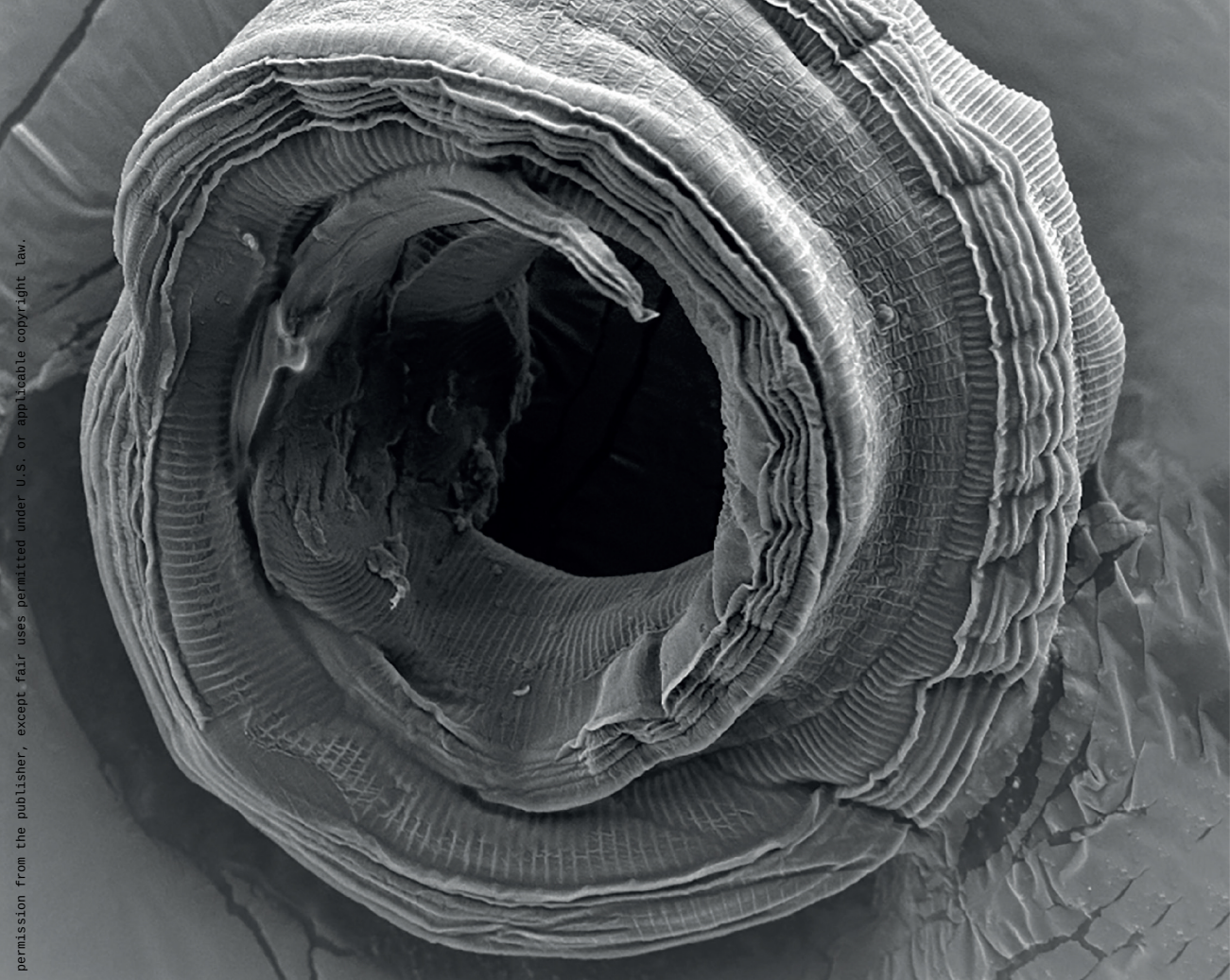




Nematodes as Model Organisms

Edited by Itamar Glazer, David I. Shapiro-Ilan,
and Paul W. Sternberg



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Preface

Along with the development of research in the biological sciences over the past two centuries, the need for a model system became apparent. That is, in order to study fundamental living mechanisms as well as for comparison of biological processes at different levels of evolutionary development, a model template is required. Model organisms are species that have been intensively and widely studied. Usually they are easy to maintain and breed in the laboratory and have particular experimental features. For example, they may have particularly robust embryos that are easily studied and manipulated in the laboratory; this is useful for scientists studying development. Alternatively, they may occupy a pivotal position in the evolutionary tree; this is useful for scientists studying evolution. Examples for model organisms include yeasts (e.g., *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*), fruit flies (e.g., *Drosophila melanogaster*), the Western clawed frog (*Xenopus tropicalis*), mice (*Mus musculus*), zebrafish (*Danio rerio*), and the nematode *Caenorhabditis elegans*.

Nematode parasites were observed and recorded as early as the 19th century. Among them is the human parasite *Ascaris lumbricoides* (O.G. Poinar, 1983: *The Natural History of Nematodes*. Prentice-Hall Inc, New Jersey, USA). In the late 19th century, Theodor Boveri already used this species as a model to describe in detail the first observations of cell division, in early *Ascaris* embryos (C. O'Connor, 2008: Cell division: stages of mitosis. *Nature Education* 1, 188). The free-living nematode *C. elegans* has attained prominence as a model to study various biological problems. The initial early work was done by E.C. Dougherty, V. Nigon, and their colleagues. However, the greatest surge of interest in this organism is due to studies on the genetics, development and anatomy of *C. elegans* by Sydney Brenner and his colleagues, who received the Nobel Prize for their outstanding contribution to science. These studies further triggered the interest of experts from different disciplines such as geneticists, cell and developmental biologists, neurologists, endocrinologists, toxicologists, nutritionists, and gerontologists, using *C. elegans* as a biological model to understand some fundamental processes of life.

As research techniques advanced and knowledge about nematodes expanded, other species of nematodes became useful tools as biological models. These include entomopathogenic nematodes belonging to the families Steinernematidae and Heterorhabditidae, the beetle-associated nematode *Pristionchus pacificus*, as well as the genus *Panagrolaimus*. Furthermore, the use of nematodes as biological models has expanded to other disciplines, such as parasitology, stress tolerance, environment, pharmacology, and more.

The last book that compiled knowledge related to the use of nematodes as model organisms was published in 1980, edited by B. Zuckerman (*Nematodes as Biological Models*, Academic Press, New York).

After more than three decades, we realized the need to present the major advancements, progress, and diversity in the use of nematodes as biological models. While considering the content of the book we recognized that there was a vast amount of knowledge, which could not all be incorporated in one volume. We therefore picked several topics from diverse disciplines: biological systems, ecology, toxicology, and biological control of pests. This book will be of great interest to a broad audience across a wide spectrum of expertise: biologists will use the book in comparative studies to investigate biological processes across organisms; medical scientists and pharmacologists will use this model for exploration of drugs and medicine (including the use of genome editing to eliminate diseases); ecologists will consider nematodes as indicators for environment changes; parasitologists for host–parasite interactions; and other scientists, teachers, and students will be able to use this book as a benchmark for the broad implications of nematology on other aspects of science.

The editors want to acknowledge their gratitude to the contributors of the various chapters. Notably, the chapters were written during the climax of the COVID-19 pandemic era, but all authors enthusiastically cooperated to bring this project to completion.

1 The Genus *Pristionchus*: a Model for Phenotypic Plasticity, Predatory Behavior, Self-Recognition and Other Complex Traits

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Introduction

Pristionchus pacificus is a soil nematode that was first described in 1996 based on an original isolate from Pasadena, California (Fig. 1.1A) (Sommer *et al.*, 1996). As a hermaphroditic species with the spontaneous formation of males, it shares many of the key characteristics that were essential for establishing *Caenorhabditis elegans* as a model organism in the first place. For example, *P. pacificus* grows well under laboratory conditions when provided with *E. coli* OP50 as food on standard agar plates. At 20°C, the generation time is about 4 days and hermaphrodites produce around 150 self-progeny, which allows about 100 generations of worms to be propagated annually in the laboratory (Fig. 1.1B). Mating of young and old adult hermaphrodites with males will result in outcrossing and worms can be frozen in liquid nitrogen indefinitely. All these features combined made *P. pacificus* a prime target as a satellite organism for studies in evolutionary developmental biology (evo-devo), which was a new discipline emerging at that time, and for a detailed comparison with *C. elegans*.

Indeed, *P. pacificus* lived up to its promise, as many of the major techniques that resulted in the success of *C. elegans* as a model system were implemented in *P. pacificus* over the years. First, forward genetics using various mutagens resulted

in the isolation of morphological and developmental mutants offering the possibility to investigate any biological process in an unbiased manner (Sommer and Sternberg, 1996; Ogawa *et al.*, 2009; Ragsdale *et al.*, 2013). This advantage is still important in the contemporary analysis of the evolution of complex traits (see below). Second, DNA-mediated transformation techniques were established, although these protocols require more sophistication than in *C. elegans* and necessitate the involvement of complex arrays (Schlager *et al.*, 2009). Third, reverse genetic tools were implemented with CRISPR-Cas9-based technologies largely outcompeting all previous methods (Witte *et al.*, 2015; Nakayama *et al.*, 2020). Fourth, a sophisticated “omics” toolkit has been developed involving genome sequencing and resequencing by single-molecule sequencing technologies (Dieterich *et al.*, 2008; Rödelberger *et al.*, 2017), proteomics (Borchert *et al.*, 2010), RNA sequencing-based transcriptomics (Rödelberger *et al.*, 2018), and epigenomics (Werner *et al.*, 2018a,b). All these genomic features are visualized on the websites www.pristionchus.org and www.wormbase.org, accessed 16 March 2022.

Given the strong focus on evolutionary questions, early *Pristionchus* research aimed to identify the ecological niche of these nematodes. While *Pristionchus* worms can be found in soil

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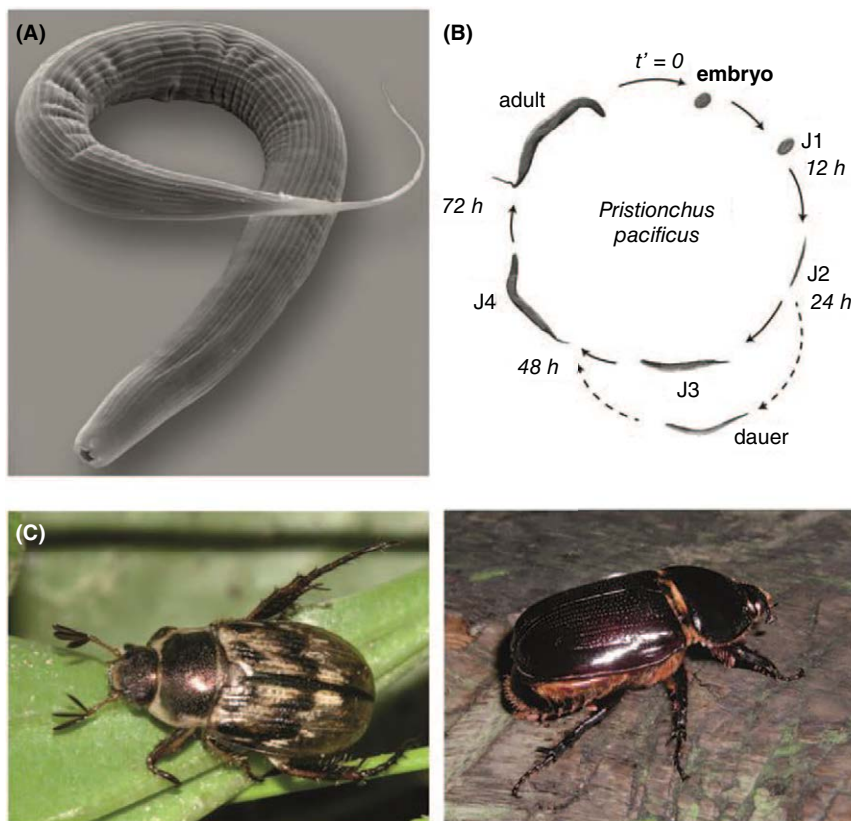


