydrous polyacrylamide so that the resulting gel attains a water activity between 0.800 and 0.995. The nematodes were partially desiccated, but survival at room temperature was low. Further, this formulation was difficult to dissolve and resulted in clogging of sprayers.

13.3.3.2. Powders

In 1988 a formulation was described in which nematodes were mixed in clay to remove excess surface moisture and to produce partial desiccation (Bedding, 1988). The formulation, termed a ‘sandwich’, consisted of a layer of nematodes between two layers of clay. This formulation was commercialized by Biotechnology Australia Ltd, but was later discontinued due to inconsistent storage stability, clogging of spray nozzles, and a low nematode-to-clay ratio.

An improved wettable powder formulation has been developed that enables storage of heterorhabditids and steinernematids at room temperature (Grewal, 1998). Nematodes are partially desiccated because of the addition of water absorbents in this formulation. This formulation is also easy to apply owing to high dispersibility in water. *Heterorhabditis megidis* can be stored up to 3 months at 22°C without loss in viability in this formulation (see Table 13.1).

13.3.3.3. Granules

Capinera and Hibbard (1987) described a granular formulation in which nematodes were partially encapsulated in lucerne meal and wheat flour. Later, Connick *et al.* (1993) described an extruded or formed granule in which nematodes were distributed throughout a wheat gluten matrix. This ‘Pesta’ formulation included a filler and a humectant to enhance nematode survival. The process involved drying of granules to

low moisture to prevent nematode migration and reduce risk of contamination. However, granules rapidly dry out during storage resulting in poor nematode survival.

A water dispersible granular (WG) formulation has been developed in which infective juveniles are encased in 10–20 mm diameter granules (Fig. 13.1) consisting of mixtures of various types of silica, clays, cellulose, lignin and starches (Georgis *et al.*, 1995; Silver *et al.*, 1995). These granules are prepared through a conventional pan granulation process in which droplets containing a thick nematode suspension are sprayed onto pre-mixed formulation powder on a tilted rotating pan (Grewal and Georgis, 1998). As nematode droplets come in contact with the powders, the granules start to form, and roll over the dry powders adsorbing more powder around them. The granules are then sieved out of the powders and packaged into shipping cartons. The granular matrix allows access of oxygen to nematodes during storage and shipping. At optimum temperature, the nematodes enter into a partial anhydrobiotic state owing to the slow removal of their body water by the formulation. The induction of partial anhydrobiosis is usually evident within 4–7 days by a threefold to fourfold reduction in oxygen consumption of the nematodes (Grewal, 2000a,b).

Water dispersible granular formulations offer several advantages over other formulations. These include: (i) extended nematode storage stability at room temperature, (ii) enhanced nematode tolerance to temperature extremes enabling easier and less expensive transport, (iii) improved ease-of-use of nematodes by eliminating time and

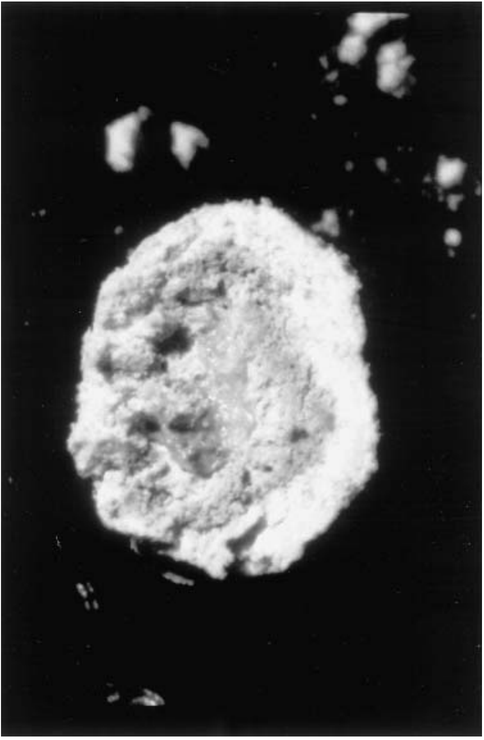

Fig. 13.1. Cross section of a water dispersible granule showing enclosed infective juveniles of *Steinernema carpocapsae*.

labour intensive preparation steps, (iv) decreased container size and coverage ratio, (v) decreased disposal material (i.e. screens and containers), and a more acceptable appearance. This is the first commercial formulation that enabled storage of *S. carpocapsae* for over 6 months at 25°C (Grewal, 2000a).

13.4. Formulations for Application

Low post-application survival in the soil and on foliage reduces nematode efficacy (Smits, 1996). Although several adjuvants have been evaluated to enhance nematode survival, limited research has been conducted to develop formulations that enhance survival. The development of formulations that provide protection to the nematodes from environmental extremes during and after application can substantially enhance nematode efficacy. This area deserves more attention and may yield interesting results. The formulations specifically intended to enhance post-application survival of nematodes in the soil and foliage are described below.

13.4.1. Desiccated cadavers

Nematodes applied in the form of infected wax moth (*Galleria mellonella*) larvae were found to be as effective as aqueous nematode suspension against soil pests (Welch and Briand, 1960; Janson and Lecrone, 1984). The application of nematode-infected insects can be superior to aqueous suspensions (Shapiro and Glazer, 1996). A formulation based on desiccated cadavers coated with clay has been developed that may allow application without cadavers rupturing or adhering together (D. Shapiro, Georgia, 2000, personal communication).

13.4.2. Capsules

Macrogels containing encapsulated nematodes have been suggested as delivery systems for the control of soil and foliar pests. Encapsulation of nematodes in calcium alginate gel beads was first reported by Kaya and Nelsen (1985) who advocated their use as baits, or for soil applications. When the alginate capsules were placed in soil with adequate moisture, most nematodes migrated out of the granules within a week. Navon *et al.* (1999) evaluated an edible-to-insects calcium alginate gel for *S. riobrave*. They reported high nematode survival for 48 h in the gel at 61% relative humidity. A gel-forming polyacrylamide used to enhance water-holding capacity of sandy soils, has been shown to enhance survival of *S. carpocapsae* when applied against the *Diaprepes* root weevil in citrus (W. Schroeder in Georgis, 1990). Chang and Gehert (1992) developed macrogels containing encapsulated *S. carpocapsae* along with a water-holding acrylamide in gellan gum. Gellan gum is liquid at room temperature and can be induced to gel with the addition of a divalent cation such as calcium.

13.4.3. Baits

Baits containing infective juveniles, an inert carrier (e.g. corncob grits, groundnut hulls or wheat bran), and a feeding stimulant (e.g. glucose, malt extract, molasses or sucrose) or a sex pheromone have been developed (Georgis, 1990). *S. carpocapsae* and *S. scapterisci* have shown particular promise in the baits; because of their 'sit-and-wait' foraging strategy they do not escape the formulation and are more tolerant of desiccation

than other species. Yet only moderate control of cutworms, grasshoppers and tawny mole cricket was achieved (Georgis, 1990). When trap stations were used that ensured nematode contact with the target pest and protected nematodes from sunlight and desiccation, the baits outperformed the standard chemical insecticide against adult house flies in pig units (Renn, 1998) and German cockroaches in apartments (Appel *et al.*, 1993).

13.5. Factors Affecting Nematode Survival in Formulations

13.5.1. Culture method

Nematodes produced *in vivo* have been found to be more stable than those produced *in vitro*. For example, survival of *S. riobrave* stored in water at 9°C was higher when cultured in *G. mellonella* larvae than in liquid media (Fig. 13.2). However, the mechanisms for these differences have not been explored and may provide clues to physiological factors affecting storage stability. Exposure of nematodes during culture to stresses including temperature extremes, oxygen deprivation, sheer stress, type and quantity of antifoam, and microbial contamination can influence nematode quality leading to reduced shelf-life. Nematodes are highly sheer sensitive and sheer stress associated with stirred fermenters can influence nematode reproduction (Friedman, 1990). Sheer stress during fermentation can also reduce nematode survival in the formulations. In batches of *S. carpocapsae* produced in stirred fermenters, a high negative correlation between impeller tip speed and nematode survival at 25°C was found (Fig. 13.3).

13.5.2. Stored energy reserves

Lipid constitutes about 60% of the dry weight of infective juveniles of entomopathogenic nematodes (Selvan *et al.*, 1993b) and is considered as a major energy reserve. The amount of lipid varies with nematode species and production

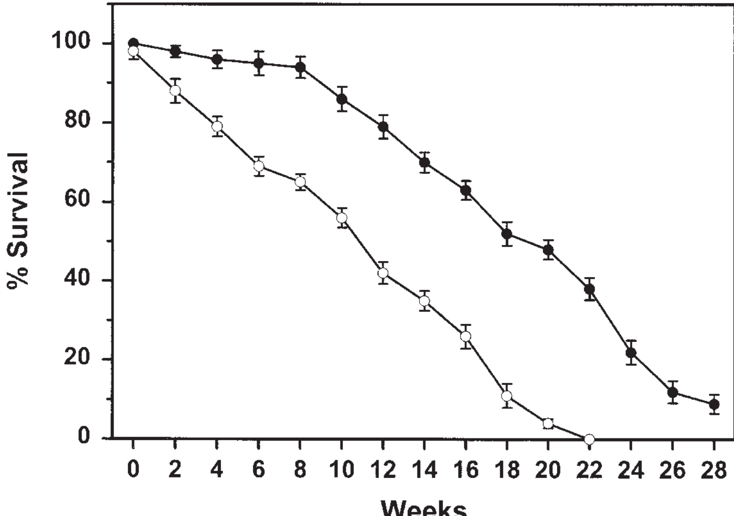

Fig. 13.2. Survival (± SE) of *Steinernema riobrave* infective juveniles in water at 9°C when cultured in *Galleria mellonella* larvae (●) or in liquid culture (○).

batches (Grewal and Georgis, 1998) and is influenced by the type and amount of media components, antifoam, temperature, and dissolved oxygen during fermentation. The rate of lipid utilization also differs among nematode species (Grewal, 2000b) and among individuals within a species (Patel *et al.*, 1997b). Other factors including temperature, oxygen availability, and nematode activity in storage can also influence the rate of lipid utilization and thus survival (Grewal and Georgis, 1998).

13.5.3. Temperature

Temperature is the most important factor affecting nematode survival in formulations. Each species has an optimum storage temperature, which is well below the optimum temperature for activity and reproduction of the species and reflects the climatic conditions of its original locality (Table 13.2). Optimum temperature for successful induction of anhydrobiosis also differs with nematode species. Most species can withstand some level of desiccation at their optimum reproduction temperature, but desiccation directly at temperature extremes can be lethal. For example, storage of *S. riobrave* at 5°C immediately after formulation in water dispersible granules resulted in 100% mortality within 2–3 weeks, as compared to less than 20% mortality at 25°C (Grewal, 1998). This sensitivity of *S. riobrave* to desiccation at 5°C was significantly reduced by preconditioning of infective juveniles in water at 5°C for 4–7 days before formulation (Grewal, 2001, unpublished results). This correlated with the accumulation of trehalose during cold preconditioning. Trehalose accumulation at low temperatures appears to be common among entomopathogenic nematodes (Ogura and Nakashima, 1997; Qiu and Bedding, 1999; Grewal and Jagdale, 2001, unpublished results), and may be a component of survival strategy during environmental stress (Jagdale and Grewal, 2001, unpublished results).

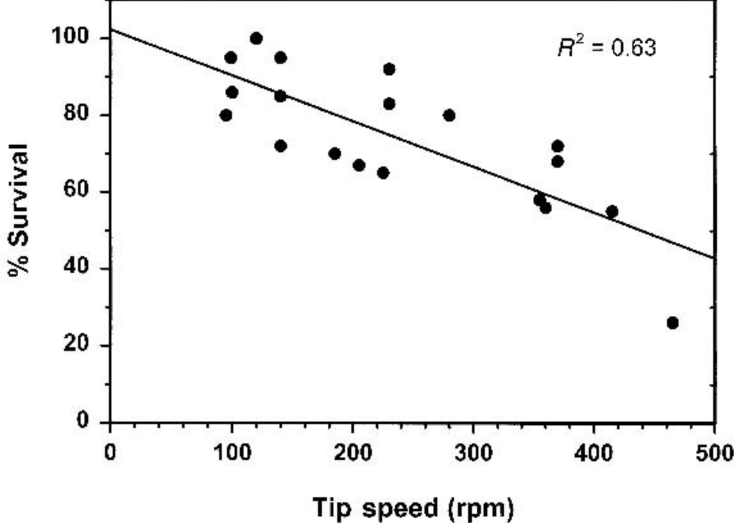

Fig. 13.3. Effect of impeller tip speed during liquid culture in a stirred fermenter on the survival of *Steinernema carpocapsae* infective juveniles in a flowable gel after 8 weeks at 25°C.

Table 13.2. Temperature (°C) preferences and original localities of *Steinernema* and *Heterorhabditis* spp.

Nematode species	Strain	Original locality (mean low and high temperature, °C)	Optimum storage temperature[a]	Optimum reproduction temperature[b]	Temperature reproduction range[b]	Temperature infectivity range[b]
S. carpocapsae	All	Georgia, USA (9–26)	5	25	20–30	10–32
S. feltiae	SN	France (6–19)	5	18	10–25	8–30
S. glaseri	NC	North Carolina, USA (3–22)	10	25	12–35	10–37
S. riobrave	RGV	Texas, USA (14–27)	15	28	20–35	10–39
S. scapterisci	Colon	Colon, Argentina (8–28)	10	28	20–32	10–35
H. bacteriophora	HP88	Utah, USA (3–22)	10	25	15–30	10–32
H. megidis	HO1	Ohio, USA (3–17)	5	18	10–25	8–32

[a] P. Grewal, unpublished data.
[b] Grewal *et al.* (1994a).

13.5.4. Anhydrobiosis

Although anhydrobiosis is an important means of achieving storage stability of entomopathogenic nematodes, comparisons of longevity between desiccated and non-desiccated nematodes were made only recently. Grewal (2000a) demonstrated that the longevity of desiccated *S. carpocapsae* in water dispersible granules was extended by 3 months as compared to those stored in water at 25°C, whereas the longevity of desiccated *S. riobrave* was extended only by 1 month. These differences in desiccation survival may be related to the differences in the rate of water loss by the infective juveniles. Patel *et al.* (1997a) reported that *S. carpocapsae* had a lower rate of water loss than *S. riobrave*. Kondo and Ishibashi (1989) reported that the differences in water loss among *Steinernema* spp. were related to differences in cuticle structure. Despite the induction of partial anhydrobiosis, nematodes require high moisture for survival in the formulations. Grewal (1998) reported that initial moisture content was positively correlated with survival of *S. carpocapsae* in water dispersible granules at 30°C.

The extension in longevity through anhydrobiosis is metabolically costly. This becomes evident when longevity of desiccated and non-desiccated nematodes is compared at low temperatures. At 5°C, the longevity of desiccated *S. carpocapsae, S. feltiae* and *S. riobrave* in water dispersible granules was shorter than in water (Grewal, 2000b). During desiccation, nematode metabolic rate increases sharply in the first 24 h, then declines slowly to a level below the normal metabolic rate within 4–7 days (Grewal, 2000a,b), and glycogen and lipid reserves are converted to trehalose (Womersely, 1990; Solomon *et al.*, 1999). Upon rehydration, trehalose is either used as an energy source or converted back to glycogen.

Entomopathogenic nematodes appear to use two distinct desiccation survival strategies: desiccation avoidance and tolerance (Grewal and Jagdale, 2001, unpublished results). Both *S. feltiae* and *S. riobrave* exhibit a desiccation avoidance behaviour as they rapidly migrate out of the granules whereas *S. carpocapsae* shows no desiccation avoidance response. Aggregation by *S. carpocapsae* (Simmons and Poinar, 1973; Womersely,

1990) and migration by certain strains of *S. feltiae* (Solomon *et al.*, 1999) to desiccation on filter papers under controlled relative humidity conditions has been observed. Duncan *et al.* (1996) reported that *S. riobrave* moved downwards in response to desiccation on the surface of soil in vertical experimental arenas. The differences in behaviour in response to desiccation in the formulations appear to be related to the foraging strategy of the nematodes. *S. carpocapsae* is an ambusher that uses a 'sit-and-wait' strategy to find hosts whereas *S. feltiae* and *S. riobrave* are classified as cruisers (see Lewis, Chapter 10, this volume). In an ambush search, infective juveniles exhibit a nictation behaviour displacing over 90% of their body in the air in search of highly mobile hosts on or near the soil surface. This behaviour exposes infective juveniles to a rapid desiccation environment as opposed to the species that cruise through the soil profile in search of the prey. Therefore, it is predicted that cruise foraging nematodes would use desiccation avoidance strategy by moving deep into the soil profile while the ambush foragers will be more adapted to desiccation tolerance.

13.5.5. Antimicrobial agents

Microbial contamination is a significant problem in nematode formulations with high moisture content. Microbial contamination can deplete the available oxygen, reduce dispersibility of formulations, cause clogging of spray nozzles, and reduce acceptability of the product. Although antimicrobial agents can be used to suppress microbial growth, they must be carefully selected as they can reduce nematode survival in the formulations. Furthermore, nematode species differ in their susceptibility to antimicrobial agents. For example, *S. feltiae* and *S. riobrave* are more susceptible to Proxel, a commonly used antimicrobial agent, than *S. carpocapsae*.

13.6. Quality Control and Standardization

Maintenance of high viability and virulence during production and formulation forms the backbone of an effective quality control strategy. Viability refers to the percentage of living infective juveniles (compared with dead and non-infective stages) whereas total viable nematodes are the total numbers of living infective juveniles in a suspension. This distinction is important as dead nematodes dissolve over time and viability alone may be misleading. Also some nematode species adopt quiescent postures that may be easily confused with dead nematodes. Therefore, motionless nematodes should be either probed or agitated by adding a drop of hydrogen peroxide to facilitate assessments. Over-packing is a method of ensuring minimum total viable nematodes during storage.

Virulence is the most important component of nematode quality. Virulence can be measured by several different methods including one-on-one bioassay (Grewal *et al.*, 1999; Converse and Miller, 1999), LC_{50} (Georgis, 1992), establishment efficiency (Hominick and Reid, 1990; Epsky and Capinera, 1994), and invasion rate (Glazer, 1992). However, assays using multiple nematodes against single or multiple hosts are considered inappropriate for quality control purposes due to host–parasite interactions such as recruitment and over dispersion of natural parasite populations (Grewal *et al.*, 1999). The one-on-one assay compares virulence of any nematode species with a pre-determined 'standard' against a susceptible host such as *G. mellonella* larvae. This method measures the proportion of infective nematodes in a population and is

sensitive to 'impaired' nematodes. This method is applicable to species that have a lethal level of one infective juvenile per larva (Table 13.3). However, lowest nematode concentrations that produce about 50% larval mortality of *G. mellonella* have been determined for other species. For example, *S. scapterisci* causes 30–70% mortality at 15 infective juveniles per larva and *H. bacteriophora* causes 40–65% mortality at 5 infective juveniles per larva (Table 13.3). *G. mellonella* is a preferred bioassay host because it is highly susceptible and is commercially available, thus supply can be assured.

Filter paper arenas are suitable for ambushing nematodes whereas sand columns are optimal for cruisers (Grewal *et al.*, 1994b). However, ambushers and cruisers perform equally well in the newly developed sand-well bioassay (Grewal *et al.*, 1999) that facilitates both ambushing and cruising behaviours by infective juveniles. This method is also easy to set up and is closer to field situation than the filter paper bioassays. Therefore, it is proposed that the sand-well bioassay be adopted as a standard quality control tool for assessing the virulence of entomopathogenic nematodes. Major steps to set up this bioassay are given below:

1. Add 1 g of dry sterilized play sand (Lonestar #60, particle size <710 μm) to each cell of the 24-cell-well plate (Falcon).
2. From a well-mixed nematode sample, extract a representative sub-sample of infective juveniles. Place the sub-sample into a tissue culture dish and add enough deionized water to spread and cover the nematodes to a depth of 2–3 mm. Loosely secure cap and store labelled sample at room temperature for 48 h.
3. After 48 h conditioning, swirl contents of tissue culture flask and transfer approximately 10 ml of deionized water. The actual quantities of sample and water used should be sufficient to separate the nematodes enough that individuals can be picked up with the transfer pipette.
4. Pick up 1, 5 or 15 living infective juvenile(s) (see Table 13.3) in 30 μl of water using a transfer pipette and a dissecting microscope. Expel the nematodes into one of the cell-wells. Using the same pipette immediately transfer 30 μl of diluted MICRO soap (International Products Corp., Trenton, New Jersey) into to the same cell. A diluted MICRO soap solution is prepared by adding 20 μl of MICRO soap to 50 ml of room temperature tap or deionized water.

Table 13.3. Parameters of the sand-well bioassay for virulence assessment of *Steinernema* and *Heterorhabditis* spp.

Nematode species	Strain	*Gallerio mellonella* : nematode ratio	Bioassay temperature (°C)	Expected larval mortality after 72 h[a]
S. carpocapsae	All	1 : 1	28	50–75
S. feltiae	SN	1 : 1	25	35–50
S. glaseri	NJ43	1 : 1	28	35–50
S. riobrave	RGV	1 : 1	28	50–75
S. scapterisci	Colon	1 : 15	28	30–70
H. bacteriophora	HP88	1 : 5	25	40–65
H. megidis	UK	1 : 5	25	35–55

[a] Mean percentage larval mortality in four 24-well plates over several nematode batches.

5. Continue transferring living infective juveniles from the petri dish into cell-wells, using the MICRO solution flush to assure transfer. As rows of cells are filled, slide the cell-well cover up so that filled cells are covered. Place infective juveniles into a total of 96 cells (4 cell-well plates).
6. Once all cell wells are filled, add one clean *G. mellonella* larva to each cell, replace cover, and wrap with parafilm. Incubate the cell-well plates at 25°C or 28°C depending upon species (see Table 13.3).
7. Inspect after 48 or 72 h (depending on species/strain) for dead *Galleria* larvae. Record the total number of dead larvae found.
8. A water (or negative) control sample consisting of 4 cell-well plates (96 cells) into which 1 g dry sand, 30 μl of room temperature tap water, 30 μl of diluted MICRO solution, and 1 *G. mellonella* larva have been added should be kept to estimate control mortality. Record the number of larvae dead after 48 or 72 h.
9. Operator calibration can be performed by having multiple operators conduct the test with the same nematode sample and *G. mellonella* lot or shipment. Each participant should carry out replicate tests. The results may be analysed statistically for differences.

As the product ages, the depletion in stored energy reserves may reduce virulence (Patel *et al.*, 1997b; Wright *et al.*, 1997), nictation ability (Lewis *et al.*, 1995), and environmental tolerance (Selvan *et al.*, 1993a; Patel *et al.*, 1997a) of infective juveniles. Therefore, time from production to formulation, formulation to packaging, packaging to shipping is usually controlled. The use of batch codes and expiry dating are useful methods of tracking and controlling the inventory life (refrigerated storage time before application). Assessment of microbial contamination is also an integral part of nematode product quality assessment. Physical characteristics such as product colour and weight, granule size distribution, formulation dispersibility, product temperature, and packaging are also monitored to reduce batch-to-batch variability and maintain consistency.

Gaugler *et al.* (2000) assessed quality of commercially produced nematodes aimed at a mail-order market in the USA. They found that most companies were accessible, reliably shipped pure populations of the correct species on time, in sturdy containers, and often with superb accompanying instructions. Nematodes were received in satisfactory condition with acceptable levels of viability. Consistency, however, was a problem, with each supplier having one or more weak spots to bolster. Most shipments did not contain the expected nematode quantity, and one shipment had no nematodes. Pathogenicity of several products against *G. mellonella* larvae was not equivalent to laboratory standards. *H. bacteriophora* was not always available when ordered. A few products contained mixed populations of *S. carpocapsae* and *H. bacteriophora*. Application rate recommendations provided by several suppliers were unsound. They concluded that the cottage industry lacks rigorous quality control, self-regulation is problematic without feedback, and consumers are rarely able to provide this feedback. Improved reliability by the nematode industry will most likely be achieved via industry-generated agreement on standards for quality.

13.7. Transport and Handling

Suboptimal storage by the vendor or end-user may be a major factor affecting nematode viability and efficacy. Overall, the nematode species isolated from cold climates

tolerate warm temperatures poorly and those from warm localities do poorly at cold temperatures (Tables 13.2 and 13.4). Desiccated nematodes are more tolerant of temperature extremes than non-desiccated nematodes, and are therefore more stable during transport and handling. Georgis *et al.* (1995) reported that *S. carpocapsae* could be stored for 2 days at 38°C without loss of viability in water dispersible granules, but not in alginate or flowable gels. Glazer and Salame (2000) also reported that desiccated *S. carpocapsae* had higher survival at 40°C than non-desiccated nematodes. As nematode species differ in their high temperature tolerance (see Table 13.4), and shipping temperature is unpredictable, refrigerated shipping should be preferred whenever feasible.

Nematodes in liquid formulations are prone to oxygen deprivation during shipping and handling. Although infective juveniles can withstand short exposure to hypoxic conditions, they do not tolerate hypoxia indefinitely. Also nematode species differ in their ability to withstand hypoxic conditions (Table 13.4). Qiu and Bedding (2000) reported that *S. carpocapsae* infective juveniles incubated in M9 buffer at 25°C under anaerobic conditions, could be revived when returned to aerobic conditions if exposure to anaerobic conditions was not more than 7 days. Refrigerated storage and bubbling air can improve nematode storage stability. This method is currently being tested to ship nematodes in refrigerated trucks from California to Florida for use in the citrus industry (M. Dimock, 2001, personal communication). Addition of a proprietary metabolic inhibitor has been shown to enhance stability of concentrated nematodes in water enabling non-refrigerated transport (see section 13.3.2.2).

13.8. Application Technology

Major target pests and commercial formulations of entomopathogenic nematodes are listed in Table 13.5. Although nematodes are generally applied as curative treatments, prophylactic applications to the soil surrounding seedlings and seeds have been advocated (Grewal and Georgis, 1998). In an attempt to inoculate *S. glaseri* in New Jersey for the control of Japanese beetle grubs, several inoculative releases of the nematodes were made from 1939 to 1942. Although re-isolated from southern New Jersey, the elimination of the bacterial symbiont by the use of antibiotics used in the *in vitro* rearing procedure, and possibly poor climatic adaptation of this neotropical nematode

Table 13.4. Relative environmental tolerance of major entomopathogenic nematode species (*Steinernema* and *Heterorhabditis*).

Nematode species	Desiccation tolerance	Hypoxia tolerance	UV tolerance	Heat tolerance	Cold tolerance
S. carpocapsae	High	High	High	Moderate	High
S. feltiae	Moderate	Moderate	Moderate	Low	High
S. glaseri	Moderate	Moderate	Moderate	Moderate	Moderate
S. riobrave	Moderate	Moderate	Moderate	High	Low
S. scapterisci	High	High	High	High	Low
H. bacteriophora	Low	Low	Low	Moderate	Moderate
H. megidis	Moderate	Moderate	Low	Low	High

limited the success of this programme (Gaugler *et al.*, 1992b). In another attempt, *S. scapterisci* originally isolated from Uruguay was introduced into Florida as a classical biological control agent for the tawny mole cricket (Parkman and Smart, 1996). The nematodes were reported to have established after treatment of 50 m^2 plots in pastures with either infective juveniles or nematode-infected mole crickets.