Fig. 1.1. An introduction to *Pristionchus*. **(A)** An SEM image of the main laboratory wild-type isolate of *Pristionchus pacificus*, PS312, now utilized in labs around the world. **(B)** *P. pacificus* life cycle with a generation time of roughly 4 days. Like all species of the family Diplogastriidae, in *P. pacificus* the J1 stage is not free-living and remains confined to the egg. There is therefore an embryonic molt with the J1 larvae molting to the J2 stage before hatching from the egg. Redrawn from Werner *et al.* (2020). **(C)** In addition to being retrieved in soil samples, *Pristionchus* nematodes show a frequent association with beetles, including the Oriental beetle *Exomala orientalis* (left) and the rhinoceros beetle *Oryctes borbonicus* (right). Pictures courtesy of M. Herrmann.

samples from around the world, the most reliable source for *Pristionchus* nematodes is scarab beetles, including chafers, stag beetles, and dung beetles (Fig. 1.1C). This association, after being first discovered in central Europe (Herrmann *et al.*, 2006), resulted in the identification of the Oriental beetle *Exomala orientalis* in Japan as a common host of *P. pacificus* (Herrmann *et al.*, 2007). After more than a decade of intense samplings around the globe, around 50 *Pristionchus* species are available and additional host species from soil settings, such as millipedes, have been discovered (Kanzaki *et al.*, 2018). One unique feature of *Pristionchus* nematodes is the independent convergent evolution of hermaphroditism at

least seven times in this genus, allowing unique insight into genome evolution (Rödelsperger *et al.*, 2018).

Alongside these studies, *P. pacificus* has been developed into a prime model organism in various research areas from developmental genetics, population genetics, genomics, and bio-informatics to evolutionary ecology, behavioral ecology, epigenetics, and neurobiology. This approach always aimed for an integrative analysis of evolutionary biology (Sommer, 2009). The following contribution will provide a brief overview on original studies in evo-devo and *Pristionchus* phylogeny, ecology, and life history. The center of this contribution is the more recent work using

P. pacificus as a model system for complex trait formation and the evolution of novelty. This approach largely uses the unique features of *Pristionchus* nematodes, such as mouth-form plasticity, predatory behavior and self-recognition, all of which are unknown from *C. elegans* and most other nematode species.

Evo-devo Beginnings and Developmental Systems Drift

The development of *P. pacificus* as a model system for comparative studies has to be seen in the context of the evo-devo movement in the late 1980s and early 1990s. Historically, zoologists had relied on developmental processes and morphological structures to explain animal phylogeny. However, with the Neo-Darwinian synthesis the importance of development for evolutionary research and for explaining evolutionary patterns had faded away. A strong revival of evolutionary developmental biology only occurred when developmental genetics started to provide mechanistic insight into development. This was first achieved for segmentation in the early embryo of *Drosophila*, which initiated a wave of comparative studies (Patel *et al.*, 1989; Sommer and Tautz, 1993). This was later followed by investigations of various postembryonic processes in multiple insects and other arthropods (for review, see Carroll *et al.*, 2001).

In nematodes, the first comparative investigation was the complete postembryonic cell lineage analysis of *Panagrellus redivivus* (Sternberg and Horvitz, 1981, 1982), to be followed by the analysis of early embryogenesis in multiple nematodes, such as *Acrobeloides* or *Diploscapter* (for review, see Schierenberg and Sommer, 2014). When the molecular mechanisms associated with *C. elegans* vulva development were being identified, systematic comparative studies were initiated using cell lineage analysis and experimental cell ablation studies, involving around a dozen species of multiple genera and families of nematodes (Sommer and Sternberg, 1994, 1995, 1996). While some of them showed striking deviations in development relative to *C. elegans*, these species were exclusively either gonochorists, with a male–female mode of reproduction, or parthenogenetic. Thus, they missed the hermaphroditic

mode of reproduction that would eventually enable easy genetic and experimental manipulation. The early choice of *P. pacificus* as a satellite species was based on the fact that this species shared with *C. elegans* hermaphroditic reproduction and other technical features (see above), but at the same time differed in various developmental features, including vulva and gonad development (Sommer and Sternberg, 1996; Rudel *et al.*, 2005). Thus, *P. pacificus* combined the prospect of mechanistic insights with evolutionary diversification of developmental processes.

Already the description paper of *P. pacificus* as a novel species provided the proof of principle of its genetic accessibility, by describing the isolation of first *dumpy* (Dpy) and *uncoordinated movement* (Unc) mutants based on *C. elegans*-type mutagenesis experiments (Sommer *et al.*, 1996). The first systematic mutagenesis experiment for *P. pacificus* vulva development was published in the same year (Sommer and Sternberg, 1996), and the first mutant-based developmental control gene to be cloned was the Hox gene *lin-39* just 2 years later (Eizinger and Sommer, 1997). During the next decade, systematic cell ablation studies, saturation-based mutagenesis experiments combined with map-based cloning and reverse genetics provided detailed insight into the molecular mechanisms of *P. pacificus* vulva development, which are summarized for the interested reader in Fig. 1.2 (Tian *et al.*, 2008; Wang and Sommer, 2011). Together, these studies resulted in the unexpected finding that vulva development in *P. pacificus* and *C. elegans* is a prime example of what is now called “developmental systems drift”: while the vulva of these two species is a homologous organ and is formed from homologous precursor cells, the underlying molecular mechanisms and regulatory logic are fundamentally distinct. Most importantly, vulva development in *C. elegans* is induced by EGF/Ras signaling (for review, see Sternberg, 2005), whereas in *P. pacificus* it relies on Wnt signaling with several different ligands that are expressed in multiple different cells and body regions (Tian *et al.*, 2008; Wang and Sommer, 2011). Developmental systems drift was first proposed as a general principle by True and Haag (2001) largely based on the evolutionary analysis of sex determination. By now, it can be considered to represent one of the two key principles of the evolution of developmental

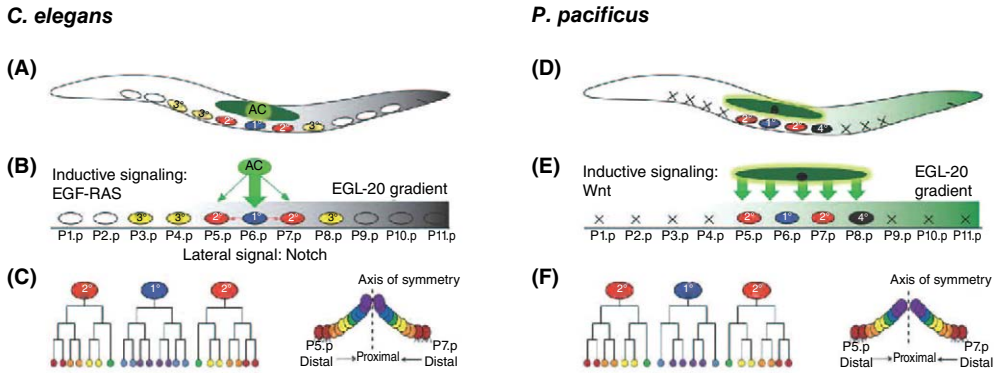


Fig. 1.2. Comparison of vulval development between *C. elegans* (left) and *P. pacificus* (right). The vulva develops from the three precursor cells P(5–7).p of the ventral epidermis (A,D). In *P. pacificus*, the anterior and posterior precursor cells P(1–4,9–11).p die of programmed cell death, whereas in *C. elegans* these cells fuse with the hypodermal syncytium hyp7. In *C. elegans*, vulva formation by P(5–7).p is induced by a signal from the gonadal anchor cell (AC) (green) and requires epidermal growth factor (EGF)-RAS signaling (green) (B). P6.p (blue) has a 1° cell fate, generates eight progeny and forms the central part of the final vulval structure (C). P(5,7).p have a 2° cell fate through *lin-12*/Notch signaling, and form seven progeny and the anterior and posterior part of the final structure, respectively. In *P. pacificus* in contrast, vulval formation is induced by Wnt signaling and requires multiple Wnt ligands, including EGL-20 from the posterior body region and Wnt ligands expressed in the gonad (green) (E). Thus, *P. pacificus* vulva induction differs from *C. elegans* in: (i) the inducing signaling pathway; (ii) number of involved ligands; and (iii) cellular basis of signal induction (B). P(5–7).p form 2°-1°-2° cell fates again and produce seven, six, and seven progeny, respectively (F). Together, the example of vulva formation indicates developmental systems drift: homologous cells form a homologous structure relying on different signaling pathways and molecular mechanisms.

processes together with *cis*-regulatory evolution. While *cis*-regulatory evolution explains the co-option of conserved developmental control genes in independent developmental processes and in multiple species and phyla (Raff, 1996; Carroll *et al.*, 2001), developmental systems drift provides a complementary perspective: even homologous structures are often regulated by non-homologous molecular mechanisms. The detailed comparison of *P. pacificus* vulva development to that of *C. elegans* helped to identify developmental systems drift as a major principle in evo-devo.

Rapid Genome Evolution and Taxonomically Restricted Genes

While the *C. elegans* genome is around 100 Mb in size and contains around 20,000 genes (WormBase), the original sequencing of the *P. pacificus* genome in 2005 revealed that it has a size of roughly 170 Mb and contains more than 25,000

genes (Dieterich *et al.*, 2008). This has subsequently been further refined through single molecule resequencing and improved gene annotation pipelines suggesting a total of around 28,000 genes in *P. pacificus* (Rödelsperger *et al.*, 2017, 2019). Most strikingly, only 25% of all *P. pacificus* genes share 1:1 orthologs with *C. elegans*, which includes many of the developmental control genes that are involved in developmental systems drift. In contrast, the overwhelming majority of genes have undergone more recent gene birth and death events (Markov and Sommer, 2015). Even more surprising was the discovery that around one-third of all *P. pacificus* genes have no similarity to genes in *C. elegans* or other nematodes (Dieterich *et al.*, 2008). This finding has survived the test of time, with more comprehensive nematode sequencing validating the high frequency of genes appearing to be unique within a species. Most recently, comparative genomics of eight *Pristionchus* species with two closely related outgroups revealed that gene evolution is an ongoing steady process (Prabh

et al., 2018). Thus, a substantial fraction of genes undergoes rapid evolutionary diversification or evolves *de novo*, resulting in so-called orphan or taxonomically restricted genes (for review, see Rödelsperger *et al.*, 2019).