13.8.1. Application equipment

Nematodes can be applied using most conventional liquid application systems designed to deliver pesticides, fertilizers, and irrigation (Table 13.6). Considerations involved in the selection of an application system are volume, agitation system, pressure and recycling time, system environmental conditions, and spray distribution pattern (Shetlar, 1999). Nematode application equipment used in different cropping

Table 13.5. Major target pests and commercial formulations of entomopathogenic nematodes (*Steinernema* spp. and *Heterorhabditis* spp.)

Common name	Scientific name	Life stage[a]	Nematode species	Formulation[b]
Artichoke plume moth		L	*S. carpocapsae*	WP
billbugs	*Sphenophorus parvulus*	L, A	*S. carpocapsae*	WG, WP
			H. bacteriophora	Sponge
Black vine weevil	*Otiorhynchus sulcatus*	L	*H. bacteriophora*	Sponge
			H. marelata	Sponge
			H. megidis	WP
Black cutworm	*Agrotis ipsilon*	L, P	*S. carpocapsae*	WG, WP
Bluegrass webworm	*Parapediasia teterrella*	L	*S. carpocapsae*	WG, WP
			H. bacteriophora	Sponge
Cat flea	*Ctenocephalides felis felis*	L, P	*S. carpocapsae*	WG, WP
Citrus root weevil	*Diaprepes abbreviatus*	L	*H. bacteriophora*	Sponge
			S. riobrave	WG, LC
Cranberry girdler	*Chrysoteuchia topiaria*	L	*S. carpocapsae*	WG, WP
			H. bacteriophora	Sponge
			H. marelata	Sponge
Cranberry rootworm	*Rhabopterus picipes*	L	*H. bacteriophora*	Sponge
Fall armyworm	*Spodoptera frugiperda*	L	*S. carpocapsae*	WG, WP
Fungus gnats	*Bradysia* spp.	L	*S. feltiae*	Vermiculite
Japanese beetle	*Popillia japonica*	L	*H. bacteriophora*	Sponge
			H. zealandica	WP
Mint root borer	*Fumibotrys fumalis*	L	*S. carpocapsae*	WG, WP
Mint flea beetle	*Longitarsus waterhousei*	L, A	*H. bacteriophora*	Sponge
			S. carpocapsae	WG, WP
Northern masked chafer	*Cyclocephala borealis*	L.	*H. bacteriophora*	Sponge
			H. zealandica	WP
Sciarid flies	*Lycoriella* spp.	L	*S. feltiae*	Vermiculite
Strawberry root weevil	*Otiorhynchus ovatus*	L	*H. bacteriophora*	Sponge
Tawny mole cricket	*Scapteriscus vicinus*	N, A	*S. scapterisci*	WP
			S. riobrave	WG

[a]A = adult; L = larva; N = nymph; P = pupa.
[b] LC = liquid concentrate; WG = water dispersible granule; WP = wettable powder.

Table 13.6. Nematode application equipment used in different cropping systems and major handling considerations.

Application system	Cropping system	Handling considerations
Surface application		
Tractor or shelf-propelled sprayer systems	Field crops	Excessive tank agitation, excessive pump pressure, temperature of the spray solution, clogging of nozzle filters and screens
Spray-irrigation systems (pivot or linear) fitted with fertigation or chemigation	Field crops	Injection holding tank agitation system, liquid flow within distribution pipes, volume to charge and empty the system
Trickle tube systems or permeable soakers	Fruits and vegetables	Nematode agitation, Filters and screens
Back pack and hand pump sprayers	Fruits and vegetables	Nematode agitation, Filters and screens
Low volume spinning disc systems	Fruits and vegetables	Nematode agitation
Hand held boom or shower droplet single nozzle systems with a long hose	Turf and ornamentals	Nematode settling in the tank and hose, temperature in the hose
Hand guns	Turf and ornamentals	Nematode settling in the tank and hose, temperature in the hose
Hand cans	Home and garden	Nematode settling
Hose-end sprayer	Home and garden	Nematode settling in the hose
Sub-surface application		
Slit injectors	Turf and ornamentals	Nematode agitation, filters and screens
Soil injectors	Trees and shrubs	Nematode agitation, filters and screens
Hypodermic syringe	Trees and shrubs	Nematode agitation

systems and their handling considerations are given in Table 13.6. For application against soil insects, a spray volume of 750–1890 litres ha^{-1} is generally recommended (Georgis *et al.*, 1995). This is satisfactory for boom and lawn sprayers equipped with large ‘shower’ nozzles. However, most boom spray systems are designed to use less spray volume, usually in the range of 200–400 litres ha^{-1}. Although nematodes can be delivered with low-volume sprayers, pre- and post-application irrigation should be adjusted to compensate for reduced volume. Pre-application irrigation moistens the soil or turf thatch thus facilitating nematode movement and post-application irrigation is essential for washing any nematodes that may be on plant surfaces into the soil. Studies indicate that 0.25–0.65 cm of post-application irrigation is sufficient to move the nematodes into the soil (Shetlar *et al.*, 1988). Post-application irrigation must be applied before the spray droplets dry. For many crop systems, this means treating smaller areas and then applying irrigation immediately.

Screens and filters need to be large enough to allow passage of nematodes. *Steinernema carpocapsae* can pass through sprayer screens with openings as small as 100 μm in diameter, but larger openings are required for larger species such as *S. glaseri*

and *H. megidis*. Therefore, removal of the filters and screens is usually recommended, although this requires recalibration of the equipment. High pressure and extensive recycling through the pumping systems can damage nematodes. Most agricultural pumping systems use membrane or roller pumps and do not develop sufficient pressure or shear to damage nematodes. However, high-pressure hydraulic pumps can develop high internal pressure and may shred the nematodes. In general, nematodes should not be subjected to pressures exceeding 300 psi (2070 kPa).

The use of application equipment in which nematodes may be subjected to temperatures exceeding 30°C either in the tank, delivery hose or nozzles should be avoided. Also most irrigation, fertigation and chemigation systems do not empty when they are not in use. Therefore, the nozzles close to the pump source will begin releasing nematodes long before the end nozzle. These systems should be calibrated to determine how much liquid must enter the system before the nematodes reach the end nozzle. Lack of oxygen in the spray tank and delivery hose can also inactivate nematodes. This situation is aggravated when the application equipment is exposed to direct sunlight that increases temperature and oxygen demand. Oxygen-deprived nematodes are more prone to sunlight and desiccation damage as they are unable to escape due to reduced mobility.

13.8.2. Soil application

Spraying the soil surface is the most common method of nematode application. Both soil type and moisture influence survival and movement of nematodes. In general, nematode activity and survival is lower in clay than in sandy-loam soils (Kaya, 1990). Nematodes require a thin film of water for movement, but are not capable of movement under flooded conditions. The effect of soil moisture on nematode host-finding depends upon species with cruise foraging *H. bacteriophora* and *S. glaseri* being more sensitive to moisture extremes than the ambusher *S. carpocapsae* (P. Grewal and T. Webber, 1995, unpublished results). Maintenance of optimum soil moisture after nematode application enhances activity and efficacy (Shetlar *et al.*, 1988). Soil temperature can also affect nematode efficacy. Warmer temperatures reduce nematode survival while cooler temperatures reduce activity and infectivity (Grewal *et al.*, 1994a). Soil temperatures between 12 to 28°C are considered favourable for application of most nematode species (see Table 13.2). If soil temperature is above 28°C, a pre-application irrigation is usually recommended to reduce soil temperature before nematode application. Natural enemies may also reduce survival of inundatively applied nematodes (see Kaya, Chapter 9, this volume).

13.8.3. Foliar application

Droplet sizes and spray distribution, are important considerations when applying pesticides in the foliar environment. Conventional hydraulic nozzles are known to produce a wide range of droplet sizes, many being too small to carry an infective juvenile. Lello *et al.* (1996) reported that the higher output hydraulic nozzles (standard fan and full cone) deposited greater numbers of nematodes onto leaves and gave up to 98% mortality of *Plutella xylostella* on Chinese cabbage. In the case of spinning disc nozzles, over 90% of the drops never had nematodes (Mason *et al.*, 1998a). In general, an increase in flow rate resulted in greater numbers of nematodes deposited per cm^2.

The deposition of the nematodes on foliage is generally increased by the addition of adjuvants to the spray solution (Mason *et al.*, 1998b).

However, the major reason for the lack of success of foliar applications of nematodes is the intolerance of infective juveniles to extremes of desiccation (Lello *et al.*, 1996), temperature (Grewal *et al.*, 1994a), and ultraviolet radiation (Gaugler and Boush, 1978; Gaugler *et al.*, 1992a). Most nematode species do not infect hosts at temperatures exceeding 32°C (Table 13.2) and differ in desiccation and UV tolerance (Table 13.4). Nickle and Shapiro (1992, 1994) demonstrated effective protection of *S. carpocapsae* from sunlight using fluorescent brightener Tinopal and viral enhancer Blankophor BBH. Lello *et al.* (1996) state that the effects of sunlight can be minimized by applying the nematodes at dusk, but maintaining high humidity (> 80% relative humidity) and free water on leaf surfaces has been more difficult to achieve. Although adjuvants generally enhance nematode efficacy (MacVean *et al.*, 1982; Eidt, 1991; Glazer *et al.*, 1992; Broadbent and Olthof, 1995; Baur *et al.*, 1997), the level of increase is generally considered insufficient for recommending foliar applications (Baur *et al.*, 1997; Mason *et al.*, 1998b).

13.8.4. Compatibility with agrochemicals

Entomopathogenic nematodes are often applied to sites and ecosystems that routinely receive other inputs that may interact with nematodes including chemical pesticides, surfactants (e.g. wetting agents), fertilizers, and soil amendments. Often it is desirable to tank mix one or more inputs to save time and money. Infective juveniles are tolerant of short exposures (2–6 h) to most agrochemicals including herbicides, fungicides, acaricides, and insecticides (Rovesti and Deseo, 1990; Ishibashi, 1993), and therefore, can often be tank-mixed. However, some pesticides can reduce nematode infectivity and survival (Zimmerman and Cranshaw, 1990; Patel and Wright, 1996; Grewal *et al.*, 1998). Surfactants and other pesticide formulation ingredients may also be toxic to nematodes. For example, neem oil is not toxic at recommended concentrations, but is usually applied with detergents that can be highly toxic (Krishnayya and Grewal, 2000, unpublished results). Nematode species differ in their susceptibility to chemical pesticides and data on one species should be cautiously used for other species. Also different formulations of the same pesticide may differ in toxicity to the nematodes. Owing to the continuous introduction of new active ingredients and formulations in different market segments and differences in susceptibility of nematode species to pesticide formulations, it is difficult to provide up-to-date information. However, heterorhabditids tend to be more sensitive to physical challenges, including pesticides, than steinernematids.

Some pesticides act synergistically with entomopathogenic nematodes and therefore improve nematode efficacy in inundative applications. Imidacloprid (Koppenhoffer and Kaya, 1998), tefluthrin (Nishimatsu and Jackson, 1998) and pathogens such as *Paenibacillus popillae* (Thurston *et al.*, 1994) and *Bacillus thuringiensis* (Koppenhoffer and Kaya, 1997) act synergistically or additively with entomopathogenic nematodes. Nematodes are also compatible with most inorganic fertilizers when applied inundatively but natural populations are negatively affected (Bednarek and Gaugler, 1997). Composted manure and urea do not negatively influence *S. carpocapsae* but fresh manure reduces virulence (Shapiro *et al.*, 1997).

13.9. Conclusions and Future Prospects

Significant progress has been made during the past two decades in developing formulations that enhance nematode storage stability. Nematodes can now be stored for over 5 months under refrigeration in several products and for 12 months in one product. Developing products with room temperature shelf-life have proven more challenging. Products based on alginate gel formulations were the first to enable storage of *S. carpocapsae* for 3–4 months at room temperature. Further improvement was made with the development of the water dispersible granules in which *S. carpocapsae* can be stored for 5–6 months at 25°C. Shelf-life in most products has been enhanced by reducing nematode activity and metabolism through physical trapping, metabolic inhibition, cold storage, or by the induction of anhydrobiosis. Products based on polyether-polyurethane sponge as an inert carrier are most widely used in the cottage industry engaged in the mail order home lawn and garden market. Due to the overnight refrigerated shipping requirement the cost of sponge-based products is high (Gaugler *et al.*, 2000). Vermiculite and water dispersible granular products are most commonly used in retail markets such as mushrooms, greenhouses, turfgrass, cranberries and citrus.

Anhydrobiosis has been the key to improving nematode stability. However, anhydrobiosis is metabolically costly, and steinernematids and heterorhabditids are capable of only partial anhydrobiosis. Even desiccated nematodes in the formulations require high moisture for long-term survival, which encourages fungal contamination. Large differences in desiccation tolerance among species suggest that searching for new species and strains adapted to extremes of desiccation may be advantageous. Success in enhancing desiccation tolerance of tobacco through genetic transformation with a trehalose-6-phosphate synthase gene (Holmstrom *et al.*, 1996) opens the possibility of employing genetic engineering techniques to improve anhydrobiotic potential of nematodes.

Improved understanding of the nematode behaviour and physiology has led to the development of improved formulations. Research on nematode formulations indicates that the evolution of foraging behaviour of entomopathogenic nematodes may have led to the dichotomy in nematode desiccation survival strategies. Ambushers tend to use a desiccation tolerance strategy, whereas cruisers use a desiccation avoidance strategy. Ambusher species are less active during storage, have a lower basal metabolic rate, are more desiccation tolerant, and are thus more stable in the formulations. Cruisers are more active during storage, have a higher basal metabolic rate, are less desiccation tolerant, and are therefore less stable in the formulations. Only through cold preconditioning, that enhances trehalose accumulation, is the migration of cruising species reduced, enabling a limited induction of anhydrobiosis.

Although nematodes can be applied to the soil or plant canopy using conventional pesticide, fertigation and chemigation equipment, post-application survival is still a black box. Factors affecting post-application survival of nematodes in soil and on exposed foliage are poorly understood, and attempts to develop formulations enhancing post-application survival have been rare. Although, considerable effort has been expended in the identification of adjuvants that enhance nematode deposition, retention, and survival on foliage, improvements have been insufficient to recommend foliar applications. This area deserves more research as most foliar pests are

highly susceptible to nematodes, and the widely used organophosphate insecticides are being withdrawn from the market due to environmental and safety concerns.

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14 Production Technology

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14.1. Introduction

Entomopathogenic nematology rests on two evolutionarily convergent nematode families. The Steinernematidae and Heterorhabditidae are unified by their common use of symbiotic bacteria to exploit insects as hosts. These primitive families made an evolutionary leap to parasitism without severing their nutritional relationship with bacteria. The practical implications of this relationship are extraordinary. Whereas other entomophilic nematodes and insect parasitoids are fastidious to culture, the bacterial

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partner of entomopathogenic nematodes converts many proteins into a substrate in which the nematodes can develop and reproduce. The resulting capability to easily produce large numbers of nematodes is responsible for their progressing in less than 20 years from obscure insect parasites to widely recognized biological control agents.

No other facet of entomopathogenic nematology has been more shrouded in secrecy, and even misinformation, than that of production technology. Research data on the development and improvement of procedures for economical mass production has overwhelmingly been conducted by industry and, therefore, is largely inaccessible. Even when conducted by public institutions, production advances have tended to be proprietary. Patents are sometimes purposely imprecise in describing developments. Moreover, there has been an increased tendency to withhold advances as 'trade secrets' rather than submitting costly patent applications, a sharp departure from the 'patent-protected technology base' espoused by early nematode production companies (Friedman, 1990). This is understandable where, unlike formulation technology, patent infringement is inapparent in the end product. In short, there is a dearth of primary literature, which provides a formidable challenge in documenting and analysing contributions to mass production technology.

Friedman's (1990) production chapter in the previous volume (Gaugler and Kaya, 1990) provides a sound foundation for the present effort by introducing us to investment models, economies-of-scale, plant capacity, and technology development strategies. Here we examine the evolution of innovation in nematode production technology, explain specific methodology, and consider challenges while providing guidelines for prospective producers.

14.2. Strain Development

Two traits are highly sought in entomopathogenic nematode strains: high virulence against the target insect(s) and ease-of-culture. Both traits must be embodied within a single strain, since a high yielding strain is useless if it possesses inferior virulence and the converse is equally true. Also desirable are superior stability (i.e. shelf-life) and versatility (i.e. effectiveness against multiple insect pests).

Strain development consists of isolation and improvement. There are two approaches to strain isolation: large-scale sampling and location of appropriate environment (Bains, 1993). Large-scale sampling is essentially a random process widely practised among entomopathogenic nematologists using a simple insect baiting method (Bedding and Akhurst, 1975) and then screening the isolates. This 'shotgun' approach has generated thousands of isolates from dozens of countries and every inhabited continent (see Hominick, Chapter 6 this volume). Because entomopathogenic nematodes are ubiquitous, even a casual search yields interesting new strains. Unfortunately, there is no evidence that the new strains have desirable traits without laborious and tedious screening in the laboratory. Academic and government researchers usually conduct isolations, so these screens focus on questions of virulence and versatility. Promising strains are then further tested in field trials. Typically only species and strains that pass these hurdles come to the attention of industry, which assesses suitability for mass production and storage. Despite its unsystematic nature and tendency to select for lepidopterous-active nematodes – *Galleria* larvae are most commonly used as bait insects – this approach has yielded

commercial strains, including *Steinernema riobrave* (Cabanillas *et al.*, 1994) and *Heterorhabditis marelatus* (Liu and Berry, 1996).

The alternate approach is a directed search of environments in which the organisms will have had to develop the desired trait. The organisms that break down methane were originally isolated from the soil around a cracked gas main. In entomopathogenic nematology, the search centres on sampling populations of a target pest. This method was used effectively in isolating the slug-killing nematode *Phasmarhabditis hermaphrodita* (Wilson *et al.*, 1993). These workers held field-collected slugs under crowded laboratory conditions and then examined individuals showing signs of disease. Similarly, *Steinernema scapterisci* was isolated from parasitized mole crickets (Nguyen, 1988). Although this approach is best when performed systematically, most commercial isolates were found by chance; that is, they are not used against the original host. For example, the All strain of *S. carpocapsae* is applied against diverse insect pests but not the grape root borer, *Vitacea polistriformis*, from which it was isolated (All *et al.*, 1981); *H. megidis* is marketed for black vine weevil but was isolated from a scarab larva (Poinar *et al.*, 1987).

The second component of strain development is strain improvement, which means to modify the genetics of an organism so that it carries out a process more effectively (Bains, 1993). This approach is heavily emphasized for microbes used in industry, including biological pesticides such as *Bacillus thuringiensis*, but has received startlingly little attention in the entomopathogenic nematode industry.

The means for improving strains, notably selection, mutagenesis, and genetic engineering, are discussed by Burnell (see Chapter 12, this volume). The only genetic approach used to date in generating a commercial strain has been hybridization to increase genetic diversity followed by selection. Gaugler (unpublished data) generated a novel strain of *H. bacteriophora* by hybridizing diverse strains showing superior fecundity and virulence. The resulting strain, designated HbNJ, was then selected for several generations against scarab larvae. The strain performed well in field trials and in fermentation trials where yields in a 3000-litre bioreactor of $2.5–3.2 \times 10^5$ infective juveniles ml^{-1} in 9 days were achieved consistently (Fig. 14.1). The strain was commercialized by Ecogen in 1994 and Bio-Integrated Technologies in 1995. Licences

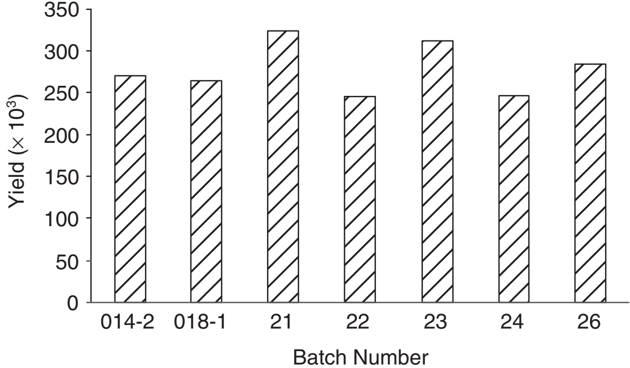

Fig. 14.1. Yields of *Heterorhabditis bacteriophora* (HbNJ strain) infective juveniles from seven 3000-litre bioreactor production batches (S. Franceschini, Italy, 2000, unpublished data).

were granted on a 'trade secret' basis rather than the more conventional, and expensive, patent route.

Strain impacts the final outcome more than any other feature of production. Thus, it is disconcerting to note the intense effort other biologically based industries devote to strain development, whereas nematode companies have all but ignored this activity. Friedman's (1990) otherwise excellent treatise on production technology, written from the perspective of the then dominant nematode production company, Biosys, does not even address strain development. Particularly troubling is that strains isolated a decade or more ago and repeatedly subcultured are on the market despite questionable virulence (e.g. HP88 strain of *H. bacteriophora*; Gaugler *et al.*, 2000).

Large-scale soil sampling will continue to be the largest contributor of genetic diversity for commercial purposes. Nevertheless, directed searches in the target host population and environment are most likely to yield useful strains, and this systematic approach establishes a host–parasite relationship, circumventing some of the screening phase of strain development. We endorse the modification of this method as used in isolating *P. hermaphrodita* from stressed field-collected hosts.

The plethora of new strains and the emergence of a nematode strain repository (CABI *Bioscience* UK Centre) provide an encouraging underpinning for future efforts in strain development. The next hurdle to exploiting this biodiversity is devising better methods for identifying promising strains, as the present trial-and-error approach of sifting through soil-isolated strains to match host to parasite is tedious, costly, and laborious. Lewis *et al.* (1997) demonstrated that host suitability can be predicted by parasite recognition behaviour. This study showed *S. carpocapsae* to be highly responsive to caterpillars, moderately responsive to white grubs, and unable to differentiate between millipedes and plastic. This corresponds with the suitability of these insects as hosts, thereby providing a measure of adaptation and a novel means of predicting nematode–insect matches.

Although the new strains that are continually being isolated offer the promise of superior traits, incremental improvements are likely to arise from wild-type strains. An exponential improvement, capable of jolting entomopathogenic nematodes from curiosity to a legitimate pest management tool will require genetic improvement.

14.3. Inoculum

Troubleshooting production problems begin with the inoculum. Monoxenic inoculum is neither necessary nor practical for *in vivo* production, a sharp contrast with rearing requirements for artificial media. Nevertheless, moderate contamination is a common cause of failed *in vivo* cultures. Sodium hypochlorite washes can reduce levels of undesirable microbes trapped under the infective juvenile sheath. Persistent contamination problems can be overcome by purifying the bacteria *in vitro* and re-introducing it into the nematode. Nematode growth and development in *in vivo* cultures depend upon the presence of symbiotic bacteria in the infective juvenile gut; yet some nematodes, notably *S. glaseri* (Akhurst, 1993), can have low bacterial retention that leads to unsatisfactory yields.

Although poorly documented, most workers have experienced 'hot' strains that through either founder effect, genetic drift, or inbreeding became ultimately laboratory adapted, with unfavourable consequences for yield, virulence, or both. Whether

the bacteria or the nematode is responsible is undetermined. Genetic deterioration is second only to contamination as a source of inoculum problems. Liquid nitrogen storage (– 196°C) preserves genetic integrity and maintains fitness and performance of nematode stocks (Popiel and Vasquez, 1991) for high volume manufacturers. However, low volume producers using *in vivo* technology tend to subculture stocks repetitively in *Galleria*. These producers should establish strain maintenance programmes to periodically evaluate seed culture fitness and consider periodically infusing new genetic material. Simply recording yields can be helpful, as declining yield is often an early sign of stock deterioration. Subtle changes in cadaver colour can also provide useful clues regarding fitness (L.S. Portales, Cuba, 2001, personal communication).

High volume manufacturers using artificial media are not immune from inoculum problems. The symbiotic bacteria show colonial, cellular, and functional polymorphism (see Boemare, Chapter 2, this volume). Control of phase variation is obligatory because only the phase I variant produces the crystalline inclusion proteins that support nematode reproduction (Bintrim and Ensign, 1998), whereas phase II lack antibiotic activity and can inhibit nematode growth. Storage of pure phase I bacteria at ultra-low temperature (–80°C) maintains bacterial phase integrity. The existence of lysogenic phages in *Xenorhabdus* and *Photorhabdus* (Boemare *et al.*, 1992, 1993) indicates that care should be taken to avoid these in stock cultures.

14.4. *In Vivo* Culture

Nematode *in vivo* production is a cottage industry of low-volume producers. With limited capital or expertise for *in vitro* culture (Table 14.1) the default strategy is to rear nematodes in insect hosts. *In vivo* mass production hinges on the availability of a highly reliable, exceptionally susceptible, and relatively inexpensive supply of hosts. Although *Tenebrio* are substantially cheaper (Blinova and Ivanova, 1987), with rare

Table 14.1. Comparison of nematode mass production technologies.

	In vivo	*In vitro* solid phase one[a]	*In vitro* solid phase two[b]	*In vitro* liquid
Capital costs	xxxxx[c]	xxx	x	x
Labour costs	x	xxx	xxxx	xxxxx
Cost-of-goods	x	xxx	xxxx	xxxx
Ease of scale-up	x	xx	xxxx	xxxxx
Technical expertise required	xxxxx	xxx	x	x
R&D requirements	xxxx	xx	xx	x
Waste disposal	xxxxx	x	xx	xxxx
Availability of toll or contract manufacturers	x	x	xx	xxxxx
Space requirements	xx	x	xx	xxxxx
Ease of harvest	xxxx	x	xx	xxxx
Reliability of raw materials	x	xxxxx	xxxxx	xxxxx

[a] Production system using Tasmania model, 1993–1995; see section 14.5.2.
[b] Production system using Ecogen-Sylvan model, 1995–1996; see section 14.5.3.
[c] Indicates assessment from poor (x) to excellent (xxxxx).

exceptions (e.g. crickets by B&R Supply in the mid-1980s) *Galleria mellonella* larvae are the host of choice. *Galleria* are reared by more companies than any other insect. No insect has been found to be more susceptible to a wider range of nematode species. When bulk purchased (> 1×10^5 larvae), they can cost as little as US$0.01 per larva plus shipping. Yields per larva range from 1 to 3.5×10^5 infective juveniles (Dutky *et al.*, 1964; Milstead and Poinar, 1978), so that up to 25,000 *Galleria* are needed to treat one hectare at the standard 2.5×10^9 nematodes ha^{-1} rate. Nematode producers have sometimes proven vulnerable to unexpected host variation due to virus infection, antibiotic use, or adverse shipping conditions (e.g. extremes of temperature).

14.4.1. Laboratory

White (1927) laid the foundation for *in vivo* culture in a classic paper on collecting vertebrate parasitic nematodes from dung. The concept was adapted by Dutky *et al.* (1964) to harvest infective juveniles emerging from hosts. The method exploits the tendency of infective nematodes to migrate from expended host cadavers into a water reservoir. Every entomopathogenic nematode laboratory uses the White trap albeit with some modifications (e.g. Lindegren *et al.*, 1993). This simple harvest method is well suited to the purpose it was designed for: production of inoculum for experiments. But it is labour intensive – the mark of a cottage industry is the substitution of 'sweat equity' for capital (Friedman, 1990).

14.4.2. Scale-up

Few processes can be run efficiently on a laboratory bench; scale-up is necessary. Scale-up is the process of taking production from the bench and up to a scale at which it is commercially useful (Bains, 1993). *In vivo* systems are difficult to scale-up. Temperature and humidity are the only wholly controllable process parameters. Spraying insect hosts with infective juveniles can accelerate inoculation or the insects can be dipped into a nematode suspension. But harvesting emerging infective juveniles is an intractable bottleneck. Scale-up here has largely consisted of simply providing larger White traps, which reduces extraction efficiency by increasing the distance of migration to the reservoir.

Carne and Reed (1964) described a harvest apparatus in which cadavers are supported on a disk resting in the mouth of a large funnel. Water level in the funnel is maintained at cadaver height by a constant-level device. Emerging infective juveniles migrate through the disk and settle to the funnel bottom where they are collected by opening the stopcock. The central innovation of the system is the perforated disks through which nematodes pass to the water reservoir, reducing the need for significant lateral migration. There are no reports that the design was tested.

Gaugler and Brown (2001) have proposed an *in vivo* production system that does not rely on migration or a reservoir. The system uses shallow perforated holding trays for inoculation, incubation, and harvest. Each tray holding several hundred hosts is dipped into a nematode suspension and then stacked in a high humidity environment. When nematode development is complete the tray moves to a harvester, which conceptually resembles the mist chambers used to extract nematodes from soil (Southey, 1986). Each tray is suspended under two rigid plastic pipes equipped with

atomizer nozzles. A timer connected to a water supply provides periodic misting cycles (3 min at 6 h intervals) for the tray; air movement is also important to moderate saturation of the cadaver and subsequent anoxia. Exposure to free water induces infective juveniles to emerge; the nematodes are rinsed with the runoff water into an angled drip tray. Each drip tray serves ten or more holding trays arrayed overhead, and directs nematodes by gravity flow into a collecting pipe, and then to a central storage tank. Harvest is complete in 48 h with a 97% cadaver extraction efficiency. Cadaver remains are discarded and the holding tray replaced for *in situ* cleaning with detergents and disinfectants delivered via the misting nozzles. Because the system is largely automated it is projected to reduce labour costs.

Regardless of the means of harvest, collected infective stages nematodes must be concentrated and separated from culture residue, bacteria, and undesired life stages before formulation. Insufficient separation inevitably results in inferior product shelf-life due to microbial activity. Nematode concentration and separation have been accomplished by centrifugation, gravity settling, or filtration. Centrifuges are too costly for companies practising *in vivo* methods. These small producers tend to let suspensions settle and then decant the supernatant as waste. Other than its inherent inefficiency, this crude method's primary deficiency is that nematodes are held in a stressful low-oxygen environment, which accelerates lipid depletion, while gravity acts. The third approach, standard filtration, is cheap but the filter soon fouls and filtration ceases. Cross-filtration, in which the linear flow of liquid across a filter membrane moves particles from the surface and keeps the membrane clean, could help this and has been suggested by Gaugler and Brown (2001). They also advocate a simple and inexpensive continuous-deflection separation method for capturing nematodes from a liquid stream, a method used successfully to remove particulates from stormwater (Schwartz and Wells, 1999). This approach tested on a lab bench-scale removes 98% of wastewater in two passes with $< 5\%$ loss of nematodes. In an encouraging study, the first of its kind, Young *et al.* (1998) describe the physical properties of nematode culture components, with the long-term goal of providing a theoretical underpinning for the design of new separation procedures. The point is that new, scale appropriate, separation technology is urgently needed for producers who are currently dependent upon gravity settling.

14.5. *In Vitro* Solid Culture

Mass production on artificial media was realized 30 years before Dutky *et al.* (1964) established effective *in vivo* methods. Solid culture was pioneered by Rudolf Glaser, who was the first to artificially culture a parasitic nematode. Glaser and his co-workers, in one of the most ambitious and least known experiments in biological control, produced and released billions of *S. glaseri* throughout New Jersey against Japanese beetles from 1939 to 1942 (Fleming, 1968). Regrettably, early workers were unaware of the nematode's bacterial partner. Nematode mass production was carried out in shallow trays of veal-pulp medium with salicylic acid and formaldehyde to repress contaminating microbes (McCoy and Girth, 1938), including apparently the natural symbiont, *Xenorhabdus poinarii* (Gaugler *et al.*, 1992). Today, the need for monoxenicity is universally recognized as one of the cornerstones of nematode *in vitro* culture (Poinar and Thomas, 1966).

14.5.1. Laboratory

Others would extend Glaser's accomplishment by developing alternative media to costly animal tissue homogenates, such as the dog food medium of House *et al.* (1965). Regardless of the growth medium, cultures were produced on the substrate surface because of the need for adequate gas exchange. That is, cultures were two-dimensional, perfectly suited for laboratory cultures but a limitation that precluded commercial scale production.

Bedding's (1981, 1984) development of practical solid culture technology was a seminal step in nematode production because it leapt from two- to three-dimensional substrates. Bedding flask cultures involved thinly coating crumbed polyurethane foam sponge with poultry offal homogenate. The porous foam afforded an outstanding surface area-to-volume ratio for growth while providing adequate gas exchange. A primer for preparing Bedding flasks is found in Woodring and Kaya (1988).

The next advance was the adoption of large autoclavable plastic bags to replace flasks as rearing vessels (Bedding, 1984). Medium and shredded foam were hand-blended and sealed into bags before sterilizing by autoclave. The bags were injected with bacterial inoculum, mixed manually, and placed on racks in an incubation room. After 24 h of bacterial growth, the bags were injected with nematode inoculum (e.g. *S. scapterisci* was introduced at 2000 infective juveniles per gram of medium) and mixed again. Holding racks were equipped with a small air compressor and gang valve leading to a network of hoses delivering air to each bag over the 2-week incubation period.

A conventional medium was also developed because poultry entrails cannot be standardized and so provide unreliable results. Spurred by Wouts (1981) development of a practical yeast extract, corn oil, and soy flour medium for the Bedding flask, a new medium was developed based on yeast extract, corn oil, corn starch and dried egg solids.

14.5.2. Scale-up phase one: Biotech Australia

Scaling up Bedding's advances to commercial production was undertaken in collaboration with Biotech Australia, which licensed the technology from CSIRO in 1985. They based their production plant of 570 m^2 in Tasmania, which took advantage of the skilled technical pool left by CSIRO when the Bedding laboratory was transferred to Canberra.

The use of bags and an improved medium permitted commercial-scale nematode production, but shortcomings were encountered that reduced effectiveness. Most troublesome was that each bag required a laborious custom fitting of costly inlet and outlet microbial filters. The compressors increased the air conditioning load in the incubation room, which became problematic for *S. scapterisci* as this species produced metabolic heat within the bag to the point that growth could be retarded. Condensation (metabolic water) sometimes saturated bag edges and was associated with poor growth. These limitations contributed to inconsistent yield.

Bedding *et al.* (1996) addressed the gas exchange issue in developing a stainless steel box as a simpler alternative. The key innovation was a foam sponge gasket lining the inside edge of the lid that provided passive ventilation. Nevertheless, Biotech Australia judged there was insufficient improvement to justify the expense of constructing the boxes, and the new system was not implemented.

Nematode extraction from the foam medium was accomplished using active migration and sedimentation as in a Baermann funnel apparatus. Harvest trays were constructed, several square metres in size, with a bottom support screen of aluminum. Water level in the trays was adjusted to the same height as the screen and a cloth fabric placed over the screen. The bags were emptied on to the cloth, and nematodes migrated through the cloth and into the water reservoir. Trays were hinged so they could be decanted after migration to remove bacteria and medium residue. The collected nematodes were then pumped to a chilled holding tank with a bacteriostat to await formulation. This manufacturing process worked moderately well for highly mobile species such as *H. bacteriophora*. By contrast, migration rates for sedentary nematodes such as *S. scapterisci* ranged from 50% to 75%, often with excessive numbers of non-infective stages that stimulated microbial activity and reduced shelf-life.

Yield depended upon nematode species and strain, with *H. bacteriophora* strain HbNJ averaging 1.4×10^9 bag^{-1} and ranging up to 2.9×10^9 bag^{-1}. By contrast, the *S. scapterisci* strain Uruguay yielded an average of 4.8×10^8 bag^{-1}, a result possibly linked to imperfect monoxenicity (Bonifassi *et al.*, 1999).

The US$100 million market Biotech Australia had projected for nematode products did not materialize and management became disillusioned with their nematode business. The Tasmanian production facility was sold in 1993 to Ecogen, a USA-based biotechnology company intent on expanding its bioinsecticide portfolio beyond *B. thuringiensis*. Production at Ecogen-Australia soon switched exclusively to *S. scapterisci* to meet the demands of the Florida mole cricket market.

14.5.3. Scale-up phase two: Ecogen–Sylvan

The next player in the evolution of nematode solid culture arrived in 1994, when Ecogen proposed coupling their nematode expertise with Sylvan Food's production expertise. Sylvan was already a leading company in using solid culture to produce mushroom spawn (equivalent to seed). The freewheeling, venture capital, long-term focused Ecogen and the risk averse, profit-driven, short-term focused Sylvan seemed an odd alliance, but self-interest prevailed. Sylvan merely wished to extend its technology beyond the food industry. Ecogen's interest was based on their markets being in the United States rather than Tasmania. USA-based production would shrink refrigerated shipping costs while ending the intermittent customs and other delays that sometimes resulted in product viability disasters. Moreover, the Sylvan approach to solid culture promised something that had previously remained elusive: true scale-up, and therefore the promise of ending chronic production shortfalls.

The heart of the Sylvan rearing system was a steam-sterilized double-cone blender capable of holding 200 kg of medium and shredded foam. The blending action assured that each foam particulate was coated (i.e. as opposed to saturated) with growth medium. Kelgum was added to the medium as a sticker to improve loading onto the foam particles. The blender was then steam sterilized, with the blending action allowing the steam to easily reach every foam pore. Thus, the Sylvan process overcame the single greatest production bottleneck in Tasmania – the capacity to sterilize a limited number of bags per autoclave cycle.

When the medium had cooled, the blender was simultaneously inoculated with 1.3×10^8 nematodes and 6 litres of bacterial suspension, and blended again to

distribute the inoculum uniformly. This was an advantage over the previous system, which tended to distribute inoculum ineffectually, contributing to asynchronicity of nematode development and hence to high levels of non-infective stages at harvest. Co-inoculation required more bacterial inoculum, but was a marked improvement over the labour-intensive two-step inoculation procedure previously followed.

The conical-bottom blender–sterilizer could now be emptied by gravity-flow through the floor and into a clean room. Clean rooms, which are essentially containment laboratories, remain clean by filtering incoming air to remove microbes. Walls, floors, and ceilings are coated with materials designed not to hold dust, and workers must wear sterile hats, lab coats, and overshoes. The clean room is essential to reduce contamination during media transfer from the blender into sterile bags.

The blender filled 70 polypropylene rearing bags, each with 2.3 kg of medium-impregnated foam. The bags (Unicorn Imp. and Mfg. Corp., Commerce, Texas, USA) included a ventilation strip of Tyvac®, a tough gas-permeable but water-impermeable woven fabric that permits passive aeration without compromising sterility – an enormous improvement over the cumbersome air pump system or Bedding's steel boxes. It was critical that the medium should not make sustained contact with the fabric, as the resulting transfer of oils blocked gas transfer. The bags were heat sealed and transferred to open racks in an incubation room (25°C, 70% RH) with good air circulation.

Bags were harvested after 2 weeks with centrifugal sifters. The sifters use rotating helical paddles within a cylindrical screening chamber to continuously propel incoming particles through apertures in the screen. The paddles do not contact the cylindrical nylon mesh screen. Water and oversize particles passing through the screen accumulate upstream into a holding tank. The shifter did not rely on nematode migration, so it was unfettered by infective juvenile behaviour and was therefore useful for all nematode species. However, the process tended to strip the sheath from infective juveniles.

A series of three centrifugal sifters, each costing US$1800, were linked together and fitted with screens of decreasing pore size. The first sifter was fitted with a large mesh screen (840 μm) and separated the nematodes from the foam, the second (120 μm) removed adult stages, and the third (fitted with a 37 μm screen) removed bacteria while concentrating nematodes into slurry for chilled tank storage pending formulation. The shredded foam could be recycled one or more times before losing integrity, reducing the disposal burden in landfills.

Despite using smaller bags, per bag yields of *S. scapterisci* at the Sylvan facility were significantly higher than at Ecogen-Australia. Developmental synchrony improved substantially so that fewer wasteful non-infective stages resulted. Where Ecogen-Australia processed 300 bags per week, Sylvan handled 700 per day. The monthly nematode production capability of the two plants was then 6×10^{11} versus 1×10^{13} infective juveniles respectively. Finally, Sylvan possessed much larger blenders and therefore had the capacity to respond to growing markets. The Tasmanian facility was swiftly closed.

Better technology is not the solution to everything. The extraordinary improvements to nematode solid culture made by the Ecogen–Sylvan collaboration ultimately proved futile, as the market share captured was insufficient to make the joint initiative viable (i.e., annual sales of 6×10^{12} nematodes annually). The companies soon returned to their core businesses of microbial pesticides and mushrooms respectively.

14.6. *In Vitro* Liquid Culture

Liquid culture and the Ecogen–Sylvan approach to solid culture share the same high capital and expertise needs, but the availability of unutilized fermenter capacity and toll or contract manufacturers lends liquid culture a decided advantage (Table 14.1). Further advantages include less space requirements, simpler harvest, less disposable waste, and compatibility with the production of other biopesticides (e.g. *B. thuringiensis*) using the same equipment. More than 95% of all nematodes are produced in liquid culture systems at companies that include Certis USA (formerly Thermo Trilogy, formerly Biosys), MicroBio, E-Nema, and SDS Biotech.

14.6.1. Medium development

Gaugler and Georgis (1991) noted that early production batches of liquid culture-produced *H. bacteriophora* had visibly low lipid reserves and showed reduced field efficacy. To assure quality, especially of *Heterorhabditis* spp., medium nutritional components must be carefully selected to enhance reserves (Fodor *et al.*, 1994; Hatab and Gaugler, 1997, 1999). Cost, availability and ease of separation are also considerations in selecting liquid media components.

Glaser (1940) developed the first liquid medium, based on kidney extract, for axenic culture of *S. glaseri*. When the nutrient function of the symbiotic bacteria was demonstrated, practical media for monoxenic culture soon followed. Friedman *et al.* (1989) proposed a medium based on soy flour, yeast extract, corn oil, and egg yolk, which provided *S. carpocapsae* yields of 1.1×10^5 infective juveniles ml^{-1}. Buecher and Popiel (1989) described a medium containing tryptic soy, yeast extract and cholesterol. Surrey and Davies (1996) reported egg, yeast extract and corn oil yielded 1×10^5 *H. bacteriophora* ml^{-1} in 15–20 days. Tachibana *et al.* (1997) patented a medium for *S. kushidai* with soluble starch, glucose, peptone, yeast extract and corn oil. They further claimed that addition of cholesterol increased the virulence of *S. kushidai*. Lipids have received more attention than other nutritional components because 60% of the total energy for the non-feeding infective juveniles is derived from metabolizing lipids (Hatab and Gaugler, 1997). Hatab and Gaugler (2001) concluded that lipid sources with high proportions of saturated fatty acids result in suboptimal yield. Yoo *et al.* (2000), describing a medium for *H. bacteriophora* containing 8% olive and canola oil (50:50), emphasized the importance of lipids rich in mono-unsaturated fatty acids. Ions supplement nutrition and are indispensable for optimal growth; they can be supplied in the form of NaCl (0.4% w/v), $MgSO_4$ (0.05%), $CaCl_2$ (0.03%), and KCl (0.03%) (Yoo *et al.*, 2000).

Liquid culture medium to produce 1×10^{12} *S. carpocapsae* costs \$0.80, which represents only 6.4% of the US\$12.38 total production cost (calculated from data in Georgis, Chapter 17 this volume). This figure is in close agreement with *H. bacteriophora* medium cost, which is estimated at 8% (Fig. 14.2). Yet medium components are among the most closely guarded of industry secrets. Nematode media components in commercial use today, nevertheless, are unlikely to differ fundamentally from those widely used in industrial fermentation. It would be difficult to envisage nematode media without yeast extract as a major nitrogen source, vegetable oil to supply lipids, grain flour (e.g. soy) for carbohydrates, and salts for ions.

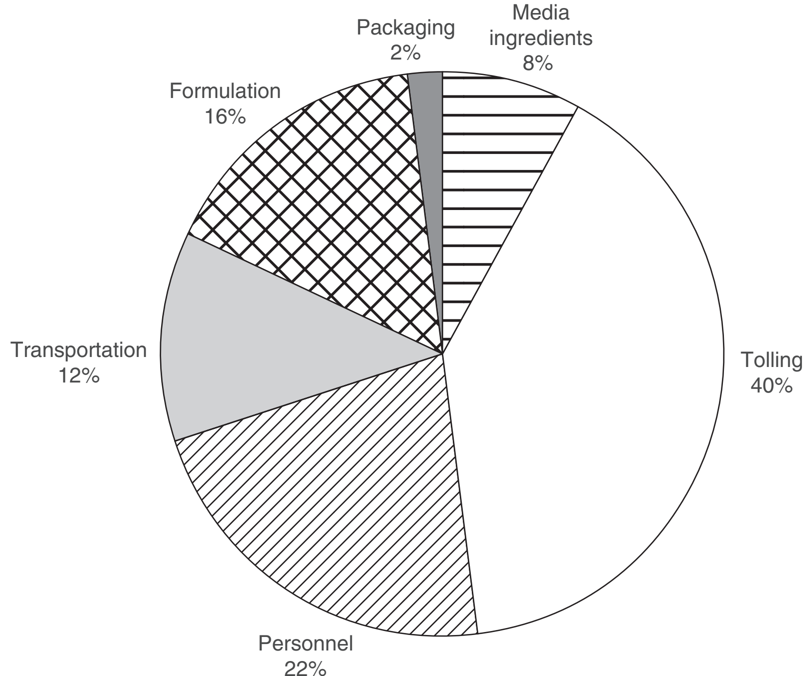

Fig. 14.2. Production cost breakdown for *Heterorhabditis bacteriophora* (HbNJ strain) for mass culture at a toll or contract manufacturer using a 3000-litre bioreactor (S. Franceschini, Italy, 2000, unpublished data).

14.6.2. Recovery

Recovery occurs when the developmentally arrested infective juveniles resume growth and feeding, a process triggered by a 'food signal' (Strauch and Ehlers, 1998). The food signal is produced by *Xenorhabdus* and *Photorhabdus* bacteria and excreted into the medium. Maximum production occurs during the late exponential phase of the bacteria. Recovery of *H. bacteriophora* is observed in the presence of cells or crystalline inclusion proteins (Bintrim and Ensign, 1998). Signal produced by *Xenorhabdus* spp. does not trigger *Heterorhabditis* recovery (Han and Ehlers, 1998), however, *S. carpocapsae* infective juveniles respond to signals from *P. luminescens* cultures (Han, unpublished data).

A food signal is produced in artificial media; however, far fewer heterorhabditid infective juveniles recover here than *in vivo* (18–90% vs. 100% respectively; Ehlers *et al.*, 1998), an outcome with harsh implications for commercial-scale production. Ehlers *et al.* (1998) consider unpredictable, unsynchronized, and low recovery of inoculated *H. megidis* infectives to be responsible for erratic results in liquid culture of heterorhabditid nematodes. Poor recovery often results in low nematode and high bacterial populations, with more first-stage juveniles therefore developing into amphimictic rather than hermaphroditic adults. Amphimictic heterorhabditids are non-productive in liquid culture, so fermentation becomes a two-generation process with extended culture time, asynchronous population growth, and reduced yield. A one-generation process can presently be achieved only by increasing inoculum density

(Ehlers *et al.*, 1998). By comparison, recovery has not been well studied in steinernematid species and conclusions concerning its impact on liquid culture cannot be drawn.

Symbiotic bacteria are not a prerequisite for recovery, because axenic infective juveniles can recover in axenic *Galleria* (Han and Ehlers, 2000). Yet food signals are not detected in common bacterial or nematode media (Strauch and Ehlers, 1998), cell culture media (e.g. Grace's), or fresh egg (Han, unpublished data).

For reasons as yet unknown, recovery is greater in solid than liquid culture (Han, unpublished data; Wang and Gaugler, unpublished data). Differences in the concentration of the food signal excreted in these culture systems may be involved. Alternatively, because the food signal is volatile (Strauch and Ehlers, 1998), evaporation should be more efficient in solid media.

Although the food signal is known to be heat-resistant, soluble in ethanol, and unstable at pH extremes (Strauch and Ehlers, 1998), the molecular structures of the chemicals have not been described. The significance of being able to synthesize food signal for nematode production can be imagined if the simple addition of these signals into media increased recovery and yield. This and other hypotheses concerning recovery remain untested for now.

14.6.3. Scale-up and bioreactor design

The difficulty with scaling up liquid systems is that billions of nematodes in a bioreactor seldom behave the same way as thousands of the same nematode in a flask. Although bioreactor (fermenter) design is an engineering issue, it is nevertheless useful to consider the dual factors influencing design needs for entomopathogenic nematodes: oxygen and shear.

Oxygen transfer is not a limiting factor for cultures in shaker flasks, whereas in commercial bioreactors this is the central problem for the bacterial symbiont; nematodes by contrast have a comparatively low oxygen demand (Burman and Pye, 1980; Gbewonyo *et al.*, 1997). Oxygen is poorly soluble in water resulting in the need for stirring impellers, gas-injecting spargers, and internal loops to increase transfer. The result is agitation, which resolves the bacterial oxygen requirement but conversely triggers shear force detrimental to the nematodes. An early approach to this problem at Biosys was to use a bubble column bioreactor for commercial production. These tall reactors use only air injected at the base for mixing. The rising bubbles drive the liquid around the reactor gently, generating negligible shear stress for nematodes. This bioreactor type proved satisfactory until product demand increased and the need for a more scalable, widely available, conventional stirred tank reactor became evident.

To resolve the conflict between high oxygen demand for the bacteria and low shear stress for the nematode within a conventional bioreactor, Pace *et al.* (1986) used low shear paddle impellers to churn the medium and a downward pointing air sparger. Friedman *et al.* (1989) discovered that shear sensitivity of *S. carpocapsae* varied with nematode stage, with the amphimictic adults and their mating behaviour most severely impacted. This was resolved by developing a 'variable agitation regime' (Friedman, 1990) which presumably was based on intense agitation to meet oxygen demands during the bacterial log phase but reduced agitation during lag phase, especially when adult nematodes were present. Because females and males of second-generation

Heterorhabditis spp. are unable to mate in liquid media (Strauch *et al.*, 1994), the influence of shear force here is less important.

A third bioreactor type for nematode mass production was introduced by Ehlers *et al.* (1998). The internal loop fermenter is a stirred tank bioreactor with an internal draft tube or central cylinder attached to the lid, which improves circulation and oxygen transfer while equalizing shear forces. A marine propeller and air sparger produce a downward flow in the inner cylinder and upward flow between the cylinder outer wall and the bioreactor wall. This design has been used for commercial production in a 500-litre reactor at E-Nema.

Nerves *et al.* (2001) describe an unconventional external-loop airlift bioreactor for producing *S. carpocapsae* and considered it 'more efficient' than previous designs. Although yield was a meager 6×10^4 per ml, the authors report a ratio of inoculum density to final yield of 120. This result was attributed to increased probability of male–female contact in the bioreactor deceleration zone of the loop where circulation was reduced. Interestingly, this novel bioreactor was sterilized *in situ* with Betadine solution.

14.6.4. Additional process parameters

The following summarizes important parameters in addition to oxygen and shear force to consider in developing liquid fermentation processes for entomopathogenic nematodes.

Foam – Another negative consequence of intense agitation to improve oxygen transfer is foaming, which can lead to blocked air exit filters and pressure build-up. Foam control is an essential element of the operation of a sparged bioreactor. Ucon defoamer was added at 1 ml per litre when *Caenorhabditis elegans* was produced in a 150-litre stirred tank bioreactor (Gbewonyo *et al.*, 1994). Potential toxicity to the bacteria or nematodes and disturbance of oxygen transfer must be considered when selecting antifoam agents. For example, silicon agents are widely used in industry for foam control but can reduce yields of heterorhabditids. Newer, nutrient-based agents provide non-toxic foam control and can impact growth positively.

Carbon dioxide – If aeration rate is low to reduce foaming, carbon dioxide can build up and become detrimental to nematode cultures (Strauch and Ehlers, 2000). However, effects are variable, as high concentration can also stimulate recovery (Jessen *et al.*, 2000). Determination of optimum carbon dioxide concentration over culture time remains undefined but worthy of study.

Temperature – Metabolizing organisms in large volume fermentations generate considerable heat that must be controlled. Moreover, temperature must be precisely adjusted for both the bacteria and nematodes. Because optimal growth temperatures of the symbiont and its nematode host do not coincide, it is advisable to coordinate bioreactor temperature with the exponential growth phase of the respective partner. Biphasic temperature adjustment can also improve culture synchrony. This may be related to recovery, as Ehlers *et al.* (2000) noted that exit from the infective stage is influenced by culture temperature.

Osmolarity – Low osmolarity can induce phase variation of the symbiotic bacteria (Krasomil-Osterfeld, 1995). This suggests the possibility of phase shift with corresponding adverse effects on nematode yield when culture osmolarity is not well regulated. This parameter tends to be poorly monitored.

Viscosity – Production of heterorhabditids, even with *in vivo* methods, results in increased medium viscosity. This is attributed to a 'polysaccharide capsule' synthesized by the symbiotic bacterium (Young *et al.*, 1998). In liquid cultures, high viscosity reduces oxygen transfer. This is one of several largely unstudied challenges contributing to heterorhabditids being generally more difficult to produce reliably than most steinernematid nematodes.

14.6.5. Culture time

Fermentation culture time is among the most important cost factors in stirred tank production systems. Unlike microbial insecticides such as *B. thuringiensis* that can be cultured in 24 h, nematode production may tie up expensive bioreactors for up to 17 days for *H. indica* (Ehlers *et al.*, 2000), 16 days for *H. megidis* (Strauch and Ehlers, 2000), and 8 days for *H. bacteriophora* (Yoo *et al.*, 2000).

Smaller species seem best because yields are greater (Fig. 14.3). Thus, the 528 μm (infective juvenile total length) *H. indica* yields on average 4.6×10^5 (Ehlers *et al.*, 2000), whereas *H. bacteriophora* at 588 μm yields 3.2×10^5 (Yoo *et al.*, 2000), *H. megidis* at 824 μm yields 7.2×10^4 (Strauch and Ehlers, 2000), and *S. glaseri* at 1030 μm yields 5.8×10^4 (Gaugler, unpublished data) infective juveniles ml^{-1}. While such yield comparisons are tenuous due to differences in culture parameters, the available data suggest that in some cases high yield can be offset by the capital depreciation costs associated with a lengthy culture cycle.