The Phylogeny of the Genus *Pristionchus*

Finding *Pristionchus* reliably in association with scarab beetles (Herrmann *et al.*, 2006), but also other arthropods, resulted in a systematic analysis throughout the world to isolate new specimens. Fortunately, the majority of soil- and arthropod-derived *Pristionchus* species which have been discovered thus far can easily be cultured in the laboratory and can be used in crossing experiments. This has made this genus a prime model for nematode taxonomy and systematics. Indeed, for reasons that are still not fully understood, *Pristionchus* nematodes show a strong positive correlation between DNA sequence divergence of the SSU ribosomal spacer and species status. Isolates with more than 1 nucleotide divergence out of a 700 bp fragment of the SSU that is commonly used for species identification usually fail to produce progeny after crossing, indicating species status (Mayer *et al.*, 2007, 2009). In all such cases, detailed morphological and by now genomic investigations later confirmed these observations (for review and recent examples, see Ragsdale *et al.*, 2015). The most recent study of this kind described nine novel species of *Pristionchus* from mainland China after two sampling trips in Yunnan and Shaanxi provinces (Kanzaki *et al.*, 2021). One of these nine novel species is another hermaphroditic species, which represents the eighth hermaphrodite in this genus, a record for nematodes. Note that, unfortunately, the positive correlation between SSU sequence divergence and species status does not hold in other nematode genera, preventing the idea of using a strict molecular barcode as early indicator of the result of crossing experiments.

The known *Pristionchus* species fall into six “clades” or “species-groups” that show striking biogeographic features (Fig. 1.3). First, the four major clades are the *maupasi*-, the *lheritieri*-, and the *pacificus*-groups, which are largely restricted to North America, Europe, and Asia, respectively,

and the *triformis*-group (Fig. 1.3). Second, biogeographic restrictions are seen for most gonochoristic species in these groups, whereas the hermaphrodites escape these restrictions and are mostly cosmopolitan. This likely results from easier dispersal mechanisms, because single dauer larvae of hermaphrodites are more likely to successfully invade a new region by hitchhiking with insect vectors. Third, all basal species of the *fissidentatus*- and *elegans*-groups are found in Asia, which suggests that the genus has evolved in Asia (Fig. 1.3). Indeed, the outgroup to *Pristionchus*, the genus *Parapristionchus*, is also found on scarab beetles in Asia (Kanzaki *et al.*, 2012). Note that a second branch of *Pristionchus* species has evolved after a basal host switch to figs and fig wasps. While these species show an extraordinary polyphenism of up to five distinct mouth-forms, these species are not culturable in the laboratory (Susoy *et al.*, 2016). Therefore, no RNA-seq-based transcriptomics are available for any of these species; thus, they are not included in the *Pristionchus* phylogeny depicted in Fig. 1.3. Finally, it is also important to note that *Pristionchus* sampling is far from saturation and completion, as the recent studies in Taiwan and China indicate (Kanzaki *et al.*, 2018; Yoshida *et al.*, 2018; Herrmann *et al.*, 2019; Kanzaki *et al.*, unpublished observation).

Due to the independent evolution of self-fertilizing hermaphroditism from gonochoristic (male/female) ancestors at least seven times throughout the genus *Pristionchus*, it is possible to assess the impact of these mating type switches on other aspects of biology. Indeed, by investigating 11 species (six gonochorists, five hermaphrodites) it has been observed that lifespan is considerably shorter in hermaphrodites relative to closely related females (Weadick and Sommer, 2016a). Also, females outlived males, with *P. exspectatus*, the sister species of *P. pacificus*, providing an extreme example as females have a maximum lifespan of 137 days and males only 76 days (Weadick and Sommer, 2016a). However, *P. exspectatus* exhibits an unusual sex-specific reproductive senescence as females lose the ability to reproduce on average after 4.7 weeks, although they expressed a median lifespan of 12.3 weeks (Weadick and Sommer, 2016b). In contrast, males lost their reproductive capabilities after 6.6 weeks with a median lifespan of 7.6 weeks (Weadick and Sommer, 2016b). These

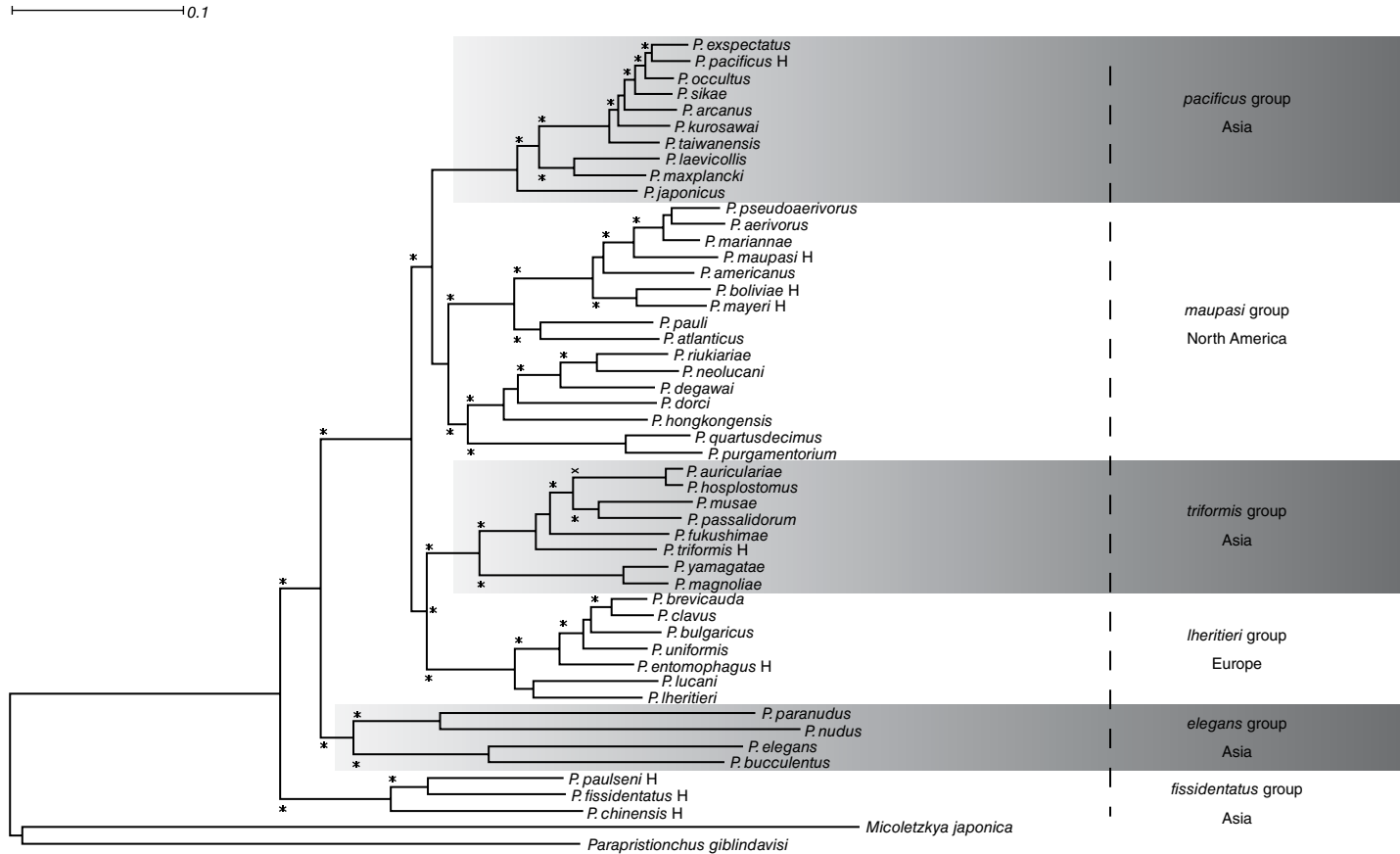


Fig. 1.3. Current phylogeny of the genus *Pristionchus* with 48 species, all of which are in living culture, and the outgroups *Paraprisionchus gibbindavisi* and *Micoletzky japonica*. This maximum-likelihood tree is based on transcriptomic datasets resulting in a supramatrix of 350,000 concatenated amino acids. Six species groups or clades can be distinguished that show biogeographic restrictions. Note that only one part of the *maupasi*-group is restricted to North America. Eight species show a hermaphroditic mode of reproduction (H). All other species are gonochorists (dioecious) with males and females. Given the distribution of these hermaphroditic species, hermaphroditism has evolved at least seven times independently in this genus, a record for nematodes. Asterisks indicate strong bootstrap support. Redrawn with modifications from Kanzaki *et al.* (2021).

findings established *Pristionchus* nematodes with their parallel evolution of mating type switching as model system for sex-specific senescence and post-reproductive survival (Weadick and Sommer, 2017).

The Life History and Ecology of *P. pacificus*

P. pacificus is a nearly cosmopolitan species that exhibits a huge genetic diversity with four major lineages as revealed by mitochondrial and microsatellite markers (Zauner *et al.*, 2007; Herrmann *et al.*, 2010). Note that Europe is the only continent where *P. pacificus* is not frequently found with only four isolates available. In contrast, samplings in North America, Bolivia, New Caledonia, South Africa, and several countries in Asia resulted in a huge collection of *P. pacificus* strains. Additionally, the first exploration of an island system revealed that the Mascarene island of La Réunion harbors *P. pacificus* in high abundance and on multiple beetle hosts (Herrmann *et al.*, 2010). La Réunion is the youngest (2–3 Ma) of the Mascarene islands and is known as a major biodiversity hotspot (Myers *et al.*, 2000; Thébaud *et al.*, 2009). Surprisingly, the genetic diversity of *P. pacificus* on La Réunion island mimics that of the rest of the world, arguing for multiple independent invasions, most likely in conjunction with different beetle hosts (Morgan *et al.*, 2012). These originally unexpected findings provided unique research opportunities in two different directions. First, intense population genetic studies coupled with mutation accumulation lines experiments revealed a nuclear mutation rate of $1.4\text{--}2.6 \times 10^{-9}$ and a diversity of the four major *P. pacificus* lineages of 4.3×10^6 generations (Molnar *et al.*, 2011; McGaughran *et al.*, 2013; Weller *et al.*, 2014). This divergence likely preceded the colonization of La Réunion island. Indeed, further studies of the different *P. pacificus* lineages on the island revealed complex demographic patterns also shaped by independent migrations (Morgan *et al.*, 2012; McGaughran *et al.*, 2013, 2016). Such migrations included recent events between La Réunion and the neighboring island Mauritius, which with 10–12 Ma is substantially older (Morgan *et al.*,

2014). Second, the geological, geographic, and ecological diversity of La Réunion with its extreme altitude and temperature differences allowed further investigations of how environmental factors influence population structure (McGaughran and Sommer, 2014). Not surprisingly, climatic variables are the most important factors influencing the genetic structure of *P. pacificus* on La Réunion. Additionally, the distinct beetle hosts with their uneven and often very local distribution strongly determine the distribution of *P. pacificus* lineages (Morgan *et al.*, 2012; McGaughran *et al.*, 2013). For example, one of the four *P. pacificus* lineages is restricted to high-altitude locations and largely to the endemic stag beetle *Amneidus godefroyi*. Detailed analysis of this association revealed a strong adaptation to the low oxygen concentrations at high altitudes resulting in oxygen-induced social behaviors (Moreno *et al.*, 2016). Taken together, these studies highlight the complex interplay between *P. pacificus* population structure, abiotic environmental factors, and beetle host biology. In particular, beetle host biology requires intense consideration to fully understand the novel complex traits seen in *P. pacificus*.

While the reliable association of *Pristionchus* worms with scarab beetles and some other soil arthropods has allowed the isolation and characterization of a large number of species in the relatively short time period of only 15 years, this type of association complicates the full understanding of the biology and the life cycle of *Pristionchus*. Adult scarab beetles, in particular chafers and stag beetles, are well known to the general public. However, their life cycle is complex, long-lasting and in large parts inaccessible. Many species have life cycles of several years with only the short-lived adult stage living above ground. After feeding and mating in trees, females go back into the soil (e.g., chafers) or dead wood (e.g., stag beetles), where they lay their eggs. After hatching, grubs spend years in the soil feeding on plant roots and debris before pupation. In many species, after hatching the young adults overwinter one more time before flying in spring; for example, the European cockchafer *Melolontha melolontha* has a 4-year life cycle, but animals are visible for only around 2 weeks as adults.