The density of inoculum contributes to final yield, culture time and production cost. Working in a liquid medium, Han (1996) found optimum production of *H. bacteriophora* required a higher inoculum density than did *S. carpocapsae*. When heterorhabditid inoculum density is low the medium is replete with nutrients, leading to a high proportion of amphimictic adults unable to copulate and therefore a second generation ending with a correspondingly high culture time (Johnigk and Ehlers, 1999).

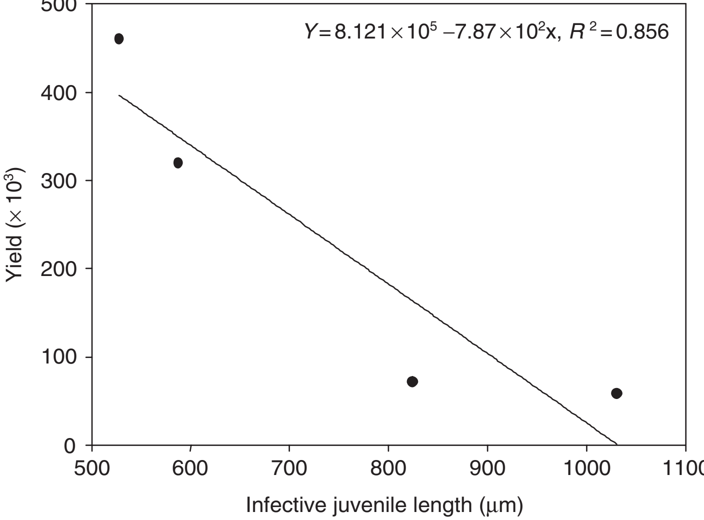

Fig. 14.3. Relationship between infective juvenile size and production yield.

14.6.6. Downstream processing

Compared with *in vivo* and *in vitro* solid culture, submerged liquid culture systems require more sophisticated downstream processing methods capable of handling large volumes; that is, centrifugation. As in the case of bioreactors, separation is best accomplished in a low stress environment. Davey *et al.* (1993) found that stress from handling associated with weighing disrupted osmoregulatory capacity in the fish-parasitic nematode *Pseudoterranova decipiens*.

Gbewonyo *et al.* (1994, 1997) separated dauer juveniles of *Caenorhabditis elegans* from spent liquid media with continuous phase density centrifugation, although this method is expensive and can cause osmotic shock. Surrey and Davies (1996) reported successful pilot-scale separation of *H. bacteriophora* using basket and scroll decanter centrifuges. Both designs provide effective separation of solids that settle rapidly; however, extraction with the basket centrifuge was labour intensive whereas decanter centrifuge separation resulted in some loss of nematode viability from shear force. The largest nematode manufacturer during the 1990s, Biosys, controlled shear force in using a horizontal scroll decanter to harvest several nematode species.

Regardless of the separation technique employed, the similar density of non-infective and infective stages makes it desirable to achieve high developmental synchrony. Synchrony may be easier to achieve with some species than others as shown by Young *et al.* (1998), who reported infective stage proportions of 98, 91.5 and 59% in shaker-flask cultures of *S. feltiae, H. megidis* and the mollusc-parasitic nematode *Phasmarhabditis hermaphrodita*.

14.7. Analysis and Future Prospects

Restrictions or outright bans of many organophosphate and carbamate soil insecticides are widely projected. As the quintessential bioinsecticide for soil insect pests, entomopathogenic nematodes should be poised to fill gaps in management tactics for minor crops where new 'softer' chemistries are less dominant. The key will be further innovations in production technology that improve cost and availability.

The *in vivo* nematode industry is not poised to contribute meaningfully to this prospective growth, as the technology is rooted in a method developed nearly 80 years ago. *In vivo* culture was not even considered by Friedman (1990) because the prevailing conventional wisdom was that this technology would die out as *in vitro* technologies came online. Yet there are more cottage industry producers than ever before, few of whom have the resources or expertise for research and development. No Glaser or Bedding has yet emerged and the resulting lack of innovation has resulted in high cost and limited capacity becoming the hallmarks of *in vivo* production. Despite the reluctance of both industry and academia to invest in *in vivo* methodology, this approach seems unlikely to disappear and, moreover, with assistance could evolve a significant role for local strains in local markets, grower cooperatives or developing regions.

Every facet of *in vivo* technology needs updating to make nematode rearing more cost-efficient. Priority needs include demonstration of scalable labour-saving methods, alternatives to gravity settling for concentration and separation, and an expanded list of inexpensive hosts. Further, mechanisms must be identified for low-volume producers to share in public-funded advances in strain development.

Ecogen–Sylvan represents the zenith of nematode solid culture innovation, offering economies-of-scale approaching that of liquid culture. It is difficult to envision the next phase of solid scale-up; but the uncommon and expensive custom equipment required suggests that solid culture may return to it roots as a capital and expertise middle ground between *in vivo* and liquid cultures. This third phase of innovation must retain the passively aerated bags, single-step inoculation, and new harvest methods previously developed, while finding less capital-intensive means of sterilization, inoculation and bag filling. Finally, fermentation parameters as they pertain specifically to solid culture require elucidation to place this production technology on the same scientific footing as liquid culture.

Only one USA company, BioLogic, currently employs solid culture technology for nematode production (Gaugler *et al.*, 2000). BioLogic is a cottage industry company, family owned and operated with limited capital, distribution, and sales, but also possesses a high degree of technical expertise. China, with Bedding (1990) acting as a technology transfer catalyst, employs solid culture for similar reasons.

In 1993, Ecogen projected US$120 M in nematode sales by 1997. This would have represented an astonishing 800+ % increase since worldwide sales by all producers at the time were less than US$15 M (R. Georgis, USA, 2001, personal communication). Ecogen's projection proved illusionary and it was central to the demise of the production facility in Tasmania. In retrospect the Tasmanian technology, albeit with needed improvements in bags, may have been the best fit for accessible markets. Ecogen's disappointment bolsters Georgis' argument (see Chapter 17 this volume) that the conventional chemical pesticide developmental model pursued by Biosys and other venture-capital funded startups developing insecticidal nematodes may have been scale-inappropriate. Available capital and expertise have been the twin constraints dictating which production technology to be implemented. To this might be coupled market size and volatility.

Investment from industry and academia in nematode liquid culture technology has culminated in commercial-scale (*c.* 10,000-litre bioreactors) production costs of US$31 (*S. carpocapsae*, see Georgis, Chapter 17 this volume) to US$42 (*H. bacteriophora*, Fig. 14.4) per hectare (2.5×10^9 nematodes ha^{-1}). Nevertheless, costs to end-users remain greater than the alternative pest management tactic in most markets: chemical insecticides (see Georgis, Chapter 17 this volume). As a result, entomopathogenic nematode applications are relegated to a handful of high-value niche markets. Growers seem unlikely to pay a premium to use nematodes when there are familiar, easy to use, low-cost alternatives.

How can substantial cost improvements in nematode liquid culture production be attained? Efforts to date have focused on economies-of-scale or yield. Achieving economies-of-scale does provide extraordinary savings (Fig. 14.4) but the prospect for further gain here is remote in view of limited product markets. As for yield, a millilitre of medium has an uppermost threshold and this may be within reach for present commercial species. Thus efforts that optimize process parameters promise desirable but overall modest improvements – with the possible exception of recovery for heterorhabditids. In other words, the resulting savings would be insufficient to close the cost-of-goods gap between chemicals and nematodes.

To understand production costs, it is instructive to compare chemical insecticides with nematodes. A stainless steel tank for chemical synthesis can be purchased for

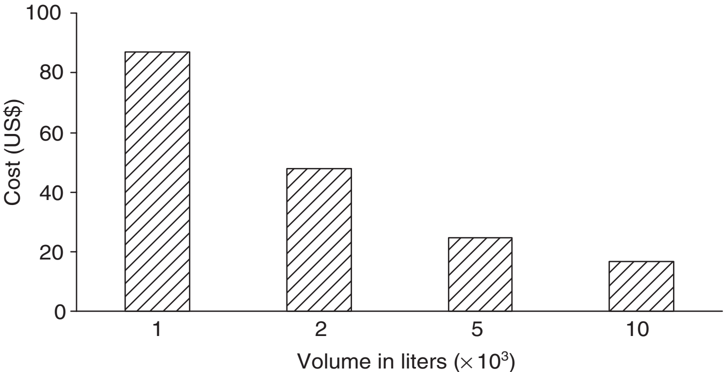

Fig. 14.4. Production costs for one billion *Heterorhabditis bacteriophora* (HbNJ strain) infective juveniles as a function of scale. Culture time was 9 days (S. Franceschini, Italy, 2000, unpublished data).

one-twelfth the cost of the same size vessel pressure rated for sterilization (Yoshioka and Fujita, 1988). Additionally, cycle time is much shorter for chemicals than nematodes. Production costs for other biologicals having a 14-day cycle time, equivalent to *S. carpocapsae*, have been estimated to be 64% capital (includes depreciation and interest) (Yoshioka and Fujita, 1988). In short, capital investment is the most important production parameter for producing nematodes in bioreactors, and therefore the greatest opportunity for cost savings.

Hsiao *et al.* (1999) proposed the production of plant cells in an inexpensive plastic-lined vessel that eliminates the need for an expensive pressure-rated tank. Their uncontrolled reactor design consists of a head plate, a plastic liner, a compression seal to secure the liner to the head plate, and a supporting vessel. The liner is gas (ethylene oxide) sterilized. Particularly impressive, a 6.5-litre working volume prototype demonstrated equivalent growth rate and yield to a highly controlled stirred tank bioreactor (cost: US$20,000) for culture of *Hyoscyamus muticus* cells. Armed with funding from the US Department of Agriculture's Small Business Innovative Research Program (i.e. social investment; Friedman, 1990), cooperative efforts are underway at BioLogic and Penn State University to adapt this technology for producing *H. bacteriophora* (Anonymous, 2000). An alternative paradigm is a disposable airlift micro-fermenter based on medical bag technology (Gaugler and Hatab, 1999). The idea is to ship kits with a micro-fermenter bag, air pump, microbiological filters, dehydrated media, inoculum and simple instructions. End-users would supply water, electricity and space to hang the unit. The intent of this design is to reduce not only capital but also formulation, storage, and transport costs. The feasibility of scaling up these uncontrolled fermentation technologies is uncertain, but targeting capital costs by these or as yet unimagined approaches is indispensable.

The outcome of suboptimal oxygen transfer, shear force, osmolarity, inoculum, antifoams, and other process parameters is suboptimal nematode quality, but quality remains an elusive and ill-defined term in nematode production. Although quality control tends to be viewed as a final product issue, process control procedures for checking nematode identity, quality of medium components, phase and quantity of

bacterial symbionts, and contamination should be established and published in an effort to develop industry-wide standards on quality.

Despite being hobbled by a paucity of basic investigation and its inherently proprietary nature, tremendous strides have been made in nematode mass production technology since Friedman's benchmark publication in 1990. The challenge now will be to maintain the pace of innovation in a period of stagnant public and private funding. The keys will be:

(i) increased academic-industry partnerships (e.g. BioLogic and Penn State University), and
(ii) a shift in mindset away from developing technologies using the conventional chemical pesticide model.

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15 Regulation and Safety

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15.1. Introduction

Entomopathogenic nematodes in the genera *Steinernema* and *Heterorhabditis* together with their symbiotic bacteria, *Xenorhabdus* and *Photorhabdus*, respectively, represent a unique insect biological control 'system'. Laboratory and field studies have shown that insects from over 17 orders and 135 families are susceptible to some degree (Smith *et al.*, 1992). This broad spectrum of potential toxicity and the commercial use of several entomopathogenic nematode species prompted the investigation of their safety to non-target organisms and development of regulatory protocols to ensure their safe usage. This chapter examines various regulatory regimes instituted around the world and the current state of knowledge of the impact of entomopathogenic nematodes and their symbiotic bacteria on non-target organisms. It also addresses non-symbiotic

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bacteria that are occasionally associated with the nematodes and the *Photorhabdus* spp. that have been isolated from human clinical specimens.

15.2. Regulatory Environment

15.2.1. Wild-type entomopathogenic nematode complexes

Commercial development of entomopathogenic nematodes in the USA in the early 1980s was aided by an exemption from registration under the Environmental Protection Agency Requirements of Registration (Federal Insecticide, Fungicide, and Rodenticide Act, Gorsuch, 1982). Canada adopted a similar approach a few years later (Anonymous, 1986), exempting all entomopathogenic microorganisms including bacteria, viruses, fungi, protozoa, and nematodes.

Numerous non-endemic species of nematodes were brought into the USA for research without going through the stringent quarantine and handling regulations required for the insect predators and parasites. Exotic species and strains such as *Steinernema scapterisci* from South America and *Steinernema feltiae* from Europe were developed and sold as commercial products in the USA. *S. scapterisci* applied for mole cricket control in turfgrass in Florida became established as 'natural' populations (Parkman and Smart, 1996). Little was known about risks associated with the introductions of entomopathogenic nematodes and concerns eventually arose about the considerable exchange of nematode germplasm occurring between laboratories (Jansson, 1993).

These issues for entomopathogenic nematodes and other entomopathogens sparked a rethinking of the mechanisms by which the USA should regulate exotic natural enemies of pests. As no specific laws existed, the use and importation of nematodes relied on interpretation of laws such as the Plant Quarantine Act of 1912, Federal Plant Pest Act of 1957, and the National Environmental Policy Act of 1969 (Kaya and Gaugler, 1993). In 1987, the US Environmental Protection Agency reviewed the registration status of entomopathogenic nematodes and once again granted a registration exemption (Anonymous, 1987). Nickle *et al.* (1988) proposed guidelines for the introduction of entomopathogenic nematodes into the USA, covering issues such as the need to import, foreign exploration, taxonomy, shipment, quarantine facilities, permits, host range tests, release, and documentation. However, as these guidelines never became official USA policy, few researchers have followed them.

In the USA there are now complex regulatory procedures and safeguards in place for the introduction of exotic nematodes (Rizvi *et al.* 1996). Each state requires that entomopathogenic nematode products be reported and that label construction conforms to its specific requirements. Permits for biological control agents (including entomopathogenic nematodes) are issued by the Biological Assessment and Taxonomic Support unit of the Organism Permitting and Risk Analysis (OPRA) branch of the Animal and Plant Health Inspection Service – Plant Protection and Quarantine (PPQ). An applicant must complete a permit form obtained from PPQ and send it to a State Plant Regulatory Official of the state to which the entomopathogenic nematodes will be shipped. The state in turn forwards the form to OPRA. Although OPRA staff are amenable to discussing recommendations for expediting this process, they warn that unless international agencies cooperate by following the recommendations before

sending live material into the USA, the permits can be rejected or the process can take several years to complete.

In Europe the situation is somewhat different (Ehlers, 1996; Ehlers and Hokkanen, 1996). In 1995 a committee of the Organisation for Economic Cooperation and Development (OECD) reviewed potential problems associated with the introduction and commercial use of non-indigenous nematodes for biological control. They concluded that all scientific evidence supported the assumption that entomopathogenic nematodes are safe, and few risks were identified. They recommended that the nematodes should not be subject to registration but that the introduction of non-indigenous entomopathogenic nematodes be regulated. Among its conclusions, the OECD Report noted that entomopathogenic nematodes 'are beneficial organisms which have been used for many years without causing any known problem' and 'are more specific and are less of a threat to the environment than chemical pesticides' (Ehlers and Hokkanen, 1996).

Regulations between various European countries vary. Indigenous, unmodified nematodes do not have to be registered for use as biopesticides in the United Kingdom; however, the introduction of non-indigenous species is strictly controlled, as are the bacterial symbionts (Richardson, 1996). Entomopathogenic nematodes are classified as 'animals' and regulation occurs through the Wildlife and Countryside Act of 1982 (Hominick and Collins, 1997). In some European countries registration is mandatory. The European Commission is seeking to harmonize procedures for the authorization of plant protection products, including rhabditid nematodes, under the provisions of Council Directive 91/414/EEC. As result of a European Commission initiative there is now a limited registration procedure that is only a paper exercise with minimum processing fees (G. Gowling, Whittlesford, England, 2000, personal communication). This limited registration requirement is now in operation in Sweden, Norway and Ireland. In contrast, some European countries (e.g. France and Germany) demand phytosanitary certification involving an investigation by governmental authorities into a company's production process. This requirement was established to satisfy concern about potential contamination issues, such as fungus growing on clay formulations. Apparently, they are rarely more than a paperwork exercise.

In 1990, a USA biological control company, Biosys, commenced working with a Japanese pesticide distributor to register the use of the *S. carpocapsae* All strain against the hunting billbug, *Sphenophorus venatus vestitus*. The Japanese Ministry of Health and the Agricultural Department both ruled that entomopathogenic nematode products should be registered. This required efficacy field trials being conducted with the cooperation of government sponsored entomologists. Efficacy equal to or better than currently registered insecticides for one rate of application with one type of formulation had to be demonstrated from 2 years of field trials. The use of safety information from published and unpublished sources was not recognized. Basic or Tier I acute toxicity studies, on the nematode and on its symbiotic bacterium, conducted by an independent laboratory were required. This was the first documented case in which the issue of safety of the symbiont was raised for registration purposes. In 1996, the Japanese government reviewed their registration programme for biologicals and provided 'lower level' status for entomopathogenic nematodes. This new status regarded these products as benign, thus speeding up and reducing registration costs. *Steinernema*

glaseri was registered for use in Japan in 2000 at a cost of approximately US$270,000 (S. Yamanaka, Ibaraki, Japan, 2000, personal communication).

The requirements for introduction of exotic entomopathogenic nematodes into Australia and New Zealand were reviewed by Bedding *et al.* (1996). As the National Registration Authority in Australia classifies entomopathogenic nematodes as macroparasites, registration is not required. However, the introduction of exotic nematodes to Australia is regulated. The Australian Quarantine Inspection Service (AQIS) and the Australian Nature Conservation Agency, which administer the Quarantine Act and the Wildlife Protection (Regulation of Exports and Imports) Act, respectively, have responsibility for importations. The procedures for importation involve a single application to AQIS, which then consults with relevant state and federal authorities before issuing the import permit. In contrast, New Zealand has defined entomopathogenic nematodes as pesticides and requires that pesticide products must be registered before commercial trials and sales are authorized. Importations must be approved by the Ministry of Agriculture and Fisheries, whereas the regulation of release of exotic nematodes comes under the auspices of the Pesticides Board.

15.2.2. *Xenorhabdus* and *Photorhabdus*

There are prospects that the symbiotic bacteria *Xenorhabdus* and *Photorhabdus* could be used as insect control agents independently of the nematodes. A patent for the use of *Xenorhabdus nematophila* against fire ants has been issued (Dudney, 1997). Activity lasting up to 6 months has been noted (K. Smith, Arizona, USA, 2000, personal observation) even though conventional research shows bacterial persistence in the environment lasting only a few days (Morgan *et al.*, 1997). In response to a recent inquiry regarding their current assessment on the registration issue of using *X. nematophila* as a stand-alone insecticide, the Environmental Protection Agency stated that a full Tier I acute toxicity study is required before registration will be considered (R. Dudney, Texas, USA, 2000, personal communication).

A wider usage of the symbiotic bacteria may arise from the incorporation of their genes into plants. Both *Xenorhabdus* and *Photorhabdus* produce two families of insecticidal proteins that are being evaluated for plant resistance to insect pests (Bowen and Ensign, 1998; East *et al.*, 1998). Transgenic plants expressing *Xenorhabdus* or *Photorhabdus* genes will be regulated with reference to the laws pertaining to transgenic plants in general. Barber (1999) has summarized the approach to the regulation of transgenic plants being adopted by the 29 members of the OECD.

In the current climate of concern, there is strong pressure to confirm the absence of potential allergenicity before allowing the use of novel proteins in foods. Although the use of transgenic plants expressing the Cry1 and Cry3 insecticidal protein of *Bacillus thuringiensis* was approved, there has been concern about the use in food of corn that expresses Cry9C (StarLink™; Anonymous, 2000). Although no allergenic responses to Cry9C have been detected and it has no epitope sequence homology to known allergens, the Cry9C protein differs from the Cry1 and Cry3 proteins in having some stability to simulated gastric digestion and to heat. In February 2000, a Scientific Advisory Panel for the US Environmental Protection Agency (USEPA) agreed that the available data did not provide evidence indicating 'that Cry9C is or is not a potential food allergen' (Anonymous, 2000). In the absence of proof to the contrary, the USEPA

labelled Cry9C a potential allergen and declined to approve Starlink™ corn for use in human food products. This experience indicates that there will probably be extensive testing required before approval for the release of transgenic plants containing genes from the symbiotic bacteria is granted.

15.2.3. Genetically modified entomopathogenic nematodes

Field trials with entomopathogenic nematodes show large variation in reduction of target pest populations. The greatest impact on the success of entomopathogenic nematodes as biological control agents is due to abiotic factors, most of which relate to the soil environment in which these nematodes are naturally found. Genetic manipulation has been employed in efforts to overcome these impediments.

Genetic efforts range from classic selection (Gaugler *et al.*, 1994) to DNA insertion (Hashmi *et al.*, 1995). The literature relating to the genetics of entomopathogenic nematodes was summarized by Liu *et al.* (2000). The authors concluded that genetic engineering

> . . . has shown promise for the rational improvement of entomopathogenic nematodes to overcome limitations related to inadequate efficacy, stability, and economics to realize their full pest control potential. There is every reason to believe that the use of entomopathogenic nematodes for insect control will increase substantially in the near future.

A strain of *H. bacteriophora* was genetically modified for enhanced thermotolerance by the introduction of the *hsp70A* gene from *Caenorhabditis elegans* (Gaugler *et al.*, 1997). This was the first release of a genetically engineered, non-microbial, insect natural enemy into the environment and is the only example of the release of a genetically engineered entomopathogenic nematode. As such, it is our only guide to understanding the current thinking on regulation.

The proponents had to approach several government agencies to obtain the necessary permits and authorizations for the field release. The USEPA concluded that the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), which excludes nematodes from regulation, does not distinguish between genetically engineered and wild-type nematodes, and that registration was not required. The USEPA further ruled that the nematode's associated bacterial partner, as an integral part and mechanism of action of the nematode, is not subject to FIFRA as a separate entity. However, the bacteria must be those occurring in nature with the nematodes and, specifically, not be genetically engineered.

The key regulatory hurdle was the US Department of Agriculture's Animal and Plant Health Inspection Service (APHIS). This agency regulates biological control agents because of their potential to indirectly harm plants. The APHIS Transgenic Arthropod Team conducted a regulatory assessment based on the applicants' submission. The assessment required provision of detailed information the transformation method, characteristics and geographical origin of donor, vector and recipient, characteristics of the modified organism, its potential environmental impact, safeguards, host and habitat preferences, dispersal characteristics, and experimental design. The Team concluded that the heat-shock protein could be expected to have little effect on nematode survival or ecological relationships. A field release permit was issued. The authors then required permission from various local regulatory agencies for final approval. The review process was completed in less than 3 months.

Gaugler *et al.* (1997) concluded that releases of transgenic nematodes in the USA will be decided on a case-by-case basis. The regulatory picture for transgenic nematodes outside the United States is unclear and will remain so for the immediate future, as the social, political and scientific consequences of the release of transgenic organisms that cannot be constrained are debated.

15.3. Effects on Non-target Organisms

Natural occurring insect pathogens, including entomopathogenic nematodes, can and do play a significant role in regulating populations of different insect species (Akhurst *et al.*, 1992; Raulston *et al.*, 1992; Strong *et al.*, 1996). It is therefore imperative to establish the extent to which nematodes applied for pest control impact on populations of non-target organisms.

15.3.1. Invertebrates

Information on natural hosts is lacking for more than half of the recognized *Steinernema* and *Heterorhabditis* species. Although early collections of entomopathogenic nematodes were made from naturally infected insects (Peters, 1996), most have been isolated by baiting of soil samples with a susceptible species (e.g. *Galleria mellonella*, Akhurst and Brooks, 1984). Similarly, while the efficacy of entomopathogenic nematodes for target species is well documented, less is known of their impact on non-target species.

Predators and parasitoids are potentially affected by entomopathogenic nematodes, through direct infection, early death of the parasitized host, or reduction in the host population. Some parasitoid species were killed by nematodes in laboratory tests indirectly though host infection (Kaya, 1978, 1984; Triggiani, 1985). Battisti (1994) found a 66% reduction in emergence of *Xenoschesis fulvipes*, an ichneumonid parasitoid of the sawfly *Cephalcia arvensis*, in field trials involving application of *S. feltiae*. In contrast to parasitoids, predators are more likely to be affected directly by infection. Lemire *et al.* (1996) reported that in Petri dish assays *S. carpocapsae* infected coccinellid beetles, causing temporary paralysis and, in some cases, death. Georgis *et al.* (1991) reported that immature stages, but not the adults, of several predatory beetles were susceptible to *S. carpocapsae* and *H. bacteriophora* (Table 15.1). In contrast, when adults and larvae of two species of predatory beetles (Carabidae) were stored in soil containing *S. carpocapsae*, the larvae were unaffected but some adults died (Ropek and Jaworska, 1994). Clearly, assessment of the impact of nematodes on predators and parasitoids should be made on all life stages exposed to nematodes.

The potential environmental impacts of entomopathogenic nematodes were reviewed by Barbercheck and Millar (2000). They concluded that the non-target species most likely to be affected are those with soil-dwelling stages active when the nematodes are applied. However, only small reductions in field populations of non-target species have been associated with applications of entomopathogenic nematodes (Ishibashi *et al.*, 1987; Buck and Bathon, 1993; Ropek and Jaworska, 1994). For example, Rethmeyer and Baton (1992) investigated the impact of *S. feltiae* and *Heterorhabditis* spp. on different coleopteran families in a beech–oak (*Betula-Quercus*) forest, a pine (*Pinus*) forest margin, a field crop and an orchard. In observing species from the families Carabidae, Staphylinidae, Chrysomelidae, Curculionidae and Elateridae, they

found few reductions in population size. However, they recovered higher numbers of some coleopteran species from nematode-treated plots than from untreated plots.

Entomopathogenic nematodes can infect and reproduce in certain non-insect arthropod species under laboratory conditions. Poinar (1989) reported penetration, mortality and development in representatives of the Symphyla, Collembola, Arachnida, Crustacea and Diplopoda in Petri dish studies. Jaworska (1991) reported 100% mortality of a terrestrial isopod species and a millipede species exposed to 3000 infective juveniles of *S. carpocapsae* or *S. feltiae* in a Petri dish and 50% mortality in

Table 15.1. Examples of reported effects of entomopathogenic nematodes on non-target invertebrates.