Through the association of *Pristionchus* with scarab beetles, a further layer of complexity is apparent as nematode beetle infestation rates vary between locality, year, and species (Herrmann *et al.*, 2006). It is, therefore, particularly difficult to perform empirical studies on the ecology of *P. pacificus*. In order to overcome this shortcoming, much empirical work has focused on the rhinoceros beetle *Oryctes borbonicus*, native to La Réunion (Herrmann *et al.*, 2010; Morgan *et al.*, 2012). *O. borbonicus* is known to have a *P. pacificus* infestation rate of roughly 90% on one particular meadow (Herrmann *et al.*, 2010). This unique prerequisite has facilitated experimental investigations by killing wild-caught beetles and burying them in specifically designed cages. These ongoing studies have resulted in a number of surprising findings. First, *P. pacificus* is exclusively found in the arrested dauer stage on the living beetle (Herrmann *et al.*, 2010; Meyer *et al.*, 2017). Second, dauer larvae of *P. pacificus* exit 1 week after the beetle's death to start feeding on the developing microbes at the carcass (Meyer *et al.*, 2017). Note that other nematodes also present on the rhinoceros beetle usually exit from the dauer stage prior to *P. pacificus*. Third, the microbiome of the nematode is largely identical to that of the living beetles but not of the surrounding soil (Meyer *et al.*, 2017). Fourth, recent studies, using larger cages with 27 sub-compartments and longer investigation periods of up to 3 months, revealed a biphasic boom-and-bust life cycle of *P. pacificus* centered around the beetle carcass (Renahan *et al.*, 2021). Reproducing animals are found only on the carcass, whereas dauer larvae disperse. Fifth, two peaks of dauer larvae formation have been observed on the carcass at 4 and 8 weeks after killing the insect, indicating an unexpected biphasic cycle. Interestingly, these cycles anti-correlate with bacterial abundance and with the abundance of reproducing nematodes. Finally, dauer larvae disperse surprisingly fast reaching the edge of the 15 × 15 × 15 cm cage after 6 weeks (Renahan *et al.*, 2021). While these experiments have helped clarify some of the basic aspects of the *P. pacificus* life cycle in association with a scarab beetle, many facets remain unknown, with current studies attempting to address these issues using increasingly complex experimental set-ups. However, these studies on the *P. pacificus* life cycle in

association with *O. borbonicus* provide a useful context for the evaluation of the complex traits to be discussed below.

Developmental Plasticity and Predatory Feeding

Mouth-form plasticity in *P. pacificus*

The most striking feature of *P. pacificus* is the plasticity of its stoma resulting in two alternative mouth-forms associated with omnivorous feeding strategies. The so-called “stenostomatous” (St) form has a narrow buccal cavity with a single dorsal tooth resulting in a strict bacterial feeding morph (Fig. 1.4A). Alternatively, the “eurystomatous” (Eu) form has a wide buccal cavity with a dorsal and a right subventral tooth permitting these animals additionally to feed on fungi and other nematodes (Fig. 1.4B). No intermediate mouth-forms exist and adult animals are fixed and can no longer switch their acquired mouth-form fate. The formation of these two alternative morphs is a prime example of developmental (phenotypic) plasticity, the ability of an organism to form distinct phenotypes under different environmental conditions. Developmental plasticity is an important concept in developmental biology and evolutionary biology, but empirical studies have long been scarce largely because controlling and manipulating plastic traits is difficult under laboratory conditions. During the past two decades, theoretical studies have pointed towards the importance of developmental plasticity for the evolution of novelty and as a previously neglected concept and mechanism of evolution (Pigliucci, 2001; West-Eberhard, 2003). As a result, experimental studies on various examples of plasticity have been initiated in the past decade. These studies not only highlight the significance of plasticity but also provide mechanistic insight into the underlying molecular, phylogenetic, and evolutionary processes (for recent review, see Sommer, 2020). Importantly, *P. pacificus*, with its hermaphroditic mode of reproduction and thus clonal propagation, is a powerful model to study developmental plasticity and the environmental influence on developmental decision making, as will be discussed below.

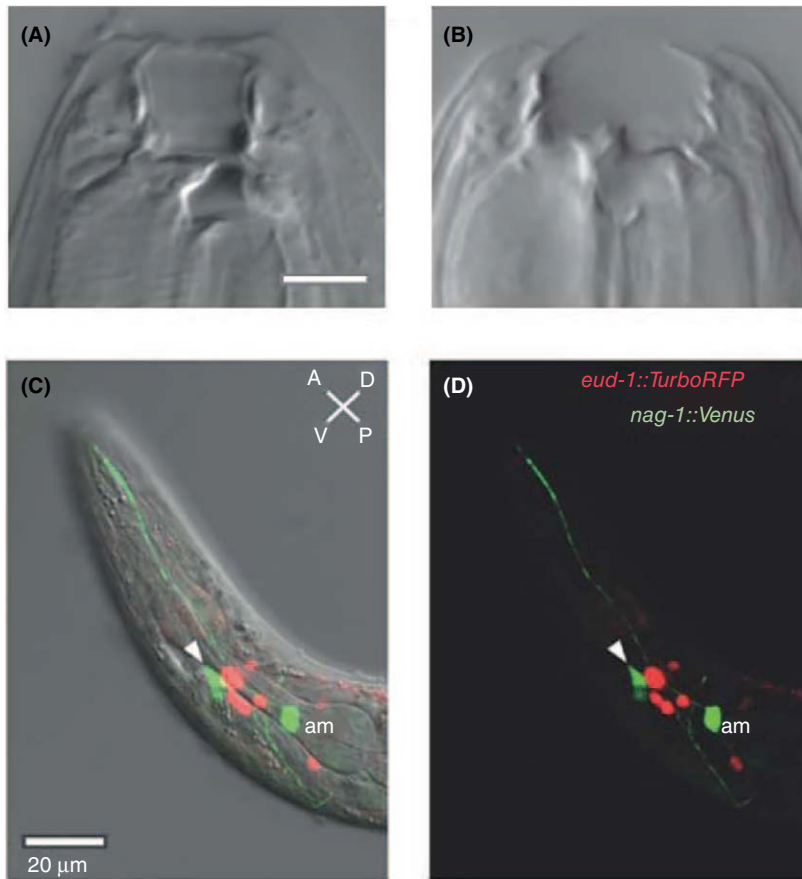


Fig. 1.4. Mouth-form plasticity in *P. pacificus*. **(A)** Stenostomatous morph animals are narrow-mouthed with a single tooth and are bacterial feeders. **(B)** Animals that acquire the alternative Eurystomatous morph have a wide buccal cavity with a dorsal and a right subventral tooth expanding their dietary range to include feeding on fungi and other nematodes. **(C)** The gene regulatory network behind the mouth-form decision is well characterized with several genes including *eud-1* and *nag-1* expressed in discrete sensory neurons. Redrawn from Sieriebriennikov *et al.* (2018).

Genetic basis and environmental influence

Most *P. pacificus* isolates form a stable strain-specific ratio of Eu:St animals independent of the phenotype of the mother. For example, the wild-type reference strain PS312 from California has a 70–90% Eu ratio. This ratio is found in progeny of both Eu or St mothers of the PS312 genotype. The mouth-form ratio of any given strain can be altered by environmental and genetic factors allowing specific manipulation and thus the elucidation of underlying molecular mechanisms. First, a simple shift of culture conditions

can reverse the mouth-form ratio of *P. pacificus* PS312. While growth on standard nematode growth medium (NGM) agar plates results in the 70–90% Eu ratio mentioned above, liquid culture conditions using the same *Escherichia coli* food source establishes a 90% St phenotype (Werner *et al.*, 2017). Note that variation in liquid culture medium, rotation speed, and other abiotic factors can result in any desirable mouth-form ratio (Werner *et al.*, 2017). Thus, the *P. pacificus* mouth-form plasticity can be very easily manipulated, making it a prime candidate for plasticity research.

Second, population density has a strong influence on mouth-form ratios and already early studies identified associated small molecules, such as the diascaroside *dasc#1* (Bose *et al.*, 2012). More recent work revealed that these population-based interactions are largely cross-generational, as *dasc#1* and other small molecules are mostly secreted by adult animals (Werner *et al.*, 2018a,b). These cross-generational interactions are likely of relevance in the wild, potentially influencing mouth-form ratios in developing progeny to better adapt to shifting environments. It is important to note that these mouth-form plasticity features show striking similarities with the regulation of dauer development, the plasticity of nematode life cycles that has been discussed in greater detail elsewhere (Sommer and Mayer, 2015).

Third, relatively constant mouth-form ratios of *P. pacificus* on agar plates allow unbiased forward genetic screens for the isolation of mutants with mouth-form deficient phenotypes. The first such genetic screen resulted in the isolation of *eud-1*, a *eurystomatous*-form defective strain that only forms St animals (Ragsdale *et al.*, 2013). *eud-1* is an unusual gene, which encodes a sulfatase. Mutants in *eud-1* are dominant so that hermaphrodites with a single mutant copy are already preferentially St. Such *eud-1* mutants are *loss-of-function*, a rare genetic phenomenon, already suggesting extreme dose sensitivity. Indeed, overexpression of *eud-1* in a *eud-1* mutant background can completely revert the all-St mutant phenotype into an all-Eu phenotype (Ragsdale *et al.*, 2013). Further, *eud-1* is located on the X chromosome; therefore, males with their XO genotype are preferentially St. However, overexpression of *eud-1* in males will result in all-Eu males indicating dose-dependence (Ragsdale *et al.*, 2013). Taken together, all these experiments indicate that *eud-1* is a classic genetic switch gene, as it is known from developmental genetic studies in multiple model organisms, including *C. elegans*. Most importantly, however, *eud-1* with its characteristics confirms a long-standing prediction of evolutionary theory and plasticity theory, namely that phenotypic plasticity relies on developmental switch genes that can sense the environment and subsequently direct alternative developmental decisions (West-Eberhard, 2003; Ragsdale *et al.*, 2013). While it was striking

that already the first genetic screen for *P. pacificus* mouth-form mutants identified such a switch gene, later research indicated that the situation is far more complicated, as indicated below.

***eud-1* and the gene regulatory network**

Subsequent investigations revealed that *eud-1* is a complex genetic locus. First, it contains an antisense transcript that acts to promote *eud-1* expression itself (Serobyán *et al.*, 2016). Second, *eud-1* is part of a multigene locus that contains two pairs of duplicated genes in a tandem inverted configuration (Sieriebriennikov *et al.*, 2018). The other gene pair, *nag-1* and *nag-2*, encode for N-acetyl-glucosaminidases, which in humans act on extracellular matrix proteins similar to sulfatases. Interestingly, mutants in *nag-1* and *nag-2* have a phenotype opposite to *eud-1*, resulting in all-Eu animals under all culture conditions. Most importantly, *eud-1*, *nag-1*, and *nag-2* are expressed in different sensory neurons (Fig. 1.4C). This finding further supports the notion that these genes are involved in sensing the environment and that the environmentally induced mouth-form switch is a network with modular organization.