Non-target organism	Nematode species	Effect	Reference
Parasitoid insects			
Apanteles militaris (Hym.: Braconidae)	*Steinernema carpocapsae* *Heterorhabditis bacteriophora*	Indirect (host death) in lab tests	Kaya (1978)
Compsilura concinnata (Dip: Tachinidae).	*S. carpocapsae*	Indirect (host death) in lab tests	Kaya (1984)
Apanteles ultor (Hym.: Braconidae	*S. feltiae* *H. bacteriophora*	Indirect (host death) in lab tests	Triggiani (1985)
Cephalcia arvensis (Hym.: Ichneumonidae)	*S. feltiae*	Reduced emergence in field	Battisti (1994)
Predatory insects			
Harmonia axyridis (Col.: Coccinellidae)	*S. carpocapsae*	Some beetles were temporarily paralysed and others were killed in Petri dish assays	Lemire *et al.* (1996)
Harpalus sp. and *Pterostaticus* sp. (Col: Carabidae); *Cicindela* sp. and *Tetracha* sp. (Col: Cicindelidae) *Philonthus* sp. (Col: Staphylinidae); *Labidura riparia* (Derm: Labiduridae)	*S. carpocapsae* *H. bacteriophora*	Immature stages but not adults killed in lab test; no effect on populations in field	Georgis *et al.* (1991)
Bembidion proerans, Pterostichus cupreus (Col.: Carabidae)	*S. carpocapsae*	Adults but not larvae killed in lab test; small reduction in field populations	Ropek and Jaworska (1994)
Platynus dorsalis and *Amara similata* (Col.: Carabidae); unidentified Staphylinidae (Col.)	*S. feltiae* *Heterorhabditis* spp.	Slight reductions in some, and increases in other, species after nematodes applied in the field	Rethmeyer and Bathon (1992)
Other invertebrates			
Longitarsus sp. and *Phyllotreta* sp. (Col.: Chrysomelidae), *Barypeithes* sp. (Col: Curculionidae), unidentified Elateridae (Col.)	*S. feltiae* *Heterorhabditis* spp.	Slight reductions in some, and increases in other, species after nematodes applied in the field	Rethmeyer and Bathon (1992)
Onychiurus (Collembola)	*S. carpocapsae*	Reductions in field populations	Poinar (1989)
Scutigerella immaculata (Symphyla)	*S. carpocapsae*	Killed in lab tests	Poinar (1989)
Armadillidium vulgare, *Porcellio scaber* (Crustacea: Isopoda)	*S. carpocapsae* *H. bacteriophora* *S. glaseri*	Killed by *S. carpocapsae* and *H. bacteriophora* in lab test but not by *S. glaseri*	Poinar (1989)

Table 15.1. Continued

Non-target organism	Nematode species	Effect	Reference
Porcellio scaber (Crustacea: Isopoda)	*S. carpocapsae* *S. feltiae*	Killed in lab tests. *S. feltiae* also killed 50% in glasshouse tests.	Jaworska (1991)
Atyia innocous, *Macrobrachium acanthurus* (Crustacea: Caridea)	*S. carpocapsae*	Not affected in lab test	Kermarrec and Mauléon (1985)
Various species of Arachnida	*H. bacteriophora*	Killed in lab tests	Poinar (1989)
Blaniulus guttulattus (Myriapoda)	*S. carpocapsae* *S. feltiae*	Killed in lab tests. *S. feltiae* also killed 50% in glasshouse tests	Jaworska (1991)
Boophilus annulatus, *B. microplus*, *Amblyomma variegatum* (Acarina)	*Steinernema* and *Heterorhabditis* spp.	*B. annulatus* was killed by 17 species of nematodes in lab tests but *B. microplus* and *A. variegatum* were not killed by any nematode species	Mauléon *et al.* (1993)
Ixodes scapularis (Acarina)	13 species of *Steinernema* and *Heterorhabditis*	Only engorged females were killed in lab test	Hill (1998); Zhioua *et al.* (1995)
Aporrectodea sp.	*S. carpocapsae*	Intact earthworms were not infected	Capinera *et al.* (1982),
Allolobophora caliginosa	*S. scapterisci*	Only dead or injured earthworms were infected. However, nematodes developed on the bacteria associated with earthworm segments	Nguyen and Smart (1991)
Aporrectodea caliginosa	*Steinernema* sp.	No effect on earthworm cocoons in lab test	Nüutinen *et al.* (1991)
Aporrectodea turgida, *Aporrectodea trapezoides* [*Aporrectodea caliginosa*], *Lumbricus terrestris*, and *Eisenia* sp.	*S. carpocapsae*, *S. glaseri*	No effect in lab tests	Potter *et al.* (1994)
Macrobiotus richtersi (Tardigrada)	*S. carpocapsae*	Infection in lab tests	Ishibashi *et al.* (1987)
Oncomelania hupensis (*Gastropoda*)	*S. carpocapsae*, *S. glaseri*, *S. feltiae*, *H. bacteriophora*, *H. zealandica*	Some mortality in lab tests	Li *et al.* (1986)
Dericeras agreste, *D. reticulatum* (Gastropoda)	*S. carpocapsae*, *S. feltiae*, *H. bacteriophora*	Killed in lab tests	Jaworska (1993)

greenhouse trials with *S. feltiae* against the same two species. Ishibashi *et al.* (1987) reported the development of *S. carpocapsae* in tardigrades.

Entomopathogenic nematodes are also pathogenic for some species of ticks. Samish and Glazer (1991) reported laboratory infection of engorged female *Boophilus annulatus* by *S. carpocapsae* 4 days after exposure. Mauléon *et al.* (1993) found that, although *B. annulatus* was susceptible to 17 isolates of *Steinernema* or *Heterorhabditis*,

neither *Boophilus microplus* nor *Amblyomma variegatum* was susceptible. Zhioua *et al.* (1995) discovered that only engorged adult *Ixodes scapularis* females became infected with *S. carpocapsae* and *S. feltiae.* Hill (1998) reported similar results with 13 species and strains of entomopathogenic nematode; no infection occurred with unfed or engorged larvae, nymphs, males, or unfed females.

Capinera *et al.* (1982), Nguyen and Smart (1991), Nüutinen *et al.* (1991) and Potter *et al.* (1994) investigated the impact of different entomopathogenic nematodes on various earthworm species. In some instances, nematode development was reported but no impact on earthworm populations was observed. In investigating improved dispersal of *S. carpocapsae* in soil column tests when earthworms (*Lumbricus terrestris* or *Aporrectodea trapezoides)* were present, Shapiro *et al.* (1993) found nematodes on the exterior and interior of the earthworms.

Some Gastropoda are susceptible to entomopathogenic nematodes. Jaworska (1993) documented the susceptibility of two different slug species (*Deroceras agreste* and *D. reticulatum*) to *S. carpocapsae, S. feltiae* and *H. bacteriophora* in Petri dish bioassays. She found that each of the entomopathogenic nematode species developed in the slug cadavers. However, Wilson *et al.* (1994) found that *S. feltiae* and a *Heterorhabditis* sp. were not able to infect and kill *D. reticulatum*. When the symbiotic bacteria were injected into slugs, only *Xenorhabdus bovienii* was pathogenic, albeit poorly. Li *et al.* (1986) discovered that up to 97.5% of *Oncomelania hupensis*, a snail intermediate host of the blood fluke, *Schistosoma japonicum,* were infected when exposed to *S. feltiae, S. glaseri* or *H. bacteriophora* at 300 infective juveniles cm^{-2} in soil pots.

The evidence indicates that impact of entomopathogenic nematodes on non-target insects and other invertebrates is limited. The nematodes have been shown capable of infecting a range of invertebrates from several phyla under laboratory conditions with high nematode dosages. However, the observable impact on field populations of non-target invertebrates has either been small and transitory or undetectable. Reports of indigenous nematode populations impacting on insect populations can be divided between relatively balanced, nematode–host associations (e.g. Strong *et al.*, 1996) and unbalanced, short-lasting epizootics (e.g. Akhurst *et al.*, 1992). The few papers reporting nematode epizootics involve studies associated with pest species; there have been no reports of epizootics in non-target, non-pest species.

An overview of the reports on the effects of entomopathogenic nematodes on non-target invertebrates (Table 15.1) shows that most studies to date have been conducted under laboratory conditions. These studies, which have generally been conducted with high dosages of nematodes, show a wide range of invertebrate susceptibility. Research on the extent and impact of entomopathogenic nematodes on non-target invertebrates in the field, using commercial dosages of the nematodes, is essential to a proper evaluation of their environmental impact.

15.3.2. Vertebrates

15.3.2.1. Entomopathogenic nematodes

A key reason why registration regulations for entomopathogenic nematodes are less stringent than for conventional pesticides is their inability to infect or reproduce in vertebrates (Table 15.2). Kermarrec and Mauléon (1985) and Kermarrec *et al.* (1991) studied the susceptibility of toads, frogs and lizards to different entomopathogenic

Table 15.2. Tests of the effects of entomopathogenic nematodes and their symbiotic bacteria on vertebrates.

Test animal	Nematode or bacterial species	Application	Effect	Reference
Guinea pig	*Xenorhabdus bovienii*	Orally, nasally, intradermally, subcutaneously, intraperitoneally	No pathology	Obendorf *et al.* (1983)
Rat	*Steinernema glaseri*	Intraperitoneally	No pathology	Jackson and Bradbury (1970)
	S. carpocapsae	Orally, intraperitoneally	No pathology or effect on weight gain	Gaugler and Boush (1979)
	X. bovienii	Orally, intradermally, subcutaneously, intraperitoneally	No pathology	Obendorf *et al.* (1983)
Mouse	*S. carpocapsae*	Orally, subcutaneously, intraperitoneally	Skin ulcers when administered subcutaneously	Kobayashi *et al.* (1987)
	S. feltiae, S. glaseri	Orally, subcutaneously, intraperitoneally	No pathology	Kobayashi *et al.* (1987)
	Heterorhabditis bacteriophora	Orally	No pathology	Kobayashi *et al.* (1987)
	S. carpocapsae, *H. bacteriophora*	Subcutaneously	No pathology	Poinar *et al.* (1982)
	X. nematophila, *Photorhabdus luminescens*	Subcutaneously, intracerebrally	No pathology	Poinar *et al.* (1982)
	X. nematophila	Topically to abraded skin	No pathology	Kermarrec *et al.* (1991)
	X. bovienii	Orally, nasally, intradermally, subcutaneously, intraperitoneally	No pathology	Obendorf *et al.* (1983)
Rabbit	*S. glaseri*	Orally, abdominally, orbital cavity	No pathology	Wang and Liu (1983)
	X. bovienii	Conjunctivally	No pathology	Obendorf *et al.* (1983)
Monkey	*S. glaseri*	Orally, abdominally, nasally	No pathology	Wang *et al.* (1984)
Chicken	*S. carpocapsae*	Orally	No pathology	Kermarrec and Mauléon (1985)
	X. nematophila, *P. luminescens*	Subcutaneously	No pathology	Poinar *et al.* (1982)
Toad	*S. carpocapsae*	In water	Young tadpoles killed	Kermarrec and Mauléon (1985)
Frog	*S. carpocapsae*, *H. bacteriophora*	In water	Young tadpoles killed	Poinar and Thomas (1988)

Table 15.2. Continued

Test animal	Nematode or bacterial species	Application	Effect	Reference
	S. carpocapsae, *S. glaseri*, *S. anomali*, *S. feltiae*, *Heterorhabditis* spp.	In water	Adults unaffected	Kermarrec *et al.* (1991)
Fish	*S. carpocapsae*	In water	No pathology	Kermarrec and Mauléon (1985)
Lizard	*S. carpocapsae*	Orally	No pathology	Kermarrec and Mauléon (1985)
	S. carpocapsae, *S. glaseri*, *S. anomali*, *S. feltiae*, *Heterorhabditis* spp.	Orally	Livers infected by bacteria such as *Vibrio* spp., *Pseudomonas aeruginosa* and *Chromobacterium*	Kermarrec *et al.* (1991)

nematode species. They found that, although adult frogs and toads were unaffected, some nematode strains could kill the early tadpole stages of the toad *Bufo marinus*. Poinar and Thomas (1988) also reported penetration of *S. carpocapsae* and *H. bacteriophora* (= *heliothidis*) through the alimentary tract of young tadpoles of the frog species *Hyla regilla* and *Xenopus laevis. S. carpocapsae* released their symbiotic bacterium and in two cases the nematodes developed into adult females before dying. Tadpole mortality was associated not with *Xenorhabdus* but with foreign bacteria entering the penetration holes made by the invading nematodes. Kermarrec *et al.* (1991) found that adult *Anolis marmoratus*, an iguanid lizard, died after 80,000 infective stage *S. carpocapsae* or *Heterorhabditis* spp. were administered orally. *S. carpocapsae* were found as live infective juveniles in the body cavity whereas the *Heterorhabditis* spp. caused necroses in the intestine but no nematodes survived. The effect of the nematodes on the lizards was apparently indirect as the livers were infected by non-symbiotic bacteria (*Pseudomonas, Chromobacterium, Vibrio*). These studies are sometimes dismissed because they were conducted under controlled conditions with high levels of constant exposure and some stress on the experimental animals. However, further investigation, in the form of field studies, is warranted.

The impact of entomopathogenic nematodes on homoiothermic species has been tested (Table 15.2). Except for mice injected subcutaneously, there was no evidence of infectivity, pathogenicity or toxicity by entomopathogenic nematodes. Kobayashi *et al.* (1987) reported that mice injected subcutaneously with 2×10^4 infective juvenile *S. carpocapsae* or *S. feltiae* developed skin ulcers. In contrast, Poinar *et al.* (1982), using a lower dosage of 10^3 infective juvenile *S. carpocapsae* or *H. bacteriophora* subcutaneously injected into mice, did not detect any pathology.

The experience gained from biopesticide production suggests that there is a very small but real risk of allergenicity associated with entomopathogenic nematodes. At Biosys, a large nematode production company where over 75 people worked with more than five species of nematodes over a 12-year period, only one case of possible

allergic responses was recorded. This person had dermal reactions from handling concentrated nematode solutions during the harvesting, cleaning and storage stages of production. In an Australian research laboratory producing at least ten species of entomopathogenic nematodes for more than 20 years, one person reported a mild allergic response whenever *S. carpocapsae* infective juveniles came into contact with his eye (R.A. Bedding, Australian Capital Territory, Australia, 2000, personal communication). He had no response from nematode contacts with other tissues and has continued working with these nematodes for the past 12 years.

15.3.2.2. Symbiotic bacteria

The safety of bacterial symbionts of entomopathogenic nematodes for vertebrates has been tested independently of the nematodes. When white leghorn chicks and adult albino mice were injected subcutaneously and suckling albino mice were injected intracerebrally with *X. nematophila* and with *Photorhabdus luminescens*, no disease symptoms were observed (Poinar *et al.*, 1982). Similarly, oral, intradermal, subcutaneous and intraperitoneal applications of *X. bovienii* to guinea pigs, mice and rats produced no signs of infectivity, pathogenicity or toxicity; conjunctival inoculation of rabbits and inhalation trials with guinea pigs and mice also produced no harmful effects (Obendorf *et al.*, 1983).

Allergenic responses to *Xenorhabdus* have been noted in one person who reacted with asthmatic symptoms that resulted from an allergy to *X. nematophila* (K. Smith, Arizona, USA, 2000, personal observation). This individual, who was developing nematode formulations, was only able to work in the main facility for a few hours each day. While the nematodes and their symbionts may, like any organism, evoke an allergic response in sensitive individuals, there is no indication that they are hyperallergenic or allergenic for many people. Anecdotal evidence based on the number of people working closely with these bacteria for many years suggests that the risk of allergenicity is low.

15.3.3. Soil microorganisms and microfauna

Xenorhabdus and *Photorhabdus* produce a variety of metabolites that are toxic to bacteria and fungi (Paul *et al.*, 1981; Akhurst, 1982; McInerney *et al.*, 1991a,b; Li *et al.*, 1997; Ng and Webster, 1997) and therefore have the potential for impact on soil microorganisms. Maxwell *et al.* (1994) showed that diffusates from cadavers killed by *X. nematophila* had a transient effect on bacterial populations in soil. Webster (2000) suggested that metabolites of the symbiotic bacteria might cause 'localized and rapid changes in rhizosphere microflora' because entomopathogenic nematodes are attracted to insects feeding on roots. The impact detected by Maxwell *et al.* (1994) was, however, only slight, with a halving of bacterial counts in the higher density treatments over 4–8 days. Since some of the water and uninjected cadaver controls also varied by twofold over this time, the significance of this variation is uncertain. The design of the experiment possibly enhanced the impact, because saturating the soil with water and suspending the cadavers in water probably promoted the rate and range of diffusion of metabolites from the cadavers.

Some metabolic products of *Xenorhabdus* and *Photorhabdus* spp. are nematicidal (Hu *et al.*, 1999; Han and Ehlers, 1999; Grewal *et al.*, 1999). This may be an

anti-competition mechanism to prevent utilization of the insect cadaver by other entomopathogenic and saprophagic species. However, in investigating the reduction in plant parasitic nematodes following application of entomopathogenic nematodes, Grewal *et al.* (1999) found that heat-killed *S. feltiae* and *S. riobrave* suppressed root penetration by *Meloidogyne incognita* and suggested that this effect may be due to the metabolites of the symbiotic bacteria. Later research showed that the effects on nematodes were limited to plant parasitic nematodes. Application of *H. bacteriophora* and *H. indica* at commercial application rates depressed the diversity (number of genera) and concentration in soil of plant parasitic nematodes but had no impact on free-living nematodes (P.S. Grewal, Ohio, USA, 2000, personal communication). The limitation of impact to plant parasitic nematodes suggests that the production of nematicidal metabolites may not be an anti-competitive mechanism. However, the advantage to the entomopathogenic nematodes and their symbionts of inhibiting plant parasitic nematodes is not evident.

15.4. Isolation of *Photorhabdus* from Humans

Farmer *et al.* (1989) reported the isolation of *Xenorhabdus luminescens* (subsequently re-assigned as *Photorhabdus luminescens*; Boemare *et al.*, 1993) from a human wound in 1986. Examination of files of the Enteric Bacteriology Section at the Centers for Disease Control (CDC, USA) revealed four unidentified strains isolated between 1977 and 1987 that were subsequently identified as *P. luminescens*. There were no further reports of the association of *Photorhabdus* until Peel *et al.* (1999) reported the isolation of *P. luminescens* from four patients in Australia between 1994 and 1998. Another *P. luminescens* was isolated from a human in Australia in 1999 (D. Alfredson, Queensland, Australia, 1999, personal communication). A subsequent check with the Centers for Disease Control revealed no further records of clinical isolates of *Photorhabdus* in the Enteric Bacteriology Section, CDC (M. Peel, Victoria, Australia, 1998, personal communication).

Isolations were made from various tissues and sites (Table 15.3). In six cases the bacterium was either isolated from multiple sites on the body or from the blood, indicating that it can occur as a disseminated infection. The infection responded quickly to antibiotic treatment except in one patient (USA4) where it was sustained at least 2 weeks and in another (Aus3) for 2 months. Three patients may have been immunocompromised, but five were not; the status of the other two is uncertain.

The route of the infection was not established for any of the patients. Two patients reported having been bitten by a spider; a third may have been (Table 15.3). One patient (USA4) did not present for treatment until 6 weeks after the bite whereas another (Aus3) presented on the same day as being bitten. The patients who confirmed spider bites responded to antibiotic treatment slowly. However, the bites could not be confirmed as the source of the infection and two other patients stated that they had not suffered any bites in the affected area.

Molecular analyses clearly differentiated *Photorhabdus* isolated from humans from those isolated from nematodes. DNA/DNA hybridization studies (Farmer *et al.*, 1989; Boemare *et al.*, 1993) showed a clear distinction between the USA clinical group, which had a very high level of within-group relatedness, and the various groups of symbiotic isolates. Analysis of the 16S rDNA gene by sequencing and PCR–RFLP confirmed the

Table 15.3. Isolation of *Photorhabdus* spp. from humans (after Farmer *et al.*, 1989; Peel *et al.*, 1999, and D. Alfredson, Queensland, Australia, 2000, personal communication).

Patient	Age/gender	Isolation site (s)		Day between isolations	Immuno-compromised	Spider bite
		Lesion	Blood			
USA1	78 yr male	+	–	2	?[a]	–
USA2	80 yr female	+	+	nr[b]	nr	nr
USA3	72 yr female	nr	+	nr	nr	nr
USA4	45 yr male	+	nr	nr	–	+
USA5	36 yr female	$+_m$[c]	nr	nr	?	nr
Aus1	11 yr female	+	nr	12	–	?
Aus2	90 yr male	nr	+	nr	–	nr
Aus3	50 yr male	$+_m$[c]	–	60	–	+
Aus4	55 yr male	+	nr	3	–	–
Aus5	78 yr male	$+_m$	nr	nr	+	nr

[a] Possibly. [b] Not reported. [c] Multiple sites.

high degree of relatedness among the USA clinical isolates (Szállás *et al.*, 1997, Fischer-Le Saux *et al.*, 1998, 1999). Fischer-Le Saux *et al.* (1999) proposed that the USA clinical isolates be classified as a separate species, *P. asymbiotica*. PCR–RFLP analysis (Peel *et al.*, 1999) and sequencing of the 16S rDNA gene (C. Beard and R. Akhurst, Australian Capital Territory, Australia, 2000, unpublished data) revealed that the Australian clinical isolates are also highly similar to each other. However, significant differences between the Australian and USA clinical isolates showed that the former are not *P. asymbiotica*. Phylogenetic analysis of the 16S rDNA sequencing data suggest that Australian clinical isolates should be classified as a new species of *Photorhabdus* (P. Janssen and R. Akhurst, Melbourne, Australia, 2000, unpublished data).

There have been no reports of *Xenorhabdus* being isolated from human clinical sources. While it is possible that *Xenorhabdus* strains do not infect humans, it is also possible that there has simply been a failure to identify clinical *Xenorhabdus*. All clinical isolates of *Photorhabdus* have been in the phase II state, at least by the time they have been identified, and so are negative for most of the characters tested in clinical laboratories. While *Photorhabdus* can be identified even in its phase II form by luminescence, phase II *Xenorhabdus* have no striking characteristic(s) that provides an easy identification. However, although clinical strains of *Xenorhabdus* might have been listed as 'unidentifiable' in the past, current methodologies used in clinical laboratories refer to databases that include both *Xenorhabdus* and *Photorhabdus*. It is too early to conclude that no members of the genus *Xenorhabdus* ever infect humans. Nevertheless, if such infections occur, they are rare.

The risk of infection by the bacterial symbionts of nematodes does not appear to be great when assessed on several grounds. First, no case of infection by *Photorhabdus* has been reported from research laboratories or production facilities in which many different strains, including the clinical strains, are studied. Second, although most clinical laboratories might have had some difficulty recognizing *Xenorhabdus* and

Photorhabdus in the past, the databases for bacterial identification systems in current usage do include *Photorhabdus* and *Xenorhabdus*. Moreover, even laboratories that have been successful in identifying *Photorhabdus* from clinical sources have made few isolations. Finally, the clear taxonomic distinction between those strains of *Photorhabdus* that are symbiotically associated with nematodes and those that have been isolated from human clinical sources suggest that the latter have evolved on a separate lineage or lineages from the nematode symbionts. On the basis of molecular phylogeny, the symbiotic species of *Photorhabdus* are much less closely related to the clinical species than *Bacillus anthracis* and *Bacillus cereus* are to *Bacillus thuringiensis*, which has been widely and safely used in bacterial bioinsecticides for more than 40 years (Helgason *et al.*, 2000).

15.5. Role of Non-symbiotic Bacteria in the Safety of Entomopathogenic Nematode Products

Bacteria other than the symbiotic bacteria *Xenorhabdus* and *Photorhabdus* are sometimes associated with entomopathogenic nematodes. Lysenko and Weiser (1974) and Boemare (1983) recovered bacteria of various other genera (*Pseudomonas, Enterobacter, Alcaligenes*, etc.) after attempting to surface sterilize infective juveniles of *S. carpocapsae*, and Mracek (1977) isolated a *Flavobacterium* sp. from sawfly larvae infected with *S. kraussei*. Bacteria of the genus *Ochrobactrum* have been reported from surface-sterilized infective juveniles of *S. scapterisci* (Aguillera *et al.*, 1993), *H. indica* and *H. bacteriophora* (Babic *et al.*, 2000). Jackson *et al.* (1995) reported that *Providencia rettgeri* was associated with the majority of *Heterorhabditis* spp. strains that they tested, including *H. bacteriophora, H. indica*, and *H. megidis*. The isolation of non-symbiotic bacteria from nematodes of several species and in several laboratories shows that their association with entomopathogenic nematodes is not rare.

None of these bacteria have been shown to have a specific association with entomopathogenic nematodes. The specific associations are limited to *Xenorhabdus* for *Steinernema* and to *Photorhabdus* for *Heterorhabditis* (Boemare *et al.*, 1993). The symbionts are carried within the intestinal lumen of infective juveniles of *Heterorhabditis* spp. (Poinar and Georgis, 1990; Poinar *et al.*, 1992) or intestinal vesicle of infective juveniles of *Steinernema* and *Heterorhabditis* spp. (Bird and Akhurst, 1983; Poinar *et al.*, 1992). The non-symbiotic bacteria associated with entomopathogenic nematodes are not carried internally but on the surface of infective juveniles (Poinar, 1979). Although the isolation of non-symbiotic bacteria from infective juveniles may be the result of inadequate surface sterilization procedures, a mechanism for the transmission of non-symbiotic bacteria into the host insect has been detected. In *S. scapterisci* bacteria were seen in the space between the second (J2) and third stage cuticles of infective juveniles (Bonifassi *et al.*, 1999). Although *Heterorhabditis* lose the J2 cuticle on penetrating the insect host (Bedding and Molyneux, 1982), some of the bacteria in the intercuticular space could be carried into the host with the nematode. Molyneux *et al.* (1983) showed that non-symbiotic bacteria carried on the nematodes could become established in the insect host.

Several of the non-symbiotic bacteria that have been found associated with entomopathogenic nematodes are opportunistic human pathogens (e.g. *Ochrobactrum* spp., *P. rettgeri*) and the potential for human infection has been realized on at least one

occasion. In 1958, Dr Jaroslav Weiser contracted a urinary tract infection as a result of working with *S. carpocapsae* reared in *G. mellonella* larvae (J. Weiser, Prague, Czech Republic, 2000, personal communication). The infection was caused by *Pseudomonas aeruginosa* and responded well to conventional antibiotic treatment.

Non-symbiotic bacteria are not a major safety issue in relation to entomopathogenic nematode products. Most commercial entomopathogenic nematodes are produced *in vitro*, using monoxenic cultures of the nematode and its specific symbiont. Since the efficiency of these cultures is severely reduced by contamination with non-symbiotic bacteria or fungi, great care is taken to prevent or remove contamination. Commercial *in vitro* products should not contain non-symbiotic, potentially dangerous, microorganisms. However, non-symbiotic bacteria can be a safety risk when the nematodes are produced *in vivo* during the isolation and research phases and for some commercial products, and during *in vitro* production when large quantities of nutrient medium could become contaminated. Although the record of 20 years of commercial production indicates that the safety risk from non-symbiotic bacteria is low, it should never be discounted entirely.

15.6. Conclusions

Many different species and strains of entomopathogenic nematodes have been used successfully in a variety of habitats to control many different species of target insect pests (Georgis, 1992). Their potential for allergenicity requires that routine precautions should be taken during production and application phases to avoid direct exposure both to entomopathogenic nematodes and their symbionts. The same precautions will also provide protection against non-symbiotic microorganisms that are sometimes associated with the nematodes. It is also evident that quality control procedures for products containing entomopathogenic nematodes should include tests for the presence of contaminating microorganisms.

Despite widespread application, no significant acute or chronic toxicity to humans or other vertebrates has been reported, and no significant long-term impact on non-target invertebrate populations has been established. We are unable to identify any safety considerations that should preclude the continued use of entomopathogenic nematodes as biological control agents.