This switch network not only senses the environment, but also directs a gene regulatory network (GRN), which finally executes one of the two alternative mouth-form phenotypes. More recent genetic studies have identified large parts of this GRN of mouth-form plasticity in *P. pacificus* (Kieninger *et al.*, 2016; Bui and Ragsdale, 2019; Sieriebriennikov *et al.*, 2020). Two conserved nuclear hormone receptors, *nhr-1* and *nhr-40*, are involved in transmitting environmental information to mouth-form decision-making processes (Kieninger *et al.*, 2016; Sieriebriennikov *et al.*, 2020). Interestingly, the nuclear hormone receptors NHR-40 and NHR-1 have only a small number of common targets, which show unexpected evolutionary patterns. All of these common targets are fast-evolving genes that have no 1:1 orthologs in *C. elegans*. In contrast, *nhr-1* and *nhr-40* themselves share 1:1 orthologs between *P. pacificus* and *C. elegans*, although, in general, NHRs evolve extremely rapidly (Sieriebriennikov *et al.*, 2020).

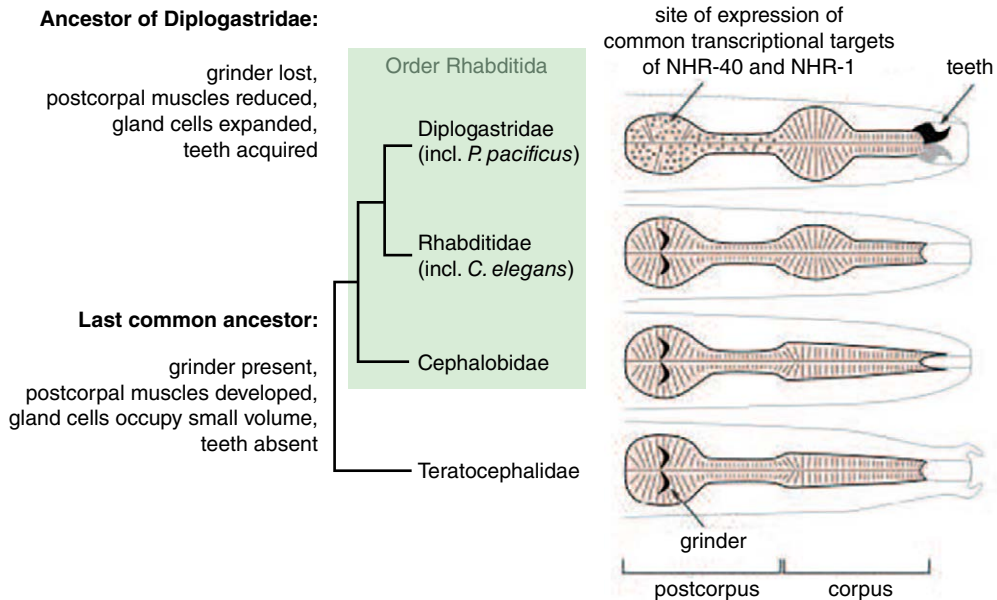


Fig. 1.5. Evolution of pharynx morphology in the order Rhabditida. The ancestral character state includes the presence of a grinder in the terminal bulb of the pharynx. In the Diplogastridae, the grinder has been lost and, concomitantly, muscle cells were reduced, gland cells expanded, and teeth are formed as an evolutionary novelty (top image). Redrawn from Sieriebriennikov *et al.* (2020).

The most remarkable aspect about the targets of NHR-40 and NHR-1 is their common expression in the single gland cell g1D (Sieriebriennikov *et al.*, 2020). Importantly, the gland cell g1D is the site of a major evolutionary innovation in *P. pacificus* and the whole Diplogastridae family (Riebesell and Sommer, 2017). Phylogenetic reconstruction indicates that the outgroup Teratocephalidae and the rhabditid families Cephalobidae and Rhabditidae represent the ancestral character state of mouth-associated characters, with: (i) a grinder; and (ii) five pharyngeal muscle cells; but (iii) no teeth (Fig. 1.5). In contrast, species of the Diplogastridae have: (i) no grinder; (ii) concomitantly gained teeth; and (iii) accompaniment of the loss of the grinder by the reduction of muscle cells in the postcorpus. This reduction of postcorpus muscle cells resulted in the expansion of three gland cells, g1D, g1VL, and g1VR, one in each sector of the laterally symmetrical pharynx (Fig. 1.5) (Riebesell and Sommer, 2017). It has been speculated that the functional remodeling of g1D, in which the target genes of NHR-40 and

NHR-1 are expressed and which, intriguingly, empties through the tip of the large dorsal tooth, may be a prerequisite for the formation of teeth and the evolution of predation (Sieriebriennikov *et al.*, 2020). Taken together, major morphological changes in the g1D gland cell accompanied the evolution of teeth, mouth-form plasticity, and predation, linking rapid gene turnover with morphological innovations and suggesting that the origin of feeding plasticity involved novelty at the level of genes, cells, and behavior.

Mouth-form plasticity and evolutionary novelty

What is the evolutionary significance of mouth-form plasticity in *P. pacificus*? Theoretical studies by Mary Jane West-Eberhard had already in 1989 suggested that developmental plasticity is a crucial step in the evolution of novel and complex traits (West-Eberhard, 1989). While one would think that the origin

of novelty has been a central question in evolutionary biology, this is actually not so. Mainstream evolutionary biology focuses largely on adaptive processes and on the ways in which genes are propagated. In contrast, the origin of novel traits in evolutionary lineages has found little attention. West-Eberhard's original proposal was heavily expanded in a monograph in 2003, which represents one of the major milestones in modern evolutionary biology (West-Eberhard, 2003). In a nutshell, she proposed three major predictions for the origin and evolution of novel and complex traits. First, novelty often starts as environmentally sensitive and developmentally plastic traits. Today, this phenomenon is also referred to as "Plasticity first evolution" or "Flexible stem hypothesis" (Gibert, 2017; Levis and Pfennig, 2019). Second, environmental sensitivity requires developmental switch genes that can integrate environmental information and finally direct alternative decision-making processes. The *eud-1* developmental switch described above was the first experimental confirmation of this important prediction (Ragsdale *et al.*, 2013). Finally, traits will not stay plastic indefinitely. Instead, environmental dependence will become genetically encoded in a process called "canalization" or "assimilation", a theory first proposed by Waddington (1975). Examples of plasticity, like the one discussed here for *P. pacificus*, can move the plasticity research field from theory to experiment and can address important challenges, which eventually will prove the significance of this phenomenon for evolution (Sommer, 2020).

Convergent evolution of predation in nematodes and organismal consequences

Finally, it should be briefly noted that *Pristionchus* and other diplogastrid nematodes are not the only group of predatory nematodes. Instead, predation has evolved multiple times by convergent evolution and is known mostly from soil but also from aquatic nematodes. One group, the Mononchida, is even exclusively predatory (Ahmad and Jairajpuri, 2010). In general, predatory

nematodes are found most frequently in undisturbed environments and many of these predators feed on other bacteriovorous nematodes; however, other invertebrates can be preyed upon as well. Mechanistically, predatory nematodes can be piercers or ingesters, as originally classified by Yeates *et al.* (1993). These different mechanisms and the convergent evolution of predation have resulted in multiple independent morphological adaptations. Therefore, the nematode head and the buccal cavity in particular have been subject to enormous evolutionary innovations, often complicating proper morphological interpretations that remain controversial. While a certain consensus has been reached in the past decades (Decraemer *et al.*, 2014), it should be noted that major uncertainties about the developmental origin and the structural composition of the nematode head remain, which is of particular importance for predatory species (Wright, 1987).

Unfortunately, most predacious nematodes cannot easily be cultured in the lab and so the studies described above for *P. pacificus* are largely unparalleled in any other predatory nematodes. Also, it is currently unknown if, when, and how often predation requires plasticity in the associated feeding structures. While current knowledge suggests that most predatory nematodes are non-plastic, this notion might result from study biases. For example, a recent investigation by Kanzaki and co-workers revealed that *Bursaphelenchus sinensis* exhibits a feeding dimorphism similar to *P. pacificus* with one predatory form that went previously unnoticed (Kanzaki *et al.*, 2019). Indeed, follow-up investigations revealed that the predatory morph of this species is similar to the morphology of *Berntsenus* Massey 1974, which thereby becomes a junior synonym of *Bursaphelenchus* Fuchs 1937 (Kanzaki and Giblin-Davis, 2020). Thus, many biological aspects associated with predation in nematodes remain to be investigated.

From Behavior to Neuroscience

Predatory behaviors

Alongside the mouth-form plasticity and the detailed ecology associated with *Pristionchus*, these

nematodes also show diverse behaviors not observed in other more commonly studied nematode species. Several of these behaviors are now being explored in earnest in order to understand the genetic, molecular, and neurobiological processes behind these behaviors and, importantly, how they may have evolved. The most obvious and dramatic of these behaviors is the previously mentioned predatory feeding (Fig. 1.6A), which depends on the development of the enlarged and paired teeth found in the Eu mouth-form to kill the larvae of other nematodes (Wilecki *et al.*, 2015). While only the dorsal tooth is moveable during predation, together the teeth structures are capable of puncturing through the cuticle of other nematode larvae with a high efficiency. As an interesting side note, in *Pristionchus* species the killing behavior appears to be entirely suppressed in the St mouth-forms; however, they do still scavenge on any available carcasses found in their vicinity (Wilecki *et al.*, 2015). Further, while predation has been observed in all *Pristionchus* Eu animals, the majority of studies have thus far concentrated on *P. pacificus*, which is the focus of the predatory descriptions below.

Upon contact with a potential prey larva, *P. pacificus* undergoes a feeding mode switch from a bacterial feeding mode involving rapid pumping of the pharynx and a stationary tooth, to a predatory feeding mode consisting of slower pharyngeal pumping and activation of the dorsal tooth in a 1:1 ratio with the pharyngeal action. Subsequent experiments have demonstrated that the predatory feeding mode in *P. pacificus* can be recapitulated in the absence of prey by the exogenous addition of the neurotransmitter serotonin and there appears to be an extra pair of serotonergic neurons in its pharynx in comparison to *C. elegans* (Fig. 1.6B) (Wilecki *et al.*, 2015). The additional function of serotonin and its importance in predatory feeding has been further emphasized through studies of mutating enzymes responsible for serotonin biosynthesis and through neuron ablation experiments. In both the serotonin-deficient mutants and in animals with ablated serotonergic neurons, the synchronicity of the pharyngeal pumping and tooth action is disrupted, which in turn also reduces the killing efficiency (Okumura *et al.*, 2017). Therefore, these studies have

begun to hint at some of the molecular and neurobiological mechanisms behind the predatory behavior with divergence and acquisition of additional functions in pathways activated through serotonin.

A further question at the heart of the predatory behaviors is understanding how *P. pacificus* senses its prey. This requires both the identification of the specific prey-sensing mechanism such as potential receptors involved and, equally importantly, the identification of the neuronal circuits that facilitate this behavior. Similar to the whole-animal connectome available for *C. elegans*, a neuronal wiring diagram consisting of the pharyngeal (Bumbarger *et al.*, 2013) and head sensory neurons (Fig. 1.6C) (Hong *et al.*, 2019) now exists for *P. pacificus*. Interestingly, these neurons show a remarkable degree of conservation in cell number and frequently cell body position between *P. pacificus* and *C. elegans*. There is, however, a clear divergence in synaptic connectivity and, in the case of the head sensory neurons, also structural differences. This is particularly apparent in the amphid neurons, which in *P. pacificus* lack the wing-shaped cilia morphology found in *C. elegans* (Hong *et al.*, 2019). Despite these differences, 1:1 homology has been assigned between *P. pacificus* and *C. elegans* pharyngeal and head sensory neurons with minimal ambiguity. Therefore, investigation of these circuits will help to enable identification of those neurons essential for predation and how they may differ from other non-predatory nematodes.

Some clues to the identification of the sensory neurons involved in the prey-recognition circuits have recently come to light through mutations affecting the growth and function of the cilia. Similar to observations in *C. elegans*, in *P. pacificus* many of the head sensory neurons also possess cilia at the end of their dendritic processes and these structures have been shown to be involved in environmental perception. Interestingly mutants defective in cilia formation, while still capable of some degree of predation, appear defective for efficient prey detection, as the frequency of prey biting is strongly reduced (Moreno *et al.*, 2019). Coinciding with this, these mutants also show abnormal mouth-form ratios, reinforcing the importance of environmental sensing for correct mouth-form development.

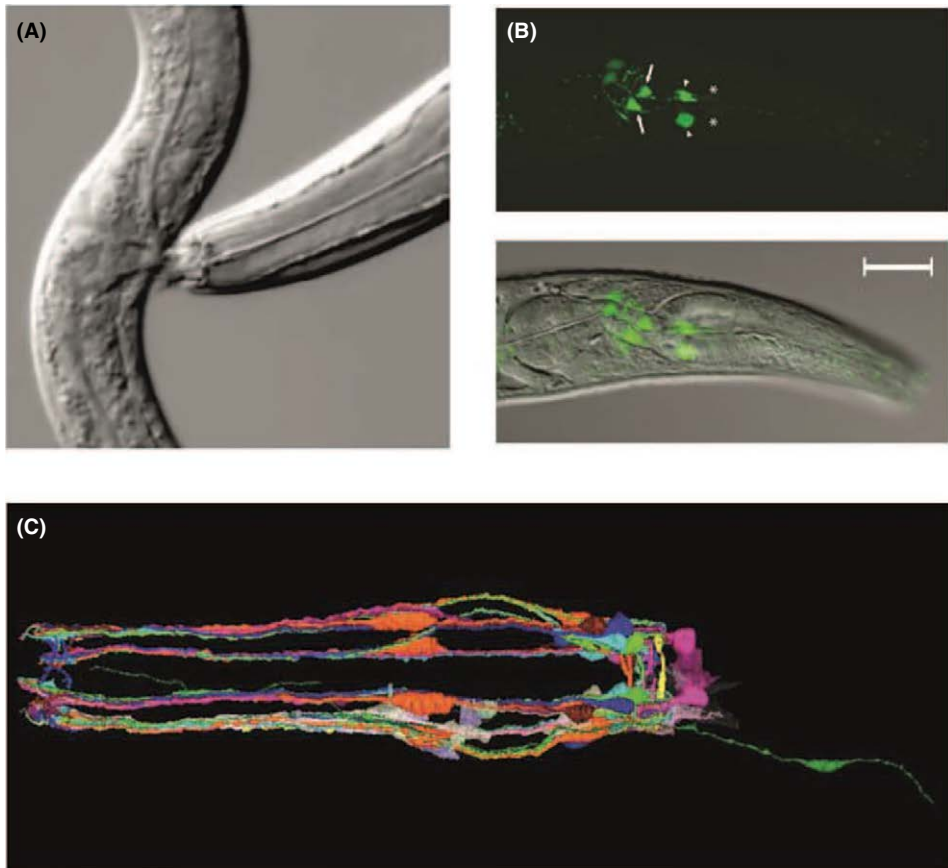


Fig. 1.6. Neurobiology of predation. **(A)** *Pristionchus* animals which develop the Eurystomatous mouth-form are voracious predators, capable of killing and feeding upon the larvae of other nematodes. In this image, a *C. elegans* larvae (left nematode) has fallen prey to a *P. pacificus* predator (right nematode). **(B)** The neurobiology behind the predatory feeding behavior is currently being elucidated, with serotonin playing a key role in regulating the predatory tooth action. Using anti-serotonin antibodies in *P. pacificus*, neurons probably corresponding to NSM (arrowheads), ADF (arrows) and I1 (asterisks) can be detected. Redrawn from Wilecki *et al.* (2015). **(C)** Connectomes provide a powerful tool for dissecting the circuits behind behaviors. Using TEM sections, the connectome for both the pharynx and the head sensory circuit (shown here) is available for *P. pacificus*, revealing distinct morphological structures and synaptic connections from *C. elegans*.

Environmental sensing and its associated behaviors

While predatory feeding offers one of the most striking and obvious behavioral differences between *Pristionchus* species and many other nematodes, there are several other intriguing behavioral distinctions. Indeed, *Pristionchus* demonstrates sophisticated environmental sensing mechanisms, which are likely adapted to the specific environments in which these species are found. Firstly,

Pristionchus displays remarkable chemosensory abilities and particularly strong attraction to the volatile pheromones associated with certain beetle species (Hong and Sommer, 2006; Hong *et al.*, 2008). It is therefore logical to assume that this chemical attraction probably facilitates the host-seeking in *Pristionchus*, aiding in the colonization and dispersal to new environments (Cinkornpumin *et al.*, 2014). Second, in a similar manner to the insect host-seeking behaviors, *Pristionchus* also finds specific bacteria attractive

or repellent. In chemotaxis experiments utilizing *P. pacificus* and bacteria found naturally associated with it, many of the most repellent bacteria were also toxic or pathogenic. In contrast, bacteria that were strongly attractive were frequently sources of high nutrition, indicating a far-ranging ability to ascertain environmental cues and generate an appropriate behavioral response (Rae *et al.*, 2008; Akduman *et al.*, 2018). Furthermore, it has been subsequently demonstrated that several of these bacteria are also capable of modulating the predatory feeding behaviors, with the production of vitamin B₁₂ by some of these bacterial species being one of the key influences increasing *P. pacificus* killing behaviors (Akduman *et al.*, 2020). Finally, experiments have also been carried out on the perception of other environmental stimuli in *P. pacificus*, in particular the occurrence of oxygen-induced social behaviors already mentioned above. In *C. elegans* the majority of wild isolates display clumping and bordering behaviors, which is thought to help avoid hypoxia under laboratory conditions. Contrary to this, most wild *P. pacificus* isolates demonstrate solitary behaviors indicating a distinct evolutionary history, genetic regulation, and sensory mechanism behind this behavior (Moreno *et al.*, 2016). Thus, *Pristionchus* shows a sensory and behavioral identity, which differs from other nematodes.

Self-recognition and Cannibalism

With contact between *Pristionchus* and other nematode larvae eliciting such an aggressive response, and as predation is so prevalent in the *Pristionchus* genus, a fundamental question has arisen from these behaviors, namely: do *Pristionchus* also kill and cannibalize their own progeny or do they perhaps have a self-recognition system to avoid this costly outcome? A recent study tested this by analyzing several *Pristionchus* species, which were fed upon larvae of either other nematode species or their own self-progeny. In all of the *Pristionchus* species assessed, it was clear that while predators showed frequent killing behavior towards other nematode species, there was no killing of self-progeny, indicating the presence of a robust self-recognition system

(Lightfoot *et al.*, 2019). This finding was all the more significant because, while thousands of researchers all around the world study many aspects of biology in *C. elegans* and other nematodes, this was the first reported example of an organismal self-recognition behavior described in nematodes.

Further experiments on mixed prey cultures with *P. pacificus* predators demonstrated that the self-recognition system requires direct nose contact with larvae and hinted at a cuticle-bound self-recognition signal while ruling out any significant role for small signaling molecules. Additionally, and perhaps most importantly, it was shown that not only are *P. pacificus* capable of distinguishing between other nematode species, but also in fact the self-recognition system shows an incredible degree of specificity, as these nematodes are also capable of distinguishing and killing other closely related *P. pacificus* strains. These results paved the way for subsequent genetic crosses and mapping experiments between different strains of *P. pacificus*, which kill one another, and from this a single gene was identified encoding one of the major components of the self-recognition signal. This gene was named *self-1* for self-recognition defective and in the main *P. pacificus* laboratory strain PS312 it was shown to encode for a small peptide of only 63 amino acids in length. Through further analysis, it was demonstrated that SELF-1 also included a hypervariable region in which it was commonly found to contain differences between diverse strains. Targeted CRISPR-Cas9 experiments reinforced the importance of this domain in generating the self-recognition specificity as even subtle amino acid changes including a Serine > Isoleucine and Isoleucine > Leucine mutation within this region are enough to disrupt the self-recognition system. Finally, through a transcriptional reporter, the SELF-1 small peptide was observed to be expressed in all the hypodermal cells throughout all life cycle stages.

The identification of *self-1* as a major part of the self-recognition signaling machinery in *P. pacificus* is a significant first step to our understanding of these behaviors in nematodes. However, *self-1* mutants show no receptor phenotype; thus, *self-1* is unlikely to be the only component in this complex behavioral mechanism. As such, the identification of any receptor

responsible for the detection of SELF-1 will be pivotal for future studies and additionally it will be important to determine whether SELF-1 is the only signal involved. Furthermore, while self-recognition behaviors are ubiquitous across the natural world and found in creatures as diverse as rotifers (Gilbert, 1976) and tunicates (De Tomaso *et al.*, 2005) to frogs (Villinger and Waldman, 2008) and octopuses (Nesher *et al.*, 2014), organisms with the necessary molecular tools required to dissect these behaviors are few and far between. Thus, the self-recognition behaviors in *Pristionchus* establish it as an emerging and potent biological system capable of providing a mechanistic understanding of self-recognition and delivering new insights into how these behaviors are regulated and how they may have evolved.

Complex Small Molecule Chemistry

Mouth-form plasticity, predatory feeding behavior, and self-recognition represent spectacular examples of *Pristionchus* biology that show the diversity of evolutionary innovations to be found in this particular nematode taxon. Associated with these key innovations are a number of chemical novelties resulting in an unexpected and extreme diversity of small molecule structures. So far, three groups of previously unknown nematode metabolites have been characterized in *P. pacificus* and its relatives. First, unlike the dauer larvae of other nematodes, *P. pacificus* and other diplogastrid dauers swim on water (Ogawa *et al.*, 2009; Penkov *et al.*, 2014). This observation suggested the presence of a hydrophobic substance that would prevent them from sinking to the bottom of a water column. Indeed, subsequent chemical investigations revealed a wax-type lipid that through chemical characterization and NRM analysis was shown to be an ester with a mass-to-charge (m/z) ratio of 866.7677 and the formula $C_{60}H_{120}O_2$ (Fig. 1.7) (Penkov *et al.*, 2014). This molecule was named “nematol” and represents one of the largest known waxes in the animal kingdom. While the biosynthesis of nematol and its secretion to the body surface are currently unknown, the wax allows the dauer larvae to stick together in larger groups.

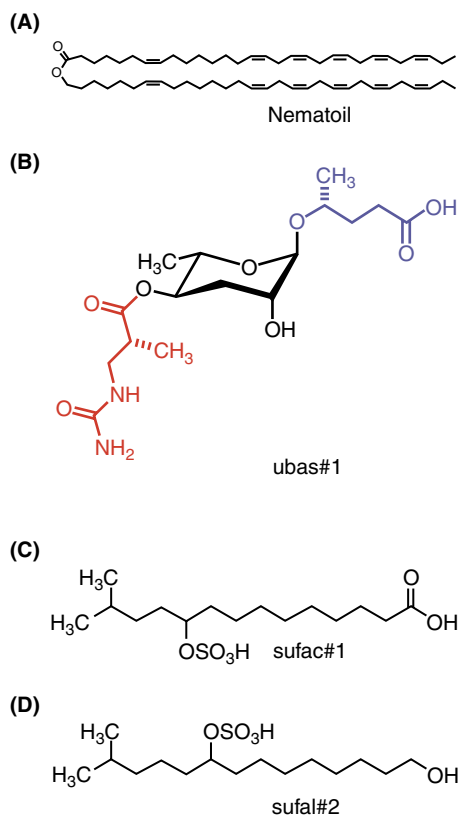


Fig. 1.7. Chemical innovation in *P. pacificus*. Structures of nematol (A), which is produced by dauer larvae and allows them to swim on water. Ubas#1 (B) is one of several complex NDMMs involved in cross-generational population density-dependent dauer induction. Sufac#1 (C) and Sufal#2 (D) are two representatives of sulfolipids in *P. pacificus*. (Courtesy of F.C. Schroeder.)