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16 Factors Affecting Commercial Success: Case Studies in Cotton, Turf and Citrus

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16.1. Introduction

Commercialization of entomopathogenic nematodes has experienced highs and lows. Successes include control of the Diaprepes root weevil, *Diaprepes abbreviatus*, in citrus (Duncan *et al.*, 1996; Grewal and Georgis, 1998), the black vine weevil, *Otiorhynchus sulcatus*, in cranberries (Georgis *et al.*, 1991), billbugs, *Sphenophorus* spp. in turf (Smith, 1994), and fungus gnats (Diptera : Sciaridae) in mushrooms and greenhouse plants

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(Gouge and Hague, 1995; Grewal and Georgis, 1998). But for every success there have been numerous failures. In many cases, success was not achieved despite the pests having shown promising susceptibility in laboratory or field trials, e.g. Colorado potato beetle, *Leptinotarsa decemlineata* in vegetables and corn (Wright *et al.*, 1987), the corn earworm, *Helicoverpa zea* in corn (Cabanillas and Raulston, 1996) and cockroaches in urban and industrial environments (Appel *et al.*, 1993). Entomopathogenic nematodes are pathogenic to over 200 insect hosts (Poinar, 1979; Klein, 1990), yet nematodes have only been successfully marketed for a small fraction of these insects. The objective of this chapter is to examine the factors that influence the success or failure of biocontrol programmes with entomopathogenic nematodes. Case studies of insects in three different cropping systems serve as a basis for our analysis. The systems were chosen to illustrate an example in which nematodes failed (cotton), have been somewhat successful (turf), and have succeeded (citrus).

16.2. Cotton

Cotton is the most important natural textile fibre in the world. The cotton plant is a perennial species but it is cultivated as an annual to reduce pest populations from year to year. Insect pests can cause significant reductions in cotton yield and quality (Schwartz, 1983). Economic thresholds depend upon a variety of factors including the cotton growth stage damaged and the predicted value of cotton at harvest. Several cotton pests are susceptible to entomopathogenic nematodes including bollworm, *H. zea*, fall armyworm, *Spodoptera frugiperda*, beet armyworm, *Spodoptera exigua*, cabbage looper, *Trichoplusia ni*, tobacco budworm, *Heliothis virescens*, and the pink bollworm, *Pectinophora gossypiella* (Raulston *et al.*, 1992; Henneberry *et al.*, 1995a; Gouge *et al.*, 1999). This section will focus on pink bollworm because most research on control of cotton pests with entomopathogenic nematodes has been conducted on this organism.

Pink bollworm is an introduced pest, first appearing in the US in 1917 in infested seed brought from Mexico (Spears, 1968). Today pink bollworm is a key pest of cotton grown in the western states, and a constant threat to the cotton regions of California's San Joaquin Valley. Currently pink bollworm is established in Arizona, California, New Mexico, parts of Oklahoma, Florida and Western Texas, but also occurs in Arkansas, Louisiana and Missouri.

Pink bollworm overwinter as diapausing larvae, then pupate and emerge in spring and early summer (Bariola and Henneberry, 1980). Feeding by larvae reduces lint and seed production, and reduces lint quality. Boll infestation also exacerbates problems caused by plant pathogens such as *Aspergillus flavus*, by offering organisms an avenue of infection. From 1966 to 1980, annual losses in California's Imperial Valley averaged 26% of the crop value; as a result cotton hectarage has declined significantly in this area (Naranjo *et al.*, 1995). Current control consists of chemical sprays targeting adults during the main boll developmental period and using transgenic varieties with insecticidal properties.

16.2.1. Development of nematodes for pink bollworm management

In the early 1990s, pink bollworm larvae were found to be highly susceptible to *Steinernema carpocapsae* and *S. riobrave* (Lindegren *et al.*, 1992, 1993a, 1994). Particular interest was generated in *S. riobrave* because it was isolated from a lepidopteran host in

an arid region of the Lower Rio Grande Valley in Texas (Cabanillas *et al.*, 1994). *Steinernema riobrave* has a better host (*Galleria mellonella*) searching efficiency compared with *S. carpocapsae* (Lindegren *et al.*, 1993a) and is more tolerant to the high temperatures (Henneberry *et al.*, 1996a) prevalent in western USA cotton agroecosystems. Small plot trials indicated potential for entomopathogenic nematodes to be a useful tool in pink bollworm management (Lindegren *et al.*, 1993b; Henneberry *et al.*, 1995a,b, 1996b). For example, treatment of Arizona fields showed that *S. riobrave* persisted in large numbers for 19 days and were recoverable up to 75 days following treatment (Gouge *et al.*, 1996). The number of infested bolls was reduced and yield increased by 19% compared with untreated fields. Similar results were obtained with *S. riobrave* and *S. carpocapsae* in Texas cotton fields (Gouge *et al.*, 1997). *S. carpocapsae* and *S. riobrave* are the only species tested under field conditions.

16.2.2. Factors affecting efficacy

16.2.2.1. Phenology

The field efficiency of biological control agents depends upon coordinating application of the agent with the susceptible insect stages. Laboratory and field studies have indicated two stages of the pink bollworm lifestage that are susceptible to entomopathogenic nematodes in soil: pre-pupae and diapausing larvae (Henneberry *et al.*, 1995a; Gouge *et al.*, 1997). Pupae are not susceptible to infection by steinernematid nematodes (Henneberry *et al.*, 1995a).

16.2.2.2. Environmental factors

Irrigation during nematode application and continued moderate soil moisture are essential for nematode movement, persistence and virulence (Georgis and Gaugler, 1991). Henneberry *et al.* (1996b) noted increased pink bollworm mortality in plots treated with *S. riobrave* after successive field irrigations. As nematode applications require irrigation, only areas growing cotton under irrigated conditions could hope to use the nematodes effectively (T.J. Henneberry, Arizona, 2000, personal communication). Except for a small hectarage in eastern New Mexico, all western cotton is irrigated. However, across the USA only 37% of cotton is irrigated, which would be of consequence if pink bollworm establishes in central and eastern areas.

Temperature limits the virulence of steinernematids by its influence on nematode activity, bacterial symbiont, or both (Kaya 1990; Grewal *et al.*, 1994). Subterranean temperatures in cotton fields rarely rise higher than 28°C, but surface temperatures may exceed 50°C. *Steinernema riobrave* infects pink bollworm at temperatures up to 36°C with maximum infection occurring at 28.5°C, whereas *S. carpocapsae* and *H. bacteriophora* have optimum infection at 25°C (Gouge *et al.*, 1999). Other nematode species are capable of infecting insects at high temperatures, including *S. glaseri, S. anomoli* (Grewal *et al.*, 1994), and *H. indica* (Shapiro and McCoy, 2000a), but have not been assessed as pink bollworm control agents.

16.2.2.3. Application methods and timing

Entomopathogenic nematodes have been applied to control pink bollworm through irrigation systems (Lindegren *et al.*, 1992; Forlow Jech and Henneberry, 1997), sub-surface application with a shanking or disc system (K.A. Smith, Arizona, 2000,

personal communication), and diverse spray equipment including aerial application (Gouge *et al.*, 1997). The methods have not been compared experimentally, but application directly on to irrigated soil or application via irrigation water would logically cause less nematode mortality due to desiccation and ultraviolet radiation compared with other methods.

Application of nematodes during pre-plant irrigation to control diapausing larvae is undoubtedly the most convenient strategy, utilizing the advantages of cooler soil temperature and uniform distribution to soil within fields unimpeded by plants. However, pink bollworm moths emerging from diapause are highly mobile (Flint and Merkle, 1981) and unless this strategy is adopted on an area-wide basis, protection of localized areas will be short-lived. After cotton plants have become established and flowering begins, movement of the moths becomes increasingly restricted (Flint and Merkle, 1981) and applications of nematodes to smaller areas is more beneficial.

Greatest pink bollworm mortality has been achieved using application rates of 2.5×10^9 infective juveniles per ha during early season applications (92.5 and 100% for *S. carpocapsae* and *S. riobrave* respectively, Henneberry *et al.*, 1996b), and 3.25×10^9 infective juveniles ha^{-1} during mid-season (100% furrow base mortality, Gouge *et al.*, 1996). The efficiency of multiple applications, with standard or reduced rates, has yet to be explored.

16.2.3. Current status and analysis

Although entomopathogenic nematodes are highly efficacious in controlling pink bollworm, they are not a viable management strategy because they cannot compete against the current management tactics. Chemical pesticides have been relied upon heavily for pink bollworm management, but current trends make transgenic cotton the most popular option. Toxin genes from *Bacillus thuringiensis* (BT) bacteria have been incorporated directly into the cotton plant creating transgenic varieties with insecticidal properties. In 2000, approximately 70% of cotton grown in Arizona was BT-cotton. BT-cotton is far more economical than nematodes, and unlike nematodes, will provide control of several major cotton pests simultaneously, including pink bollworm, bollworm and tobacco budworm. Cotton growers are charged an US$87 fee per ha for transgenic cotton-seed, whereas nematode costs, using a single application rate of 2.5×10^9 ha^{-1}, are US$312.50 ha^{-1} for *S. riobrave*. Furthermore, the ecological advantage of entomopathogenic nematodes relative to chemical insecticides is essentially irrelevant since BT-cotton also appears to have minimal impact on non-targets and the environment. Finally, another advantage BT-cotton and chemical insecticides have over nematodes is ease of use. Small wonder that entomopathogenic nematodes are not used in cotton.

The future for entomopathogenic nematodes in pink bollworm management does not look bright. Transgenic cotton varieties have had a profound impact on the way pests are managed throughout the cotton belt. The major concern of BT-cotton has been widespread development of insect resistance to the BT toxins, but this has so far proven to be minimal, and new transgenic cotton varieties are under development which use gene stacking technology, i.e. multiple genes coding for different modes of action. The future of transgenic cotton appears stable for some time to come. Thus, pink bollworm appears to be a prime example of an insect that can be managed using

entomopathogenic nematodes but for practical reasons nematodes will remain unused in conventional cotton.

The only niche where entomopathogenic nematodes could play a role in cotton pest management is in the organic, coloured cotton market. Coloured, organic cotton values are approximately two to three times that of traditionally grown cotton making use of nematodes more feasible. However, interest in growing organic coloured cotton fell in the mid 1990s, and unless this trend is reversed, it is likely the potential for using nematodes in cotton will disappear completely.

16.3. Turf

Turfgrass comprises a variety of grass species grown as a permanent or semi-permanent managed ground cover under a range of management systems (e.g. lawns, parks, cemeteries, sod farms, golf courses, athletic fields). Between systems there are large variations in value, input, demands, damage thresholds and, consequently, tolerances for pests. Damage thresholds are generally low; therefore numerous insects are considered pests (Potter, 1998; Vittum *et al.*, 1999). Among the important insect pests several are amenable to control by entomopathogenic nematodes. This section will concentrate on those pest species that have received the most attention as targets for nematodes, i.e. white grubs (Coleoptera: Scarabaeidae), mole crickets (*Scapteriscus* spp.), billbugs (*Sphenophorus* spp.) and the black cutworm (*Agrotis ipsilon*). Other pests that have been controlled with nematodes include annual bluegrass weevil (*Listronotus maculicollis*), cutworms, armyworms, sod webworms and European crane fly (*Tipula paludosa*).

16.3.1. White grubs

White grubs, the root-feeding larvae of scarab beetles, are serious pests of turfgrass throughout the world. Most important species have an annual life cycle with adults emerging in summer. The eggs are laid in the soil below the turf. By late summer most larvae have developed into the third and final instar. After overwintering the larvae resume feeding in spring until pupation in early summer. The extensive feeding activity of the larger larvae can kill large areas of grass especially under warm dry conditions. In addition, vertebrate predators can tear up the turf to feed on the grubs even at relatively low larval densities.

16.3.1.1. Development of entomopathogenic nematodes for control of white grubs

No other turfgrass pest has been studied as extensively as a target for nematodes as white grubs (Klein, 1990, 1993). As soil insects, white grubs are predisposed to nematode attack and many nematode species and strains have been isolated from white grubs (Peters, 1996). However, as a result of their coevolution with soil pathogens, white grubs have developed various defensive mechanisms that have conferred to them varying degrees of nematode resistance among grub species.

The isolation of *S. glaseri* from Japanese beetle, *Popillia japonica*, larvae in New Jersey resulted in the first effort to use entomopathogenic nematodes for pest control (Glaser, 1932; Fleming, 1968). Even though a large-scale colonization programme failed due to lack of awareness of the nematode's symbiotic bacteria (Gaugler *et al.*, 1992), the effort built the base for further development of entomopathogenic nematodes as biological control agents.

Renewed efforts to develop nematodes for white grub control were triggered by the commercialization of entomopathogenic nematodes in the early 1980s. Generally *Heterorhabditis* spp. and *S. glaseri* were found to be more effective than *S. feltiae* and *S. carpocapsae* (Klein, 1990, 1993). Most field tests in the USA concentrated on *S. carpocapsae* and *H. bacteriophora* because these species were readily available. An analysis of 82 field trials conducted against *P. japonica* between 1984 and 1988 (Georgis and Gaugler, 1991) concluded that *H. bacteriophora* strains used under the right conditions were as effective as standard insecticides, whereas the most widely used species, *S. carpocapsae*, was ill-adapted for white grub control. The 1990s were characterized by more in-depth studies of factors affecting nematode efficacy against white grubs (see below), and advances in production technology. The development of liquid culture for *Heterorhabditis* spp. (see Chapter 14) increased production efficiency making the use of nematodes for white grub control more feasible.

16.3.1.2. Factors affecting efficacy

Nematode efficacy against white grubs is affected by various interacting biotic and abiotic factors. The thickness of thatch, an accumulation of organic matter between the soil and turfgrass foliage, is negatively related to nematode efficacy because thatch restricts nematode downward movement (Georgis and Gaugler, 1991; Zimmerman and Cranshaw, 1991). Nematodes, especially *H. bacteriophora*, become increasingly ineffective for white grub control as soil temperatures drop below 20°C (Georgis and Gaugler, 1991). Irrigation volume and frequency and soil moisture are positively related to efficacy (Shetlar *et al.*, 1988; Georgis and Gaugler, 1991). Nematodes are more effective in fine-textured soils because finer soils retain moisture better and restrict nematode movement to the upper soil layers where most of the white grubs can be found (Georgis and Gaugler, 1991).

As a result of their co-evolution with soil pathogens, white grubs have developed defence mechanisms including infrequent carbon dioxide output, sieve-plates over their spiracles, frequent defecation, defensive and evasive behaviours, a dense peritrophic membrane, and a strong immune response. To optimize nematode efficacy, it is crucial to identify nematode species that have adapted to white grub ecology and biology. Generally, highly mobile cruiser-type nematodes, e.g. *Heterorhabditis* spp. or *S. glaseri* are better adapted to infecting sessile subterranean insects such as white grubs than ambushers like *S. carpocapsae* (Gaugler *et al.*, 1997a; see Chapter 10). These differences in search strategy explain the superiority of *H. bacteriophora* and *S. glaseri* over *S. carpocapsae* in Japanese beetle control (Georgis and Gaugler, 1991; Georgis and Poinar, 1994). Differences have also been found in the interactions between the nematode–bacterium complex and the white grubs' immune system (Cui *et al.*, 1993; Wang *et al.*, 1995; see Chapter 4). While all nematode species elicit a strong immune response in *P. japonica* larvae, most *H. bacteriophora* are killed by melanotic encapsulation, but *S. glaseri* escape encapsulation.

Although direct comparisons are rare, it is apparent that nematode efficacy against white grubs varies with scarab and nematode species. For example, the Oriental beetle, *Exomala orientalis*, is less susceptible to *H. bacteriophora* than *P. japonica*, whereas the European chafer, *Rhizotrogus majalis*, or the Asiatic garden beetle, *Maladera castanea* are resistant to this nematode (A.M. Koppenhöfer, unpublished data). A recently isolated *Heterorhabditis* spp., however, was highly effective against the Asiatic garden

beetle but less effective against Oriental beetle and European chafer (R. Cowles, Connecticut Agricultural Experiment Station, 2000, personal communication). Larval stage also affects nematode susceptibility but the trend varies with scarab species (Deseö *et al.*, 1990; Fujiie *et al.*, 1993; Smits *et al.*, 1994). Differences in the expression of defensive mechanisms may contribute to differences in nematode susceptibility. Defensive and evasive behaviours are much stronger in *P. japonica* than in masked chafer, *Cyclocephala hirta* (Koppenhöfer *et al.*, 2000), yet the latter are less susceptible to nematodes suggesting differences in other defence mechanisms.

16.3.1.3. Current status and analysis

Despite considerable efforts in research and development, nematode use against white grubs is limited. The diversity of white grub pests, and their varying degree of susceptibility to different nematode species, has added to the difficulty. However, there has been some success. Two companies in Japan have recently started to market *S. glaseri* (SDS Biotech K.K.) and *S. kushidai* for white grub control on golf courses in Japan and a third company in Germany is marketing *H. bacteriophora* (E-Nema Gmbh) for the same market in Germany. In the USA, several small companies produce *Heterorhabditis* spp. for use against white grubs but the extremely high price (in the range of US$1000 ha^{-1} or more) of these *in vivo* produced nematodes restricts their use to small area application such as in a homeowner setting.

Low economic thresholds and competition from chemical insecticides have played major roles in hindering wider nematode success. The control efficacy obtained under the right conditions against the Japanese beetle alone should have led to a quicker and more extensive acceptance of nematodes. But at a cost of upwards from US$500 ha^{-1}, *in vitro* products containing *Heterorhabditis* spp. are more than four times as expensive as similarly effective organophosphate and carbamate insecticides. The phasing out of these ‘harder’ chemicals by regulatory agencies could have been a boon for nematode products, but the arrival of a new generation of ‘low-impact’ insecticides continues to impede commercialization of nematodes for white grub control. The cost of these new chemicals (e.g. imidacloprid and halofenozide) is around US$250 ha^{-1}. Because their efficacy declines with advancing grub development, these chemicals must be applied on a preventative basis before white grub outbreaks can be identified, and thus involve the treatment of large turf areas that may need only partial or no treatment. Even though the preventative use makes these chemicals ultimately more expensive than the curative use of nematodes, they are extremely effective (close to 100% control) and relatively safe and therefore an attractive management option, especially where cost is not a major issue (e.g. many golf courses). Consequently, nematodes are presently used for white grub control only in countries where chemical alternatives are restricted (e.g. Germany, Japan) or in small commercial segments such as the homeowner market.

Despite potential for improving nematode utility in the future (e.g. through reduced production costs, more pathogenic nematode species and strains, and better understanding of white grub–nematode interactions), the success of nematodes as biopesticides for white grubs is likely to remain limited by competition from chemical insecticides. A more promising future for nematodes in white grub management may lie in developing alternative approaches to their use as biopesticides. For example, conservation and, even better, manipulation of the widespread

natural nematode populations in turfgrass could be used to buffer white grub outbreaks.

16.3.2. Mole crickets

Mole crickets were introduced into Florida from South America around 1900 and have since become the most important turfgrass pest in the southeastern USA. Adults and nymphs cause damage by feeding on grass roots and shoots and through their extensive tunnelling activity. After egg-laying in spring the adults die off. During summer the crickets are in the nymphal stages until adults appear in late summer. Overwintering occurs primarily in the adult (tawny mole cricket, *S. vicinus*) or nymphal stage (southern mole cricket, *S. borellii*).

16.3.2.1. Development of entomopathogenic nematodes for control of mole crickets

Initial efforts showed that *S. carpocapsae* could provide some control of mole crickets in the field (average of 58% at 2.5×10^9 nematodes ha^{-1}) (Georgis and Poinar, 1994). However, exploration for natural enemies in South America led to the isolation of a new nematode species, *Steinernema scapterisci*, from *Scapteriscus* mole crickets in Uruguay and Argentina. The ensuing introduction of *S. scapterisci* into the USA for mole cricket control (Parkman and Smart, 1996) is the first successful use of an entomopathogenic nematode in classical biological control. After laboratory studies indicated excellent control potential and nontarget safety of *S. scapterisci*, the nematode was released on 1-ha plots in pastures in 1985 (Parkman *et al.*, 1993a) and on golf courses in 1989 (Parkman *et al.*, 1994). The nematode established successfully and mean cricket trap catches had declined by 98% within 3 years. The nematode was slowly spread from release sites by infected mole crickets (Parkman *et al.*, 1993b). Establishment was also achieved by applying *S. scapterisci*-infested cadavers and using electronic mating callers to attract mole crickets to the site of application (Parkman *et al.*, 1993a). These experiments demonstrated *S. scapterisci*'s ability to establish itself permanently or act as an inoculative agent, rather than an inundative one.

When *S. scapterisci* became commercially available, in 1993, its potential as an inoculative agent was enhanced. *S. scapterisci* provided the same control levels as standard insecticides (75% at 2.5×10^9 nematodes ha^{-1}) (Georgis and Poinar, 1994). The widespread use of *S. scapterisci* on golf courses, other turf areas, and pastures greatly accelerated the spread of *S. scapterisci*.

16.3.2.2. Factors affecting efficacy

Laboratory and field studies showed that the efficacy of *S. scapterisci* was affected by mole cricket species and developmental stage (Hudson and Nguyen, 1989a,b; Nguyen and Smart, 1991; Parkman and Frank, 1992). The short-winged mole cricket, *S. abbreviatus* was less susceptible than *S. vicinus* and *S. borellii* in laboratory studies. In addition, *S. borellii* was more susceptible than *S. vicinus* in field studies, probably because the greater activity arising out of its predatory behaviour increases it chances of contact with the ambusher *S. scapterisci*. Nymphal mole crickets were substantially less susceptible to infection than adults, and small nymphs were not affected by *S. scapterisci*. In addition to *S. scapterisci, S. riobrave* has been marketed for mole cricket control in turf (Grewal and Georgis, 1998). *S. riobrave* has shown the same efficacy for

mole cricket control and is also ineffective against mole crickets nymphs, but it does not recycle in infected mole crickets (K. Smith, University of Arizona, 2000, personal communication).

16.3.2.3. Current status and analysis

Perhaps the most significant aspect of the *S. scapterisci*–mole cricket system is the nematode's ability to recycle. It is likely that the nematode will become established throughout most of the mole crickets' area of distribution in the USA (Frank and Parkman, 1999). *S. scapterisci* is an ideal control agent for pastures and turfgrass areas that can tolerate some mole cricket damage. Because of the nematode's slow spread from inoculation sites, widespread use or an inoculation programme are necessary to accelerate its spread. This, however, has been hampered by the nematode's limited availability. There have been several periods when *S. scapterisci* has not been commercially available. Economics played a major role in the nematode's rocky development. At a cost of about US\$240 ha^{-1}, *S. scapterisci* was far more expensive than insecticides commonly used in pastures but comparable to some of the new insecticides used on turf. More importantly, the limited effect of *S. scapterisci* on mole cricket nymphs requires its application in spring or fall when adults are present, while control measures are typically necessary in summer against nymphs. In addition, the performance of *S. scapterisci* was variable in southeastern Florida where the less nematode-susceptible *S. abbreviatus* is abundant. Finally, lack of aggressive promotion and limited supplies contributed to poor acceptance in the market-place. In the last several years the role of nematodes in mole cricket control has dwindled to nothing (neither *S. riobrave* nor *S. scapterisci* are available for mole cricket control at this time). However, sparks of interest have been rekindled. Recently MicroBio, a company with a proven ability to mass produce entomopathogenic nematodes in liquid culture, obtained an exclusive license to produce and sell *S. scapterisci* (G. Gowling, MicroBio Cambridge, UK, 2000, personal communication). Time will tell if this company (or another) will enable nematodes to play a more prominent role in mole cricket control.

16.3.3. Billbugs

Billbugs, *Sphenophorus* spp., are important turfgrass pests throughout much of the USA and Japan. The younger larvae feed inside the stem and crown and older larvae feed externally on the below-ground parts of the plant. Seasonal life cycles vary depending on species and latitude. No detailed studies on billbug–nematode interaction have been published. Field tests in Ohio indicated that the bluegrass billbug, *Sphenophorus parvulus* can be controlled with *S. carpocapsae* (average 78%) or *H. bacteriophora* (average 74%) (Georgis and Poinar, 1994; Smith, 1994). In Japan, *S. carpocapsae* has been more effective for control of the hunting billbug, *S. venatus vestitus* than standard insecticides (average 84% versus 69% control), (Smith, 1994; Kinoshita and Yamanaka, 1998). Use of nematode products containing *S. carpocapsae* and *H. bacteriophora* against billbugs is limited in the USA, whereas *S. carpocapsae* is the primary means of billbug control on golf courses in Japan. The main reason for this difference is the availability of effective insecticides for billbug control in the USA and lack thereof in Japan. In addition, favourable environmental conditions (temperature and rainfall) and the adoption of 'nematode-friendly' application protocols, i.e. immediate watering after

spraying and generally very careful following of label instructions may have optimized nematode efficacy in Japan (K. Smith, University of Arizona, 2000, personal communication).

16.3.4. Black cutworm

The black cutworm is a perennial problem on the close-cut bentgrass of golf course greens and tees throughout the world. The larvae dig a burrow in the thatch or soil and emerge at night to chew down the grass blades and stems around the burrow. There are multiple generations per year. Georgis and Poinar (1994) reported that *H. bacteriophora* has not provided satisfactory control (average 62%), whereas *S. carpocapsae* is highly effective for black cutworm control (average 95%). Despite this high efficacy, nematodes are not widely used for black cutworm control. Economics does not play a significant role on these high-profile turf areas. Rather, damage thresholds on golf course tees and especially greens are so low that golf course superintendents will prefer to use chemical insecticides that provide even better and more consistent cutworm control than nematodes. This will continue until the attitude of their clientele changes.

16.4. Citrus

Citrus is a long-lived perennial evergreen that is grown in an orchard cropping system. Insect pests can cause substantial reductions in citrus yields and fruit quality (Browning, 1999). Economic thresholds depend on whether the pest causes direct or indirect damage, how severe the damage is, and whether the fruit is intended for fresh marketing or processing (into juice or other citrus products). A number of citrus pests have been tested for susceptibility to entomopathogenic nematodes including the citrus leafminer, *Phyllocnistis citrella* (Beattie *et al.*, 1995), the Mediterranean fruit fly, *Ceratitis capitata* (Lindegren *et al.*, 1990), the Caribbean fruit fly, *Anastrepha suspensa* (Beavers and Calkins, 1984) and the fuller rose beetle, *Asynonychus godmani* (Morse and Lindegren, 1996). By far, the greatest amount of research on entomopathogenic nematode control on citrus pests has been towards suppression of the weevils that threaten citrus root systems in Florida and the Caribbean (Duncan *et al.*, 1999; McCoy *et al.*, 2000a), primarily *Pachnaeus* spp. and *Diaprepes abbreviatus*. The remainder of this section and analysis will focus on this citrus root weevil complex emphasizing *D. abbreviatus*, which is the most serious insect pest of citrus in Florida (Duncan *et al.*, 1999).