Indeed, large so-called “dauer towers” are seen on old agar plates when many animals have entered the dauer stage. Penkov and co-workers have speculated that these structures might be helpful in the wild for dauer larvae to find an appropriate host (Penkov *et al.*, 2014).

Second, detailed analysis of lipid chemistry in *P. pacificus* identified a large number of sulphated lipids, unknown from *C. elegans* and other nematodes (Fig. 1.7) (Liu *et al.*, 2018). Liu and co-workers found that *C. elegans* when exposed to these *P. pacificus* sulfolipids will initiate an immediate escape behavior. Thus, a predator-evoked signal consisting of sulfated lipids induces a

response behavior in potential prey. However, it is currently unknown if only *C. elegans* or also other potential prey of *P. pacificus* will initiate similar escape behaviors. It is interesting to note that the large number and amount of sulfated lipids produced by *P. pacificus* coincides with a strong increase in sulfatases and sulfotransferase encoded in the *P. pacificus* genome. Relative to *C. elegans*, *P. pacificus* has roughly three times as many genes encoding for these enzymes (Dieterich *et al.*, 2008).

Finally, nematode derived modular metabolites (NDMMs) that were first characterized in *C. elegans* and shown to regulate many life history traits, including dauer induction (for review, see Schroeder, 2015), are also known from *P. pacificus*. Indeed, dauer development and mouth-form plasticity are controlled by population density relying on specific NDMMs. However, the NDMMs characterized in *P. pacificus* showed an unexpected diversity of molecular building blocks including amino acid, nucleoside, and fatty acid modules (Bose *et al.*, 2012; Yim *et al.*, 2015). For example, ubas#1 was one of the first of these NDMMs to be synthesized and subsequently demonstrated to regulate dauer induction (Fig. 1.7) (Bose *et al.*, 2012). Most recent studies indicated that these NDMMs undergo extremely rapid evolution. Specifically, the analysis of around 30 *Pristionchus* species revealed strong species-specific patterns of NDMMs, with many of the most complex NDMMs being found in only two of the four clades of *Pristionchus* (Dong *et al.*, 2020). In contrast, basal *Pristionchus* species and other diplogastrids do not form many of the complex NDMMs known from *P. pacificus*. These surprising findings suggest rapid evolutionary innovations in small molecule chemistry (Dong *et al.*, 2020). Note that, similar to other groups of organisms, including plants and insects, the majority of natural products synthesized by these nematodes awaits functional characterization. However, a handful of NDMMs has been functionally characterized in *P. pacificus* revealing roles in dauer formation and mouth-form development (Bento *et al.*, 2010; Bose *et al.*, 2012, 2014) and similarly, the biosynthetic pathways involved in the formation of these complex NDMMs is beginning to be investigated (Markov *et al.*, 2016; Falcke *et al.*, 2018). Taken together, *P. pacificus* combines major innovations

and novelty in biology and chemistry and might represent a rewarding system for further chemical investigations.

Conclusions and Outlook

The development of *P. pacificus* as a model system for integrative evolutionary biology encompassing parallel investigations in evo-devo, population genetics, and ecology was initiated in the 1990s by the senior author of this contribution as a research fellow with Paul W. Sternberg at the California Institute of Technology in Pasadena, California (USA). By that time, the undescribed *P. pacificus* strain PS312 in the Sternberg lab collection drew immediate recognition due to the many parallels it shared with *C. elegans*, in particular with regard to its growth and propagation in the lab. Additionally, the comprehensive taxonomy and systematics of nematodes by Andrassy (1984) indicated that *Pristionchus* belongs to a nematode family with exciting associations with a diversity of insects and other arthropods. While the family of Diplogastridae was clearly understudied relative to the Rhabditidae at the time, studies initiated in the late 1990s would reveal that, in contrast to the rhabditids, the diplogastrids represent a monophyletic taxon (Holterman *et al.*, 2006; von Lieven and Sudhaus, 2000). Subsequent and parallel investigations by the laboratories of James Baldwin (Riverside), Robin Giblin-Davis (Fort Lauderdale), Natsumi Kanzaki (Kyoto), our own one, and those of others have provided a wealth of knowledge on these excitingly diverse nematodes, their spectacular associations and nearly never-ending evolutionary innovations. While a monograph published in 2015 gave a detailed overview about the knowledge on *P. pacificus* at the time (Sommer, 2015), this contribution focuses on the more recent research on complex and novel traits.

The examples of mouth-form plasticity, predatory behavior, and self-recognition, together with beetle host finding and associated neuronal circuits, represent a rich collection of conserved and divergent patterns and processes in animal biology. Furthermore, with its major advancements in omics approaches, biochemistry,

reverse genetics through CRISPR-Cas mediated tools, and sophisticated laboratory culturing set-ups, *P. pacificus* has an almost unrivaled tool kit with which to obtain a molecular-level comprehension of these biological events. However, a full understanding of the range of morphological, physiological, and behavioral adaptations of *P. pacificus* will require detailed comparative

studies involving the diversity of around 50 *Pristionchus* species currently available as living or frozen cultures and the more than 1 500 *P. pacificus* isolates. Therefore, with these ongoing investigations and future work exploring *P. pacificus* and its relatives, this genus of nematodes will undoubtedly be contributing to many more significant discoveries in the future.

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2 Aging

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Introduction

What is aging? There are many definitions of aging, including: what happens over time; the natural changes in tissues, organs, and cells over a period of time; the increased likelihood of death; or simply the time between birth and death (adapted from Arking, 1998). In the scientific world, the biology of aging consists of two separate ideas: organismal aging and cellular senescence. This chapter focuses on the use of *Caenorhabditis elegans* in studying the aging process and understanding organismal aging. *C. elegans* is known as a model system such that studies using this organism will model humans, thereby gaining insights into the biology of organismal aging. Over the past three decades, studies using *C. elegans* have significantly advanced our understanding of the aging process. Research on organismal aging has been asking the question: how long does an organism live? This contrasts with cellular senescence, for which the focus is on addressing questions pertaining to the individual cell. Since *C. elegans* hermaphrodite adults have only 959 somatic cells in the entire organism, studies have addressed the underlying mechanism for the aging process at the organismal level. However, in studying the aging process, additional questions need to be addressed. What organism are we going to use to study the aging process in the laboratory? What do we want to learn about the aging process?

This chapter considers how *C. elegans* has provided seminal knowledge for our understanding of the biology of aging. We examine the identification of genes and regimens that influence longevity, including the insulin/insulin-like growth factor (IGF)-1 signaling (IIS) pathway and more recent studies that have expanded our knowledge and provided insights into the contributions of tissues, cellular processes, and trans-generational control. We provide a historical overview of the discovery of the IIS in longevity regulation and examine caloric restriction, a second major nutrition-related intervention that modulates lifespan. We realize that we have not covered every aspect of the aging process. We refer to some excellent reviews on additional subjects (Kapahi *et al.*, 2017; Kim and Webb, 2017; Maynard and Weinkove, 2018; Guest, 2019; Xia *et al.*, 2019; Kinser and Pincus, 2020; Zhang *et al.*, 2020).

Advantages of Worms for Aging Studies

What would make an organism a good system for aging studies? There are many criteria to answer this question, but the central dogma seems to be: what is the best system to help us understand human aging? *C. elegans* is one example of

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a model organism or model system. The model refers to a “model” for humans that can be experimental and used in a laboratory. For studies on the biology of aging, typically one first examines lifespan. Unfortunately, usually, the closer one gets evolutionarily to humans, the longer the lifespan, and, accordingly, the shorter the lifespan the further one gets evolutionarily from humans. The nematode *C. elegans*, a roundworm, has been a premier system for studies on the aging process. The following are some of the reasons why.

1. *C. elegans* can be easily grown and cheaply grown in the laboratory.
2. *C. elegans* has a short lifespan (2–3 weeks, depending on the temperature).
3. Points 1 and 2 are especially important for aging, as the researcher can easily and with a modest budget identify mutants that shorten or lengthen average lifespan by as little as 10–15% and still be of statistical significance.
4. Genetic tools are ample, including the *Caenorhabditis* Genetics Center (vast collection of strains available) and an RNAi library where approximately 80% of the genes in the genome are represented, which, together, allows one to generate transgenic strains, perform gene editing, and conduct genetic screens easily and cheaply.
5. Molecular tools include a sequenced and annotated genome, and the *C. elegans* database, WormBase (<https://www.wormbase.org>, accessed 16 March 2022).

Therefore, there are many benefits to using *C. elegans* as a model system for studies on the aging process.

Why has *C. elegans* been used so successfully for aging research? As suggested by Sydney Brenner in 1963, the ability to grow large quantities of worms in the lab easily and cheaply is very helpful for aging research, in particular when identifying long-lived mutants, where animals must be kept alive for a long period of time. The relatively short and largely invariant lifespan of *C. elegans* is a great benefit.

Aging and Lifespan

What do we want to know about aging? Most studies on aging have used the measurement of lifespan as a proxy for aging, since lifespan is

a single measurement that is easily defined. The measurement of lifespan is also easily translatable across different organisms. Lifespan in *C. elegans* is measured simply by counting the days the animal is alive. The animal is prodded/poked by a platinum wire every 1–2 days and recorded if it responds. Notably, the assay is quite reproducible and has resulted in the identification of hundreds of genes, regimens, and chemicals that modulate lifespan, resulting in either lifespan extension or lifespan reduction. Genes that alter lifespan have been identified through large-scale genetic mutation and RNAi screens (Klass, 1983; Lee *et al.*, 2003a; Hamilton *et al.*, 2005; Hansen *et al.*, 2005; Lee, 2006). To date, lifespan has been used as a proxy for measuring aging, since there is no laboratory/experimental definition of aging.

However, lifespan and aging are not the same. Importantly, aging is not a singular event. It is a process and should not be defined as a single measurement. Lifespan does not give any indication of the quality of life, be it frailty or active living. For example, lifespan gives little indication of how the organisms live: are they healthy, or are they frail? Therefore, lifespan and aging should not be used interchangeably (reviewed in Lithgow, 2006; Tissenbaum, 2012). Nonetheless, this chapter focuses on lifespan regulation because, thus far, lifespan has been used uniformly to measure aging in *C. elegans*.

Initially, there was a large push to identify a biomarker of aging. What would such a biomarker look like? According to a review by Johnson (2006), a biomarker of aging would essentially be a predictor of functional capability of the organism at some later age, rather than simply its chronological age. However, more than a decade's worth of research failed to identify an evolutionarily conserved marker that would be easily testable and would monitor a basic process that underlies the aging process and predicts the rate of aging.

Healthspan

Lifespan as a single measurement gives no indication into the health of the animal. Therefore, although many genes and regimens modify lifespan, it is unclear whether any of them impact the health of the worm; for example, does a

long-lived animal spend more time active and free from disease or live in a state of prolonged frailty? It is for these reasons that studies have more recently begun to evaluate the health or “healthspan” of an animal in addition to lifespan.