Diaprepes abbreviatus was first reported in Florida in 1964 (Woodruff, 1964) and now infests more than 15% of Florida citrus (Duncan *et al.*, 1999). The life cycle is reviewed by McCoy (1999). Adult weevils emerge from soil throughout the year with a significant peak in the spring (March–June) and occasionally another peak between October and December (Stansly *et al.*, 1997). These adults feed and oviposit on foliage, and first instars drop to the ground where they burrow into soil and feed on roots. Economic damage is caused by larval feeding and is often exacerbated by the fungal disease *Phytophthora* spp., which can enter the roots at points of larval feeding (Duncan *et al.*, 1999). Although chemical insecticides can be applied (e.g. carbaryl) as an adulticide, or as a barrier treatment for neonates, the only recommended control for *D. abbreviatus* larvae that have established themselves in the orchard is application of entomopathogenic nematodes (Bullock *et al.*, 1999a).

16.4.1. Development of entomopathogenic nematodes for control of *D. abbreviatus*

Steinernema carpocapsae was the first nematode shown to be pathogenic to *D. abbreviatus* (Laumond *et al.*, 1979) and to be developed commercially for citrus root weevil control (Smith, 1994). Laboratory, greenhouse studies and field studies demonstrated some potential for *S. carpocapsae* to control *D. abbreviatus* and indicated higher virulence relative to *S. glaseri* and *S. feltiae* (Schroeder, 1987; Figueroa and Roman, 1990; Table 16.1). Reported mortality levels of *D. abbreviatus* in field studies, however, were not high (i.e. not > 70%), varied greatly, and often involved excessively high rates of application (Table 16.1).

The commercialization of entomopathogenic nematodes for citrus root weevil control changed course when a nematode that had been recently discovered, *S. riobrave*, was tested for efficacy towards *D. abbreviatus. Steinernema riobrave* caused greater *D. abbreviatus* mortality than *S. carpocapsae* in laboratory, greenhouse and field tests (Schroeder, 1994; Duncan *et al.*, 1996; Bullock *et al.*, 1999b). Several studies reported ≥ 90% suppression of *D. abbreviatus* in field studies with *S. riobrave* (Duncan and McCoy, 1996; Duncan *et al.*, 1996; Bullock *et al.*, 1999b). The encouraging research led to the commercial development of *S. riobrave* for control of *D. abbreviatus* replacing *S. carpocapsae* as the nematode of choice (Grewal and Georgis, 1998). In 1999, approximately 19,000 ha of citrus were treated with *S. riobrave* to control citrus root weevils (M. Dimock, Thermo Trilogy Corporation, Columbia, Maryland, 2000, personal communication). In addition to *S. riobrave*, *H. bacteriophora* and *H. indica* have been commercialized for use against *D. abbreviatus*, based on efficacy reported in laboratory or field studies (Shapiro *et al.*, 1999; Table 16.1).

Table 16.1. Field efficacy of *Steinernema* and *Heterorhabditis* nematodes against *Diaprepes* root weevil.

Nematode	Application rate (cm^{-2})	Percentage mortality	Reference
H. bacteriophora	127	78	Suggars Downing *et al.*, 1991
H. bacteriophora	255	63	Suggars Downing *et al.*, 1991
H. bacteriophora	637	63	Suggars Downing *et al.*, 1991
H. bacteriophora	100	62	Schroeder, 1992
H. bacteriophora	250	ns[a]	Duncan and McCoy, 1996
H. bacteriophora	175	54	Duncan *et al.*, 1996
H. bacteriophora	255	57	Duncan *et al.*, 1996
S. carpocapsae	250	65	Schroeder, 1987
S. carpocapsae	25	42	Schroeder, 1990
S. carpocapsae	100	50	Schroeder, 1992
S. carpocapsae	637	48	Suggars Downing *et al.*, 1991
S. carpocapsae	153	ns	Duncan *et al.*, 1996
S. carpocapsae	306	ns	Duncan *et al.*, 1996
S. glaseri	250	35	Schroeder, 1987
S. riobrave	250	77–90	Duncan and McCoy, 1996
S. riobrave	120	93	Duncan *et al.*, 1996

[a] ns, no suppression, i.e., mortality was not significantly different from the untreated control.

16.4.2. Factors affecting efficacy

Although entomopathogenic nematodes have been successfully commercialized to control *D. abbreviatus* in Florida citrus, levels of suppression have varied greatly (Table 16.1). The remainder of this section will focus on factors influencing the success (or failure) of entomopathogenic nematode applications for citrus root weevil control.

When applying entomopathogenic nematodes to control any insect pest, the choice of nematode species is perhaps the most critical aspect to achieving an efficacious application. (Georgis and Gaugler, 1991; Gaugler, 1999). Control of *D. abbreviatus* with nematodes may be considered a case in point. If the superiority of *S. riobrave* to *S. carpocapsae* had not been discovered it is likely that the market for nematodes in Florida citrus would have eventually failed. Thus far, *S. riobrave* has proven to be the most effective nematode for *D. abbreviatus* control. In laboratory studies, *S. riobrave* was found to be more virulent to seventh or eighth instar *D. abbreviatus* than eight other nematode species (*H. bacteriophora, H. indica, H. marelatus, H. megidis, H. zealandica, S. carpocapsae, S. feltiae, S. glaseri*) and 17 strains (Shapiro and McCoy, 2000a). In another laboratory study, however, *H. indica* was found to be more virulent than *S. riobrave* against younger instar *D. abbreviatus*, i.e. fourth–fifth instar (Shapiro *et al.*, 1999). In a greenhouse study, using potted citrus, *S. riobrave* caused higher *D. abbreviatus* mortality than *H. bacteriophora* (against seventh and 11th instars) and *H. indica* (against seventh instars) (Shapiro and McCoy, 2000b). In field studies, *S. riobrave* is the only nematode reported to have caused 90% or more mortality of *D. abbreviatus* (Bullock *et al.*, 1999b; Table 16.1).

In addition to virulence, nematode persistence could be an important factor in determining which nematode to apply (Shields *et al.*, 1999). Stability and favourable soil conditions (moisture, aeration, texture) make Florida citrus groves amenable to entomopathogenic nematode recycling and persistence (see Kaya, 1990). Indeed, endemic nematode populations exist (Beavers *et al.*, 1983) and can provide significant *D. abbreviatus* suppression (e.g. 7–42%) (McCoy *et al.*, 2000b). However, inundative applications of nematodes (i.e. *H. bacteriophora, H. indica, S. carpocapsae* and *S. riobrave*) to citrus have resulted in poor persistence, reaching pre-treatment levels within 2 weeks post-application (Duncan *et al.*, 1996; McCoy *et al.*, 2000b). Discovery of other species that are both virulent and persistent would be beneficial.

Formulation and culture method (*in vivo* versus *in vitro*) may also affect entomopathogenic nematode efficacy (Gaugler and Georgis, 1991; Baur *et al.*, 1997). With regard to control of *D. abbreviatus* with *S. riobrave*, however, no significant effects of culture method or formulation (liquid versus granular) were detected (Duncan and McCoy, 1996; Shapiro and McCoy, 2000c). In contrast to virulence, the viability of granular formulated *S. riobrave* has been reported to be variable (McCoy *et al.*, 2000b; Shapiro and McCoy, 2000c). Variations in viability may not be detrimental to efficacy because the manufacturer of the granular *S. riobrave* (Thermo Trilogy Corporation) packs an excess of nematodes to each unit to ensure that at least the labelled amount of viable nematodes are available during the shelf-life of the product (M. Dimock, Thermo Trilogy Corporation, Columbia, Maryland, 2000, personal communication). Additionally, low viability does not appear to affect the virulence of nematodes that remain alive during the product's shelf life (Shapiro and McCoy, 2000c). Nonetheless, variation in viability can be a considerable hindrance to grower acceptance of the

product; low nematode viability in several batches of *S. riobrave* (detected by the producer, distributors, or researchers) has resulted in product recalls and subsequent lowering of consumer confidence. *In vivo* produced *Heterorhabditis* spp. formulated in sponge or as paste and *in vitro S. riobrave* in liquid formulation tend to have high viabilities (e.g. > 90% and > 75%, respectively; McCoy *et al.*, 2000b).

Mortality of *D. abbreviatus* is positively related to rate of nematode application (McCoy *et al.*, 2000b). In general, a minimum of 25 infective juveniles per cm^2 is required to achieve adequate pest suppression (Georgis and Hague, 1991; Gaugler *et al.*, 2000). In citrus, however, high levels of *D. abbreviatus* control (i.e. > 85%) have only been achieved with application rates exceeding 100 infective juveniles per cm^2 (Table 16.1). Industry recommended application rates for *D. abbreviatus* control are substantially lower, i.e. approximately ten infective juveniles per cm^2 for *Heterorhabditis indica* (Grubstake™, Integrated BioControl Systems Incorporated, Lawrenceburg, IN) and 22 per cm^2 for *S. riobrave* (Biovector®, 355 Thermo Trilogy Corporation, Columbia, Maryland).

16.4.3. Current status and analysis

Factors that have contributed to the commercialization of entomopathogenic nematodes in citrus for control of *D. abbreviatus* and other root weevils are biological, ecological, and economical in nature. From a biological standpoint, successful commercialization has been achieved because a proper match between the nematode and target-pest was made. From an ecological standpoint, Florida citrus groves contain many benefits for applying entomopathogenic nematodes. The soils in these groves have a high sand content (Shapiro *et al.*, 2000) facilitating nematode mobility (Georgis and Poinar, 1983; Barbercheck and Kaya, 1991) and oxygen availability for nematode survival (Kung *et al.*, 1990). Because *D. abbreviatus* occurs primarily under the tree canopy, nematodes only need to be applied within the drip line (Duncan *et al.*, 1999; McCoy *et al.*, 2000a) where shade protects them from harmful ultraviolet radiation (Gaugler and Boush, 1978). Additionally, most Florida citrus groves are irrigated, thus moisture, which is necessary for nematode survival, (Kaya, 1990), is provided.

The importance of economic factors leading to successful commercialization of nematodes for *D. abbreviatus* control cannot be underestimated. To begin with, *D. abbreviatus* is a key pest, and thus grower demand for control is high. Furthermore, in management of *D. abbreviatus* larvae, nematodes face little or no competition from other control agents (Bullock *et al.*, 1999a; McCoy, 1999). Inexpensive chemical insecticides (e.g. chlorinated hydrocarbons) have been eliminated due to regulatory pressures and, thus far, the newer chemicals that have been tested for *D. abbreviatus* larval control have not been able to compete with nematodes in price or efficacy. Indeed, perhaps the most important success-contributing factor is nematode cost. Because *D. abbreviatus* only occur under the canopy there is no need to apply nematodes between rows or between trees. Therefore the number of nematodes (and the cost) required to treat 1 ha of citrus may be three to ten times lower than crops that require broadcast applications to the entire soil surface (e.g. cotton and turf). The low cost of nematode application (e.g. as low as US$62 ha^{-1}) combined with the high value of citrus (US$6000 gross ha^{-1} for oranges, Muraro *et al.*, 1999), adds greatly to the success of entomopathogenic nematodes in Florida citrus.

Entomopathogenic nematodes are likely to continue to be an integral part of pest management in Florida citrus. The role of nematodes in controlling *D. abbreviatus* will be enhanced by continued research and improved quality control. Factors that affect efficacy such as those described previously must continue to be studied. Additionally, the role endemic entomopathogenic nematodes play in the citrus ecosystem has only begun to be examined. How non-indigenous nematodes that are applied interact with the endemic nematode populations, as well as with other soil biota (e.g. ants, McCoy *et al.*, 2000b), must be explored. The influence of soil characteristics (Shapiro *et al.*, 2000) and timing and methods of nematode application must also be investigated further (Duncan *et al.*, 1999; McCoy, 1999; Shapiro *et al.*, 1999). Finally, perhaps the most important issue yet to be resolved will be to determine the minimum application rate necessary to ensure efficacy and protect trees from economic damage.

16.5. Conclusions

Based on the case studies and other literature we have examined (e.g. Klein, 1990; Georgis *et al.*, 1991; Grewal and Georgis, 1998; Table 16.2), we can make certain generalizations. We conclude that two basic elements are necessary for nematodes to be successful: a suitable nematode for the target pest and favourable economics. For example, nematodes associated with control of *D. abbreviatus, O. sulcatus*, and sciarid pests are all highly suited to their hosts, are applied in relatively high value commodities, and face little or no competition from other control measures. Where entomopathogenic nematodes have not succeeded, the causes are generally due to a poor match of nematode and host, or poor economic conditions. For example, *S. riobrave* proved to be highly efficacious toward pink bollworm, but in cotton, nematodes could not compete on an economic level with other control strategies. Other insects in row crops fall into the same category, e.g. *H. zea* (Cabanillas and Raulston, 1996), and *Diabrotica* spp. (Jackson, 1996) in maize. In other instances a suitable match of nematode to target pest could not be found, e.g. wireworms (Coleoptera: Elateridae) (Eidt and Thurston, 1995) and imported fire ants *Solenopsis invicta* (Drees *et al.*, 1992), in which case strategies to employ entomopathogenic nematodes are futile. Other factors that may affect the success of commercial ventures with entomopathogenic nematodes include efficacy of pest suppression relative to other available control tactics, and reliable provision of a nematode product of high quality.

Proper match of the nematode to the host entails virulence, host finding, and ecological factors. If a nematode does not possess a high level of virulence toward the target pest there is little hope of success. In rare cases persistence may compensate for moderate virulence (Shields *et al.*, 1999). Matching the appropriate nematode host-seeking strategy with the pest is also essential (Lewis *et al.*, 1992; Gaugler, 1999). Nematodes that have an ambush strategy are most suitable for controlling mobile insects near the soil surface (e.g. *S. carpocapsae*), whereas nematodes with more of a cruiser strategy (e.g. *H. bacteriophora*) are most suitable for suppressing less mobile insects below the soil surface (Lewis *et al.*, 1992). Ecological factors such as relative ability to withstand desiccation or temperature tolerance are also important in choosing the best-adapted nematode for a particular pest.

Historically, poor host suitability has been the most common cause of failure in entomopathogenic nematode applications (Gaugler, 1999). The list of pests that

Table 16.2. An analysis of host suitability for entomopathogenic nematodes against various insect pests.[a]

Pest	Nematode species	Host suitability[b] (% suppression)	Number of references in analysis
Black vine weevil (*Otiorhynchus sulcatus*)	*Heterorhabditis bacteriophora*	Good (71)	7
	Steinernema feltiae	Good (75)	3
	S. carpocapsae	Fair (58)	5
Colorado potato beetle (*Leptinotarsa decemlineata*)	*S. carpocapsae*	Fair (57)	3
Corn rootworms (*Diabrotica* spp.)	*S. carpocapsae*	Good (61)	7
Japanese beetle (*Popillia japonica*)	*H. bacteriophora*	Excellent (80)	7
	S. carpocapsae	Fair (47)	6
	S. glaseri	Good (63)	3
White grubs (*Phyllophaga* spp.)	*H. bacteriophora*	Good (72)	3
Chafers (*Cyclocephala* spp.)	*H. bacteriophora*	Fair (59)	3
Sciaridae (*Lycoriella* spp. and *Bradysia* spp.)	*S. feltiae*	Excellent (89)	5
Leafminer (*Liromyza trifolii*)	*S. carpocapsae*	Good (66)[c]	3
Black cutworm (*Agrotis ipsilon*)	*S. carpocapsae*	Excellent (86)	5
Diamondback moth (*Plutella xylostella*)	*S. carpocapsae*	Fair (56)	3
Corn earworm (*Helicoverpa zea*)	*S. riobrave*	Excellent (90)[d]	4
Borers (*Synanthedon* spp.)	*S. feltiae*	Excellent (86)	4
Spodoptera spp.	*S. carpocapsae*	Poor (27)[e]	3
Imported fire ant (*Solenopsis invicta*)	*S. carpocapsae*	Poor (25)	3

[a] Only pests (by genus or species) with at least three refereed publications on field efficacy for each nematode species were included, and only if the rate of nematode application was not excessive (i.e. not > 125 nematodes cm^{-2}).
[b] Host suitability ratings: Excellent, Good, Fair, and Poor are based on suppression levels of 80–100, 60–79, 40–59, and < 40%, respectively. The suppression levels were calculated by averaging results from field trials in the associated references.
[c] Indoor applications in high humidity.
[d] Soil applications.
[e] Above ground applications.

commercial nematode-producing companies advocate as targets has frequently been inflated (Gaugler *et al.*, 2000). Mortality caused under laboratory conditions has often been inappropriately extrapolated to field efficacy (Georgis *et al.*, 1991). Perhaps the

greatest example of exaggeration in host range has been with *S. carpocapsae* which, for many years, was practically seen as a 'cure all' (Gaugler, 1999).

The definitive test of host suitability is efficacy under field conditions. We suggest a minimum of three solid field trials to establish host suitability. An analysis of nematode host suitability (based on field efficacy) for some of the most extensively studied insect pests is illustrated in Table 16.2. The table only includes host–nematode combinations with at least three refereed publications on field efficacy. The best matches tend to be for nematodes that have high virulence toward hosts in a protected environment, e.g. Japanese beetle, fungus gnats, borers, etc. Nematode applications to environments exposed to ultraviolet radiation or desiccation, e.g. *Spodoptera* spp., are prone to failure. Behavioural aspects of the target host may also be a factor, e.g. fire ants' ability to relocate after nematode treatment (Drees *et al.*, 1992).

If economic factors are not favourable even a strategy involving the most suitable match of nematode to target pest is doomed to failure. Economic factors include the grower's perceived need to control the pest, the relative cost of nematodes compared with other management options, the value of the commodity (e.g. per ha), and the overall importance of the commodity in the agricultural market. Exemption of entomopathogenic nematodes from the Environmental Protection Agency pesticide registration has clearly benefited commercialization efforts. Niche markets tend to be amenable to entomopathogenic nematode use not only because the crop value is high, but also because the commodity occupies a small enough segment of the agricultural market for would-be competitors to shy away from registration costs and seek alternatives. On the other hand, major row crops, such as maize, cotton, soybeans and wheat are, and likely to always be, unreachable for entomopathogenic nematode marketing because the crop value is low, and the market segment is huge.

A number of measures can be taken to improve the success of entomopathogenic nematodes. In the past 10 years new opportunities in pest control have arisen due to the discovery of several new entomopathogenic nematode species, including *S. riobrave* (Cabanillas *et al.*, 1994) against the *Diaprepes* root weevil (Table 16.1) and plant parasitic nematodes (Grewal *et al.*, 1997), *H. indica* (Poinar *et al.*, 1992) for the *Diaprepes* root weevil (Shapiro *et al.*, 1999), *H. marelatus* (Liu and Berry, 1996) for *Otiorhynchus* spp. (Berry *et al.*, 1997) and *S. scapterisci* (Nguyen and Smart, 1990) for mole crickets (Parkman *et al.*, 1994). Continued discovery of novel entomopathogenic nematodes, or novel uses for them, is certain to lead to new and improved pest control. Use of nematodes may also be expanded by increasing host suitability through genetic improvement (Gaugler, 1987; Gaugler *et al.*, 1997b; Shapiro *et al.*, 1997) or better formulation. Improvements in production technology, distribution, and application will be a key to reducing nematode costs and insuring quality. In this vein, Gaugler (1997) proposed local level cooperatives to produce nematodes cheaply and effectively for on-site use. Application of nematodes in infected hosts instead of aqueous suspension may also be a potential approach (Shapiro and Lewis, 1999), which could reduce costs of *in vivo* production because several labour-intensive steps would be avoided.

The continued pressure for regulating harmful chemical pesticides, particularly from the Food Quality Protection Act, will favour development of entomopathogenic nematodes and other biocontrol agents. Conversely, discovery of novel 'environmentally friendly' chemicals such as imidacloprid and halofenozide will continue to inhibit wider nematode use. The future of entomopathogenic nematodes in

existing markets is likely to remain stable for some time, and several new markets with favourable host suitability and economics will arise. Target pests that may become successful markets for nematodes in the near future include adult pecan weevils, *Curculio caryae* (Shapiro-Ilan, 2001) in pecans, and plant parasitic nematodes (Grewal *et al.*, 1997) in turf and vegetables. Furthermore, a new frontier is being opened by using entomopathogenic nematodes, and more so, their symbiotic bacteria or associated metabolites, as anti-microbial agents in pesticide and pharmaceutical applications (Li and Webster, 1997). However, major leaps in technology will be necessary before nematodes become successful biopesticides in markets presenting the most challenging barriers such as low value row crops and outdoor above-ground applications.

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17 The Biosys Experiment: an Insider's Perspective

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17.1. Introduction

A seminal step in entomopathogenic nematology occurred in 1983 with the formation of Biosys, a California-based company with sufficient capital and focus to devise efficient mass production technologies. The easy availability of large quantities of *gratis* nematodes coupled with Biosys funding stimulated a burst of field efficacy testing, which in turn galvanized broader basic and applied interest in these nematodes. Biosys became something of an icon for the discipline. Nevertheless, despite the infusion of

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tens of millions of dollars the company struggled financially and was never profitable. The acquisition of Biosys in 1997 by Thermo Trilogy corporation in Columbia, Maryland, concluded in the company dismantling its nematode research group and focusing on only a few of the most profitable existing markets.

The author joined Biosys in 1985 as a scientist in charge of applied research and field development. Later he occupied various managerial positions, including the director of product development in 1988 and the vice president of research and development in 1993. He headed Thermo Trilogy's research and development between 1997 and 1998. His responsibilities at Biosys and Thermo Trilogy included formulation, pilot plant production, field research, product development, and quality assurance. This chapter addresses Biosys' technological and marketing achievements, its role in strengthening the use of nematode-based products, and Biosys' failure to reach its potential.

17.2. Building a Company

Biosys was founded in 1983 to meet the growing social need for environmentally safe alternatives to chemical insecticides. Yet as the company developed and refined the technologies needed to turn its ideas for biological pesticides into commercial products, it also took the time to carefully define and develop the very structure of its business. By identifying four elements critical to building a successful company, Biosys created a long-term business strategy that was aiming to contribute as significantly to the Company's success as its product lines.

The first element in Biosys' business strategy was the use of entomopathogenic nematodes to control soil-borne insect pests. The company recruited experienced personnel and advisors to develop and position nematode-based products in the market. Using its expertise in nematology, microbiology, chemical engineering, biochemistry and cellular physiology, Biosys created and patented an *in vitro* fermentation process that simulated the nematode's natural breeding environment. The company then turned its attention to developing and patenting several formulations and packaging technologies for keeping nematodes alive and effective through the production, distribution, sale and application chain. Additionally, in collaboration with researchers from the US Department of Agriculture, several agricultural universities and potential distribution partners, Biosys tested its products through extensive series of field trials throughout the world. The successful outcome of these multi-site, multi-season tests brought a number of products into the market (Georgis, 1992; Georgis and Manweiler, 1994; Georgis and Poinar, 1994). The second element of Biosys' business strategy involved identifying ways to maximize the company's growth potential while keeping capital outlays modest. As a result, the company identified and implemented several capital management strategies. Rather than build its own fermentation manufacturing facility, for instance, Biosys entered into a cost-effective long-term contract agreement with a contract manufacturer to provide fermentation facilities.

The third part of Biosys' strategic plan was to establish corporate alliances with several leading international agrochemical companies. By tapping into the established infrastructures of these firms, Biosys was able to launch many of its products without spending the time and money to create its own sales and distribution networks. Furthermore, because these partners were among the leaders in their market segments and

geographic territories, Biosys was able to quickly develop a presence for its products in USA and European markets.

Biosys recognized that diversification is important to a growing company, so it included a strategy for increasing its technological base beyond entomopathogenic nematodes as the fourth element in its business strategy. Between 1993 and 1996, the company acquired three agbiotechnology companies (AgriSense, AgriDyn, and Crop Genetics International) which provided new technology bases in pheromones, botanical insecticides, and baculoviruses. By acquisition of complementary products, Biosys planned to quickly and economically expand the depth and breadth of its products and markets throughout the world.

17.3. Technology Assessment

Between 1986 and 1995, Biosys had 22 to 31 personnel involved in fermentation, formulation, and field evaluation of entomopathogenic nematodes. The goal was to develop products that are competitive to standard insecticides on the basis of cost, ease-of-use and performance. A product development path was adopted by the company which proved later to be a reliable approach for developing nematode-based products in 3–5 years (Table 17.1).

17.3.1. Liquid fermentation

By 1988, Biosys had developed semi-automated *in vivo* and solid media mass production methods for the production of *Steinernema carpocapsae*, *S. feltiae* and *Heterorhabditis bacteriophora*. At the standard application rate of 2.5×10^9 nematodes ha^{-1}, the cost of treatment with nematode-based products was 2.5 to 5 times higher than standard insecticides. Labour cost was high and the space required to produce enough nematodes for

Table 17.1. Product development pathway used by Biosys for steinernematid and heterorhabditid nematodes.

Development path	Action
Stage 1: Basic research	Nematode taxonomy and biology
	Symbiont taxonomy and biology
	DNA-based identification
Stage 2: Applied research	Host range
	Impact on non-target organisms
	Temperature optimum and limits
	Optimization of *in vitro* production
	Formulation research
	Application techniques
	Efficacy and field evaluation
	Quality control
Stage 3: Commercialization	Production scale-up
	Formulation scale-up
	Standardization and quality assurance
	Demonstration trials
	Marketing

the market was impractical. Biosys focused its research in the development of liquid fermentation in 1986. Areas of investigation included nematode and bacterium inoculum, interactions between the nematode and the bacterium, growth media, process development (e.g. shear sensitivity, optimum aeration, population dynamics and timing), downstreaming process, and storage. By 1989, this effort led to the production of the three nematode species in 10,000 to 15,000 litre fermenters. In 1991 and 1992, the company entered into 12 years of manufacturing agreements with Archer Daniels Midland Company (ADM) for the production of nematodes and other biological products. Biosys had access to ten 80,000 litre fermenters. At this capacity the company successfully produced 10^{12} *S. carpocapsae* every month to meet market demand in 1994–1997 (Table 17.2). Failure to produce viable and predictable volumes of *H. bacteriophora*, led the company to discontinue its production of this species. The death of infective juveniles, hours prior to harvest and the low conversion rate of the nematode inoculum to first generation males and females, were major issues associated with production of *H. bacteriophora*. Although *S. feltiae* production was less predictable than *S. carpocapsae* (Table 17.3), the company continued its production for the high value markets in mushroom houses and greenhouses (Table 17.2)

In 1993, Biosys was licensed to develop two new species of *Steinernema*. The first was a strain of *S. glaseri*, developed at Rutgers University. The strain was highly effective in controlling white grubs (Scarabaeidae), a common turfgrass pest. The other species, for which Biosys received a co-exclusive licence, was *S. riobrave*. Isolated by scientists at the U.S. Department of Agriculture, this species is effective against mole crickets (*Scapteriscus* spp.) and citrus weevils complex (see Shapiro *et al.*, Chapter 16 this volume). The production of *S. riobrave* was efficient using a 80,000 litre fermenter. However, *S. glaseri* production was discontinued in 1995. Issues related to the low conversion rate (below 60%), of these nematodes to infective juvenile stages before

Table 17.2. Nematode-based products developed by Biosys (1994–1997).

Nematode species	Product	Market segment	Company
Steinernema carpocapsae	BioSafe	Home lawn and garden	Ortho Solaris Group, USA
		Turf	SDS Biotech, Japan
	Exhibit	Turf and ornamentals	Ciba-Geigy (now Syngenta), USA
		Ornamentals	Ciba-Geigy, W. Europe
	Boden-Nutzlinge	Home lawn and garden	Celeflor, Germany
	Sanoplant	Home lawn and garden	Dr. R. Maag, Switzerland
	Biosafe	Home lawn and garden	Pan Britannica Ltd, UK
	BioFlea, Halt, Defend, Interrupt	Pet/vet (fleas)	Farnham Company, USA
S. feltiae	BioSafe–N	Cranberries	Biosys, USA
	Magnet	Mushrooms	Amycel Spawnmate, USA
S. glaseri	X-Gnats	Nurseries	
S. riobrave	Vector-WG	Turf (white grubs)	Biosys, USA
	BioVector 355	Citrus (weevils)	Biosys, USA
	Vector MC	Turf (mole crickets)	Lesco, USA

harvest and low conversion of the nematode inoculum to first generation, males and females were reasons for *S. glaseri's* low probability of success (Table 17.3).

Since physiological, biochemical and morphological differences exist among nematodes, different growing media, as well as process and downstream processes were developed for each species. Although extensive resources were allocated for laboratory and pilot-plant research, efficient production processes were developed only for *S. carpocapsae* and *S. riobrave*.

17.3.2. Formulation

Inability to formulate products that are easy to use and have acceptable shelf-life has hindered the development of entomopathogenic nematodes (Grewal and Georgis, 1988; Georgis and Kaya, 1998). There are physiological differences between species of steinernematids and heterorhabditids. Therefore, Biosys researchers studied the oxygen and moisture parameters required by each species to select the optimum formulation type, ingredients, packaging size and storage conditions.

Biosys' original formulation suspended the nematodes within a gel-coated nylon screen, providing several months of shelf-life when the products were stored at room temperature or for 1 year when refrigerated (Table 17.4). This formulation enabled the company to enter new market segments such as home and garden, cranberries and mushrooms.

In 1993, Biosys developed the water dispersible granule formulation (WDG) in which the nematodes become partially desiccated several hours after formulation (Georgis and Dunlop, 1994). The WDG-formulated products dissolved quickly when water was added. Additionally, the nematodes required less oxygen which made the formulation stable at room temperature for several months (Table 17.4).

Unfortunately, with the exception of *S. carpocapsae* and *S. riobrave*, research effort with various variations of this formulation failed to produce reliable and stable formulations for other steinernematid and heterorhabditid nematodes. In most of these variations, the nematodes escaped from the granule, failed to enter into a partial desiccation stage, or the granule did not dissolve in water (trapping the nematodes in the granule). This formulation was not suited for highly active cruiser species.

One of the major drawbacks of this formulation was contamination. Although certain fungicides such as Kathan (used at low rates to avoid government regulations)

Table 17.3. Entomopathogenic nematodes produced by Biosys[a]

Nematode	Yield[b] (nematodes) ml^{-1}	Probability of success (%)[c]
Steinernema carpocapsae	154×10^3	92
S. feltiae	93×10^3	83
S. glaseri	62×10^3	71
S. riobrave	157×10^3	91
Heterorhabditis bacteriophora	142×10^3	69

[a] Semi-continuous fermentation process; 300 to 1500 to 80,000 litre.
[b] Mean of 10 successful productions from 80,000 litre fermenter.
[c] Based on yield, lipid content, and pathogenicity from 20 productions from a 80,000 litre fermenter.

Table 17.4. Historical review of the development of nematode-based formulations by Biosys[a].

Period	Formulation strategy	Nematode status	Oxygen used (ml) per 10^6 nematodes per day[b]	Shelf-life (months)[c]
1985–1987	Placement on moist substrate such as vermiculite, polyether-polyurethane sponge, peat or cedar shavings	Active	3.2	0.5–1.1
1987–1990	Placement on moist gel materials such as calcium alginate, xanthene, or polyacrylamide	Immobilized	0.67	3–4
1993	Encasement in 10–20 mm diameter water-dispersible granule consisting of silica, clays, cellulose compounds, lignine and starches	Partially desiccated	0.21	5–6

[a] Based on 10^6 *Steinernema carpocapsae* per 1.2 litre container (Georgis and Kaya, 1998).
[b] Calculated 3 days post-formulation.
[c] Based on > 90% nematode viability and unchanged pathogenicity at 25°C from the day of formulation.

and hygiene production processes were used to produce the formulation, maintaining clean products for months was unsuccessful in markets such as home and garden which demand a room temperature shelf-life of at least 6 months. Nevertheless, the formulation opened new markets for nematodes, resulting in improved revenues and profit margins.

17.3.3. Quality control

Biosys realized that market acceptance of nematode-based products depends heavily on their stability during shipping and storage, as well as ease-of-use and consistent performance under field conditions. Stability refers to maintenance of nematode quality through all stages of the production process. The first step in standardization was aimed at producing reliable and pathogenic nematodes. Inoculum batches from *in vivo* cultures were produced from stocks of nematode strains and bacteria that were stored by cryopreservation (Popiel and Vasquez, 1991) to minimize variation in nematode pathogenicity among *in vitro* production lots. Subsequent steps were focused on maintaining the viability and pathogenicity of the nematodes from fermenter harvesting to end-user application. To ensure this process, LT_{50} (lethal time needed to kill 50% of the test insects) or LC_{50} (lethal concentration needed to kill 50% of the test insects) performance standards of infective juveniles were used to measure product stability (Georgis and Kaya, 1998)

Another aspect of product assurance was timing production according to seasonal market needs. Most production is accomplished from January to March for products needed in Japan and Europe, whereas those needed for the USA are produced from May to August. For August to December markets, nematodes were produced from March to June. This production spans just 6 months.

Determining lipid contents of the infective juveniles was a standard procedure adopted by Biosys to monitor the quality of its products. Lipid is the major energy reserve for infective juveniles (Selvan *et al.*, 1993) and initial lipid level has a direct impact on shelf-life. To assure stable products, nematodes were stored at 5–10°C in large, aerated tanks and were formulated within 4 weeks of harvest (Georgis and Kaya, 1998).

17.4. Market Assessment

Product efficacy, cost, profit margins, competition, shelf-life and ease-of-use are the most important issues that impact the successful introduction and positioning of any insect control product into the marketplace. Between 1985 and 1995, Biosys was engaged in developing nematode-based products in multiple markets. Despite years of research, one or more issues forced the company to discontinue the research or withdrawal certain products from the market (Tables 17.5, 17.6).

17.4.1. Product efficacy

Through cooperation with various academic researchers and agricultural chemical companies, approximately US$4.8 m was spent to support hundreds of field efficacy trials between 1985 and 1995. During this period, the field development unit consisted of seven to nine personnel. As a result, Biosys became the leader in increasing the awareness of academic researchers, industry, distributors and growers to the use of nematodes in pest management strategies. The overall programme results are highlighted in Tables 17.5 and 17.6. Some of the major disappointments included:

1. Inconsistency and low efficacy against corn rootworms. Insecticides are applied with corn seeds to protect the roots from larval infestion, which occurs 4–6 weeks after planting. Various tests showed the inability of *S. carpocapsae* and *H. bacteriophora* to remain at high populations 4–6 weeks after their application with corn seeds. Low persistence and the modest susceptibility of first and second instar larvae provided inconsistent results, compared with insecticides that kill larvae by contact and have more than 6 weeks' half-field life.
2. Low efficacy against onion maggots and cabbage root maggots compared with standard insecticides. Within a few hours of eggs hatching, the first instar larvae enter the plant roots, making them inaccessible targets for insecticides. Therefore, insecticides are applied to the soil surface to control adults during oviposition and larvae once they emerge from eggs. The quickness of larval infestation and their resistance to infection render them unsuitable targets for nematodes.
3. No efficacy against wireworms compared with standard insecticides, due to resistance of larval stages to the entomopathogic nematodes. Nematodes were found dead and damaged in their guts.
4. Withdrawal of nematode-based products from the pet-vet market, due to product contamination and the introduction of new chemical products against cat fleas.
5. Withdrawal of nematode-based products for white grub control in the turfgrass market, due to the introduction of new chemical products and low yields in the fermenters (Table 17.3) that led to low profit margins.

Table 17.5. Product introduction decision in markets where steinernematid nematodes provided acceptable insect control.

Market segment	Common name	Scientific name	Product introduction (1992–1995)[a]
Artichoke	Artichoke plume moth	*Platyptilia cardiuidactyla*[b]	No
Citrus	Blue green weevil	*Pachnaeus litus*	Yes
	Sugarcane rootstalk borer	*Diaprepes abbreviatus*	Yes
Corn	Black cutworm	*Agrotis ipsilon*	No
	Western corn rootworm	*Diabrotica virgifera virgifera*	No
Cranberry and berries	Black vine weevil	*Otiorhynchus sulcatus*	Yes
	Cranberry girdler	*Chrysoteuchia topiaria*	Yes
	Crown borers	Sesiidae	No
	White grubs	Scarabaeidae	No
	Strawberry root weevil	*O. ovatus*	Yes
Greenhouse and nursery plants	Beet armyworm	*Spodoptera exigua*[b]	No
	Black vine weevil	*O. sulcatus*	Yes
	Leafminer	*Liriomyza trifolii*[b]	No
	Sciarid flies	Sciaridae	Yes
	Strawberry root weevil	*O. ovatus*	Yes
Mint	Cutworms	Noctuidae	No
	Mint flea beetle	*Longitarsus waterhousei*	No
	Mint root borer	*Fumibotys fumalis*	Yes
	Root weevils	*Otiorhynchus* spp.	Yes
Mushroom	Phorid fly	*Megaselia halterata*	No
	Sciarid fly	*Lycoriella* spp.	Yes
Peanut	Granulate cutworm	*Feltia subterranean*	No
	Lesser cornstalk borer	*Elasmopalpus lignosellus*	No
	Southern corn rootworm	*Diabrotica undecimpunctata*	No
Sugar beet	Sugar beet weevil	*Cleonus mendikus*	No
Sweet potato	Sweet potato weevil	*Cylas formicarius*	No
Turf	Armyworm	*Pseudaletia unipuncta*	Yes
	Billbugs	*Sphenophorus* spp.	Yes
	Black cutworm	*A. ipsilon*	Yes
	Bluegrass webworm	*Parapediasia teterrella*	Yes
	European crane fly	*Tipula paludosa*	No
	Mole crickets	*Scapteriscus* spp.	Yes
	White grubs	Scarabaeidae	Yes
Vegetable and field crops	Cutworms	Noctuidae	No
	Cucumber beetles	Chrysomelidae	No
	Flea beetles	Chrysomelidae	No

[a] Product introduction decision was based on cost effectiveness, formulation stability, and competition. All soil applications and target stages are immatures unless otherwise specified. Modified from Georgis (1992).
[b] Above-ground applications: cryptic or greenhouse environments.

On the positive side, environmental issues and the lack of available registered insecticides created opportunities for Biosys to gain markets in the following segments:

Table 17.6. Programmes discontinued by Biosys due to low efficacy of steinernematid nematodes in field and laboratory trials[a].

Common name	Scientific name	Factors[b]
American cockroach	*Periplaneta americana*	Nymphs and adults are relatively non-susceptible
German cockroach	*Blattella germanica*[c]	Early immature stages are relatively non-susceptible
Grape phylloxera	*Phylloxera* sp.	Nymphs and adults are relatively non-susceptible. Insect located deep in soil
Housefly	*Musca domestica*	Nematodes are unable to survive in manure
Imported fire ant	*Solenopsis invicta*	Insects avoid contact with nematodes by moving their colonies
Pecan weevil	*Curculio caryae*	Prepupal stages form cells located up to 15 cm below soil surface
Rice water weevil	*Lissorhoptrus oryzophilus*	Nematodes settle quickly to the bottom in flooded soil. Early instars are relatively non-susceptible
Root maggots	*Delia* spp.	Larval stages are relatively non-susceptible
Wireworms	Elateridae	Larval stages are relatively non-susceptible

[a] Modified from Georgis (1992).
[b] Relatively non-susceptible insects are those with LC_{50} > 100 nematodes/insect using soil or Petri dish bioassay methods (Woodring and Kaya, 1988).
[c] Using traps containing nematodes and attractants.

1. Turfgrass (billbugs, cutworms, and webworms) in Japan.
2. Ornamental and greenhouses (black vine weevil and fungus gnats) in USA and Europe.
3. Cranberries (black vine weevil and cranberry girdler) in USA.
4. Citrus (citrus weevil complex) in USA

17.4.2. Product usage

The introduction of the calcium alginate formulation in 1987, which was based on immobilizing the nematode without desiccation, was a major breakthrough. It was the first room temperature formulation with a shelf-life of up to 3–4 months (250×10^9 nematodes per 4 litre container) under ideal conditions. As a result, a number of agrochemical companies introduced the formulation into various market segments including turfgrass, greenhouses, berries, and home and garden. Unfortunately, it took 20–30 min to extract the nematodes from the calcium alginate before they could be applied through standard application equipment. Time of extraction (30 min) limited market penetration, however, especially when large-scale treatment was required. Furthermore, the disposal of the framing material (i.e. plastic screen) that held the calcium alginate was burdensome and the number of containers required to treat large areas was considered impractical.

For all the above reasons, research in the early 1990s focused on developing more compact product configurations and simpler mixing and application for further market penetration. These efforts led to development of a WDG product that allowed

the infective juveniles to enter a partially desiccated state, enhancing survival and preserving pathogenicity for up to 5–6 months at 25°C and providing better tolerance of nematodes to 36°C (Table 17.4). The product is applied without time-consuming preparation steps.

Inducing nematodes to enter a partial desiccation state means that less oxygen is required. Thus, a higher nematode population per litre of container space was attained (Table 17.4). To obtain a 5–6 month shelf-life, a 1.2-litre container is adequate for 250×10^6 desiccated *S. carpocapsae* in the WDGs. In contrast, a 4-litre container is needed for the same number of immobilized nematodes in calcium alginate with a shorter shelf-life.

The introduction of the WDG formulation immediately captured US$6.3 m of the market which accounted for 90% of Biosys' 1994 sales of nematode-based products. Furthermore, the market size of nematode products increased by 52% that year as a result of the new formulation.

17.4.3. Market acceptance

In the late 1980s, entomopathogenic nematodes emerged in an atmosphere of environmental concern and growing insect resistance to conventional chemicals, but products fell short of expectations. Many products were rushed to market before developing predictable efficacy and before companies were ready to effectively produce, market or support these products. Prices were higher than conventional chemicals and products had inconsistent formulation and active ingredient content. The lack of key cooperative extension and distributor support meant there was little information available on proper usage and application and as a result, end-users were not properly instructed on the characteristics and mode-of-action of these products.

Biosys made tremendous product improvements in the 1990s and their nematode products were competitive with traditional chemical controls in selected markets. The products showed improved price performance due to advancements in strain selection, production, formulation and quality control. For example, products had more concentrated nematodes and consistent performance as well as longer residual and shelf-life formulations. Biosys repositioned its products in high-value markets such as ornamentals, citrus, berries and turfgrass. Additionally, extensive efforts, including seminars, workshops and field days, were provided to educate growers and distributors on the use of its products.

17.5. Biosys Failure

Fuelled by lavish venture capital money, market projection and unrestrained enthusiasm for agricultural biotechnology, a number of start-up biopesticide companies including Biosys, went pubic in the 1980s to exploit the extraordinary potential biologicals offered as environmentally benign alternatives to chemical pesticides. Although in the 1990s, various microbial insecticides competed favourably with conventional insecticides, market size and profit margins never reached earlier predictions. This had a significant impact on the structure of the biopesticide industry (Gaugler, 1997). Biosys (nematodes, viruses), Entotech (bacteria), EcoScience (fungi), and Crop Genetics International (viruses, bacteria) disappeared through merger or

acquisition. Ecogen (bacteria, nematodes) has undergone repeated downsizing and Mycogen (fungi initially, bacteria subsequently) survived essentially by reinventing itself as a seed company. Companies such as Microbio (nematodes, bacteria) and Mycotech (fungi) have survived through maintaining a small infrastructure and gradual market penetration. In 2000, Microbio was acquired by the USA-based Becker Underwood company. The Biosys acquisition by Thermo Trilogy in 1997, culminated a period of rapid consolidation in the USA biopesticide sector. In under 4 years, five companies effectively rolled into one. Between 1993 and 1996, Biosys acquired three companies. In late 1996, the company filed for bankruptcy.

There were complex reasons that led Biosys to file for bankruptcy. Generally, the company failed to raise the necessary funds from investors to continue operation. In the mid-1990s, most investors diverted their funding into internet start-up companies with projected returns of over ten times. The projected return of investment in biopesticide companies, including Biosys was two to three times. Low revenues and limited market share also contributed to the company's failure to raise capitals. Total revenues from nematode, pheromone, botanical and baculovirus products did not exceed \$15 m in 1996.

17.5.1. Production cost

The high cost of goods sold continued to be a problem for Biosys. The manufacturer cost of US\$30.97 for 2.5×10^9 *S. carpocapsae* in WDG ha^{-1} (based on 150×10^6 nematode yield per litre, US\$0.12 media cost per litre, US\$1.30 labour and processing cost per litre and US\$1.60 for 900 g 250×10^6 nematodes in WDG per 2.5 litre container) precluded attaining early goals to penetrate low-value markets such as corn, cotton, potatoes and most vegetables. Even at a low profit margin of 30% for Biosys and the distributor, grower cost of US\$52.33 ha^{-1} was high relative to chemical insecticides. In general, grower cost for chemicals is US\$10–20 ha^{-1}, with over 40% profit margins for the producer and the distributor. As a result, Biosys redirected its effort to medium to high-value markets. The new direction eliminated the company's early projection of greater than US\$100 m in revenue. It became obvious to investors that revenue from the remaining markets could not exceed US\$50 m.

Biosys had successfully produced *S. carpocapsae* and *S. riobrave* in 80,000 litre fermenters with production exceeding 150×10^3 nematodes ml^{-1} (Table 17.3). However, contamination, viability of the infective juveniles and nematode reproduction were recurrent problems that kept the probability of production at 90%.

These factors had even greater impact on the production of *S. feltiae, S. glaseri* and *H. bacteriophora*, where the probability of achieving successful production did not exceed 85% in 80,000-litre fermenters. Furthermore, yields of *S. feltiae* and *S. glaseri* were less than 1×10^5 infective juveniles ml^{-1} (Table 17.3). Therefore, at rates of 2.5×10^9 ha^{-1}, profit margins were substantially lower for both steinernematids.

17.5.2. Product coverage

Although the WDG formulation is well suited for a wide variety of consumer and horticultural applications, in markets such as turfgrass the amount of product needed to treat a hectare was four to eight times more for chemical insecticides. To obtain a 5–6

month shelf-life, a 1.2-litre container is adequate for 250×10^6 desiccated nematodes in the granules. For mole cricket control, it requires six 1.2-litre containers per hectare. In contrast, for standard insecticides, a 4-litre container will treat over 10 ha. Thus, product coverage was considered inconvenient by many users relative to standard insecticides.

17.5.3. Product stability

Generally, insecticide products used in medium- and high-value markets have at least 2 years' shelf-life with no requirement for refrigeration. The WDG formulation has a shelf-life of 5–6 months and requires refrigeration to extend its shelf-life. Such requirements were difficult to accommodate by many distributors and end-users. This was most apparent in markets where insect activity exceeds 5-month periods such as fungus gnats (ornamentals), mole crickets (turfgrass) and fleas (outdoor applications).

High relative humidity was needed to maintain the viability of the desiccated nematodes, environments which encourage the growth of fungi and bacteria. Although fungicides were included in the formulation, contamination was a problem in many products. The contamination, in most cases, did not affect nematode viability, but it was a cosmetic problem that was unacceptable to the end-user. For example, contamination was a key reason that led distributors to discontinue marketing nematode products against fleas. Other reasons were related to profit margins and the introduction of a novel insect growth regulator for indoor applications.

17.5.4. Developing optimum products

Even though steinernematid and heterorhabditid nematodes can parasitize a wide range of insect species in the laboratory, certain nematode species or strains control specific insects in the field better than others. For example *S. glaseri* and *H. bacteriophora* actively move vertically in the soil and are most efficient in penetrating and parasitizing scarabaeid larvae, providing efficacy comparable to that of organophosphate and carbamate insecticides (Georgis and Gaugler, 1991; Georgis and Poinar, 1994). Inability to develop a cost-effective liquid fermentation process and a stable formulation for both nematode species hinder Biosys' marketing development effort to penetrate the large turfgrass market.

Reasons related to insect susceptibility, ecological behaviour, formulation, ease-of-use, storage and product cost have limited the use of nematodes to certain insects and markets. The greatest disappointment was lack of efficacy against corn rootworms, root maggots and wireworms (Table 17.6). The insecticide global markets for these soil insects at the manufacturer level exceeded $350 m in 1995.

17.5.5. New competition

Agricultural niche markets are known for their relative lack of competition compared with major markets. With the exception of many *Bacillus thuringiensis* products, microbial insecticides, including nematode-based products, were forced to concentrate their marketing efforts on niche markets because they lacked the price performance characteristics necessary to compete in the larger pesticide markets (Georgis, 1997).

Reduced toxicity, increased specificity, resistance issues and knowledge-based integrated post management are key concepts that drove the development of biopesticides. The biopesticide market was dominated by the new generation of companies such as Biosys and by notable large companies such as Abbott Laboratories (now Valent) and Novartis (now Syngenta). These companies made great strides in improving product technology in response to the ineffectiveness of conventional chemicals (mainly pyrethroids), the regulatory environment and demand for safer products.

However, the information gained from broad advances in molecular biology, insect physiology, genetics and biochemistry enabled scientists to develop a new generation of control technologies. The new technologies competing with microbial insecticides, nematodes and conventional chemistries can be broadly classified as new chemical products and transgenic plants. These products have already started to have a huge impact on the structure of the agrochemical industry with major implications for biological insecticides. New chemical products have novel modes of action, very low quantities of active ingredients and narrow specificity compared with conventional insecticides.

In the 1980s and early 1990s, the efficacy of certain species and strains of entomopathogenic nematodes against scarabaeids and mole crickets was comparable to standard organophosphates and carbamate insecticides (Georgis and Gaugler, 1991; Georgis and Poinar, 1994), causing pest suppression ranging from 65 to 95%. The new chemical insecticides such as methoxyfenozide, fipronil, and imidacloprid provide more consistent results with over 90% reduction in insect populations. For this reason, products based on *S. glaseri* were withdrawn from the turfgrass market. It became apparent that the market for this nematode against white grubs will be restricted to certain golf courses that are committed to non-chemical control approaches. In contrast, there are few insecticides that are registered for white grubs control in Japan. The environmental issues associated with these insecticides created opportunity for SDS Biotech to introduce *S. glaseri* in the Japanese turfgrass market.

17.5.6. Strategic partners

To reach its market projections of greater than US$100 M, Biosys' strategy was to establish marketing agreements with major agrochemical companies. Between 1985 and 1993, research and development agreements were signed with companies such as Ciba (now Syngenta), Sandoz (now Syngenta), Shell (now Dupont), Bayer, Chevron (now Sumitomo), American Cyanamid (now BASF), FMC, Scotts and SDS Biotech. The target insects included: corn rootworm (maize), root maggots (cucurbits, onion, carrot, sugar beet) and wireworms (maize, potato, sugar beet). Although promising results against certain insects were obtained by most companies, the efficacy of the nematodes was not as consistent as that obtained with chemical insecticides. During that period, Biosys fell short of meeting the standard set by the agrochemical companies to develop formulations and product cost comparable to the existing market standards.

Biosys' inability to reach global-marketing agreements with major agrochemical companies was a signal to the investment community that the market projection was low. In the medium- and high-value markets, Biosys was able to sign a number of

strategic marketing agreements which brought annual revenues of approximately US$7 M in 1994, 1995, and 1996. However, these revenues were significantly lower than originally projected by Biosys. Difficulties accelerated when the Biosys 'home and garden' product, was discontinued in 1994. This product was marketed by Ortho, a division of Chevron Chemical Company. Ortho was acquired by Solaris Group of the Monsanto Company and the new company decided to terminate the biopesticide product line that had been developed by Ortho.

17.6. Conclusions and Recommendations

Biosys' progress in nematode commercialization during the 1990s was phenomenal. Development of large-scale mass-production technology and innovative, easy-to-use formulations led to the expanded use of nematodes. Improvements in the quality and mass production of several species and the development of alginate and granular formulations were among the major milestones accomplished that enhanced consumer acceptance of nematode products. The emphasis to use proper rates per ha and the leadership role to adopt standard quality control procedures (e.g. 1 : 1 biossay), provided opportunities for researchers and growers to generate reliable results. These developments led to the use of nematodes for the control of mole crickets and billbugs in turf, root weevils in citrus, sciarid flies in mushrooms and greenhouses, and black vine weevil and cranberry girdler in cranberries. This progress was made possible by the collective efforts of Biosys working with universities and federal agencies, coupled with a socio-political atmosphere favouring a reduction in the use of chemical pesticides. Despite this progress, the reality is that insecticidal nematodes are not dominant products in the market, except in citrus (citrus weevil complex in USA) and turfgrass (billbugs and webworms in Japan). Limited market is attributed to a product cost, limited efficacy and host range in the field, ease-of-use, product stability and lack of information on their usage.

Although Biosys spent over US$30 M between 1985 and 1995 in researching and developing entomopathogenic nematodes, the company was unable to develop products competitive with standard insecticides for USA and European markets in row crops and vegetables. As a result, the company failed to reach the revenue and profitability expectations of stockholders. The current worldwide market for nematode-based products at the manufacturer level do not exceed US$10 M (Georgis, 1997). Low revenues and market potential will discourage current companies such as Therm Trilogy and Microbio from allocating the funds necessary to improve formulation, quality control, and production processes. It is likely that future research and development will come from government and academic institutes. Discovery of new species and strains, mass production of new species, and further improvement in formulations will continue. Genetic engineering techniques will be harnessed to enhance nematode biological control potential (Gaugler, 1988, 1993). Increased emphasis will need to be placed on the training of extension agents and end-users in the proper use of nematodes.

The development of nematodes for effective insect control in the context of sustainable agriculture will be a major challenge. A truly integrated approach is required, in which all agricultural practices will be required to obtain maximum effect from a given intervention or practice without interfering with the effectiveness of

other practices. Because of the low environmental impact and selectivity of nematodes, they will be ideal components in integrated pest management programmes.

In the past few years, a noticeable change has occurred in the commercialization of nematodes. As opposed to the conventional chemical pesticide development model followed by Biosys, a distinct small-scale cottage industry has emerged in the United States and Europe. These small businesses cater mainly to the needs of the high-value horticulture and home-lawn-and-garden markets and rely heavily on *in vivo* nematode production. Declines among venture capital funded agricultural biotechnology companies provides further impetus to this trend and will be expected to continue well into the early 21st century. However, emphasis on quality production and rigorous quality control standards are important steps in the evolution of nematodes as legitimate pest management tools (Gaugler *et al.*, 2000). Making nematode production a local or regional business may create new opportunities and potential for nematode use in pest control. Appropriately scaled businesses serving local markets may gain advantages in speed of delivery, cooperative extension support and reduced storage problems (Gaugler, 1997).

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