Healthspan, as opposed to lifespan, does not have a distinct definition or a defined assay not within the worm or across phylogeny. Healthspan implies the length of time an animal is healthy, but how to define health? How to define health in the laboratory? How to define health in the laboratory in *C. elegans*? One definition of healthspan is the length of time an animal exhibits “youthful” behavior, or the absence of defined signs of aging (Blackwell *et al.*, 2019). But how can we measure this?

Thus far, several *C. elegans* studies have looked at age-associated phenotypes such as changes in movement capacity with age (Herndon *et al.*, 2002; Huang *et al.*, 2004; Collins *et al.*, 2008). For healthspan, the following phenotypes have been used either individually or in combination: (i) movement; (ii) pharyngeal pumping (feeding); (iii) lipofuscin accumulation; and (iv) neuronal changes (Herndon *et al.*, 2002; Huang *et al.*, 2004; Pan *et al.*, 2011; Tank *et al.*, 2011; Keith *et al.*, 2014; Bansal *et al.*, 2015). It should also be noted that the term healthspan is not a singular measurement but should refer to the animal's health over the time the animal is alive. Additionally, the metric should change over time or over the lifespan of a wild-type animal. The limitations of healthspan analyses are that, without a defined established set of assays, manuscripts pick and choose somewhat at random the health metric that accompanies lifespan. To date, research on the aging process still relies heavily on lifespan.

History of *C. elegans* for Aging Research

Studies on the aging process initially focused on the ability to isolate synchronous cultures of aged worms and measure lifespan (Klass, 1977; Hosono, 1978a,b). Therefore, from the beginning, lifespan was used to measure aging. Klass (1977) first examined how temperature and food quantity could alter lifespan. Klass (1983) performed pioneering research with the first genetic screen to identify mutants with changes

in lifespan. This study identified a long-lived mutant that was later revealed to be the gene *age-1* (Friedman and Johnson, 1988). Therefore, the initial identification of a gene that alters lifespan was *age-1* by the Johnson Laboratory (Friedman and Johnson, 1988).

Five years later, a second gene that altered lifespan was identified: the gene *daf-2* (Kenyon *et al.*, 1993). Both *age-1* and *daf-2* mutants exhibited lifespan extension (Friedman and Johnson, 1988; Kenyon *et al.*, 1993). With the identity of two mutants that altered lifespan, the idea that the aging process had a genetic component (lifespan) was beginning to emerge and *C. elegans* became the choice model system to pursue studies on the aging process.

The Insulin/IGF-1 Signaling Pathway

The power of *C. elegans* genetics was the first tool for investigation into the aging process. Phenotypes that were easily visualized under a dissecting microscope helped to propel the studies. Both *age-1* and *daf-2* mutants were originally isolated genetically based on a different phenotype: dauer formation with *age-1* also isolated as the gene *daf-2.3*. Under normal growth conditions, *C. elegans* develop into self-fertilizing, reproductive hermaphrodites. In contrast, under conditions where there is an increased amount of the continuously secreted pheromone, such as crowding, food scarcity, and high temperature, worms enter an alternative developmental stage at L3, forming dauer larvae. It should be noted that high levels of pheromone are necessary for the formation of dauer larvae. The *age-1* and *daf-2* genes were originally identified as dauer constitutive mutants where these mutants would form a dauer larva even under favorable growth conditions (Albert *et al.*, 1981; Albert and Riddle, 1988).

Prior genetic analyses by several laboratories had positioned the genes *age-1* and *daf-2* and another gene, *daf-16*, on separate pathways based on genetic epistasis analysis with the dauer phenotype. *Daf-16* mutants are classified as “dauer defective”, such that growth in poor conditions will not promote worms to form a dauer (although when forced with a high amount of pheromone, some *daf-16* alleles can form dauers) (Thomas *et al.*, 1993; Gottlieb and Ruvkun, 1994; Ogg *et al.*, 1997). Genetic epistasis analysis

revealed that a mutation in *daf-16* could suppress all the mutant phenotypes, including dauer, and longevity of either an *age-1* or *daf-2* mutant (Thomas *et al.*, 1993; Gottlieb and Ruvkun, 1994; Dorman *et al.*, 1995).

In 1996–1997, molecular cloning of *age-1*, *daf-2*, and *daf-16* confirmed what the genetic analysis had revealed: *age-1*, *daf-2*, and *daf-16* encoded for an IIS pathway. Molecular studies revealed that the *daf-2* gene encoded for a receptor that was equally identical to both the insulin receptor and the IGF-1 receptor (Kimura *et al.*, 1997) and therefore this pathway is often called the IIS pathway. Molecular cloning revealed that *age-1* encoded for the catalytic subunit of phosphatidylinositol (PI); PI 3-kinase (p110) (Morris *et al.*, 1996) and *daf-16* encoded a forkhead box O transcription factor, most closely related to mammalian FOXO3A/FKHRL1 (Lin *et al.*, 1997; Ogg *et al.*, 1997). Therefore, these identifications suggested an IIS pathway-regulated lifespan (Wolff and Dillin, 2006; Kenyon, 2011).

Although the genome encodes for a single IIS receptor, there are 40 different insulins each with distinct expression patterns (Ritter *et al.*, 2013). Genetic and expression analysis has provided valuable insight (Cornils *et al.*, 2011). However, without biochemical analysis showing a direct interaction between an insulin and the DAF-2 receptor, it is unclear what the precise insulin ligand is. Genetic and molecular analysis has suggested that the insulins INS-18, INS-7, DAF-28, INS-4, and INS-1 are putative DAF-2 ligands. Interestingly, recent work from the Gill laboratory (Martinez *et al.*, 2020) suggests that alternative splicing of DAF-2 results in a second isoform, DAF-2B.

Studies suggest that although DAF-2B is a non-signaling isoform, IIS can be modulated through the ability of DAF-2B to sequester various insulin peptides (Martinez *et al.*, 2020). This contributes to our understanding of the large number of insulin peptides and the complexity of the IIS pathway.

Downstream of the DAF-2 insulin/IGF-1 receptor (Kimura *et al.*, 1997) is a well conserved PI 3-kinase signaling cascade that includes the serine/threonine kinases AGE-1/AAP-1, PDK-1, AKT-1, AKT-2, and SGK-1, and also includes the phosphatases DAF-18 and PPTR-1, which function to control and counterbalance the kinase activity. *C. elegans* lipid phosphatase DAF-18,

homologous to the mammalian phosphatase and tensin homolog (PTEN) (Ogg and Ruvkun, 1998; Gil *et al.*, 1999; Mihaylova *et al.*, 1999; Rouault *et al.*, 1999), functions as a negative regulator of the IIS pathway; and PPTR-1 is a PP2A regulatory subunit that functions to dephosphorylate AKT-1 (Padmanabhan *et al.*, 2009) and subsequently modulates the *C. elegans* insulin/IGF-1 signaling pathway. Ultimately the IIS pathway functions to negatively regulate the translocation in and out of the nucleus of *C. elegans* FOXO/DAF-16 (Ogg *et al.*, 1997; Henderson and Johnson, 2001; Lee *et al.*, 2001).

Molecular genetic analysis has revealed that under normal growth conditions, as in wild-type, the DAF-2 receptor is bound by ligand and the upstream kinases AKT-1, AKT-2, and SGK-1 sequester DAF-16 in the cytoplasm by direct phosphorylation (Lin *et al.*, 2001; Hertweck *et al.*, 2004) and therefore DAF-16 is unable to bind its target genes. In contrast, in low signaling conditions, such as in a *daf-2* mutant, there is no binding of ligand. The upstream kinases will not phosphorylate DAF-16 and therefore DAF-16 can enter the nucleus to bind its thousands of direct target genes, which can result in the activation or repression of these target genes (Henderson and Johnson, 2001; Lee *et al.*, 2001; Lin *et al.*, 2001; Hertweck *et al.*, 2004; Oh *et al.*, 2006). Phenotypically, reduction-of-function mutations in *daf-2* and the kinases upstream of DAF-16 lead to changes such as increased lifespan, dauer formation, fat deposition, and/or increased stress resistance (Kenyon, 2004, 2005, 2011; Wolff and Dillin, 2006; Antebi, 2007). Therefore, DAF-16 is the major downstream target of the IIS.

DAF-16, a transcription factor and member of the forkhead box O transcription family, directly binds to thousands of targets to regulate many different biological processes in addition to lifespan, including metabolism, stress resistance, dauer formation, and genomic stability (Lee *et al.*, 2003a; Schuster *et al.*, 2010; Kumar *et al.*, 2015; Webb *et al.*, 2016; Farina *et al.*, 2017). DAF-16 itself is modulated by direct phosphorylation, and interacts with many proteins, including 14-3-3 proteins. In addition, for lifespan modulation, DAF-16 has been shown to interact directly with JNK-1 (the *C. elegans* homolog of c-Jun N-terminal kinase) (Oh *et al.*, 2005) and CST-1 kinase (the *C. elegans* homolog

of mammalian ste20-like kinase (MST)) (Lehtinen *et al.*, 2006).

Although DAF-16 is directly phosphorylated by multiple kinases, including AKT-1, AKT-2, SGK-1, and AMPK, a DAF-16 phosphatase has yet to be identified. DAF-16 thus far relies on the ubiquitin system for degradation. Reduction of function mutation in the E3 ubiquitin ligase *rle-1* (Li *et al.*, 2007) extends lifespan whereas reduction of function mutation in the deubiquitylase, *math-33*, similar to a reduction of function mutation in *daf-16*, suppresses the longevity of *daf-2* mutants (Heimbucher *et al.*, 2015).

There have also been several “co-factors” of DAF-16 that exhibit a similar longevity phenotype to a reduction of function mutation in *daf-16*, namely the ability to suppress the extended longevity of *daf-2* mutants including heat shock factor transcription factor 1, or *hsf-1*, *skn-1* (*C. elegans* homolog of nuclear respiration factor 2/Nrf2) and *pqm-1* (C2H2-type zinc finger and leucine zipper-containing protein). For more details see reviews by Sun *et al.* (2017) and Tissenbaum (2018).

The IIS has been coupled to tissue requirements. Several studies have examined the importance of tissues including muscle, hypodermis,

intestine, and the nervous system. Experiments involving tissue-specific RNAi and generation of transgenic strains showed that the primary tissue for lifespan regulation by the IIS is the intestine and that the nervous system accounts for 10–15% (Wolkow *et al.*, 2000; Libina *et al.*, 2003).

The evolutionary conservation of the IIS has furthered its importance for longevity regulation (Fig. 2.1). Following the identification of the *C. elegans* IIS in lifespan regulation, studies revealed that insulin signaling could affect lifespan in flies (*Drosophila melanogaster*). Similar to *C. elegans*, modulation of *Drosophila* FOXO3 (dFoxo) dosage could either decrease or increase the lifespan of the organism (Lin *et al.*, 1997; Ogg *et al.*, 1997; Giannakou *et al.*, 2004; Hwangbo *et al.*, 2004). Subsequently, additional components of the *Drosophila melanogaster* insulin pathway have been identified (Fig. 2.1).

Remarkably, just over a decade after the molecular identification of the IIS pathway in *C. elegans*, multiple groups of human populations have shown a link between single nucleotide polymorphisms (SNPs) in human *daf-16* or FOXO3 and human lifespan extension (Lunetta *et al.*, 2007; Willcox *et al.*, 2008; Anselmi *et al.*, 2009;

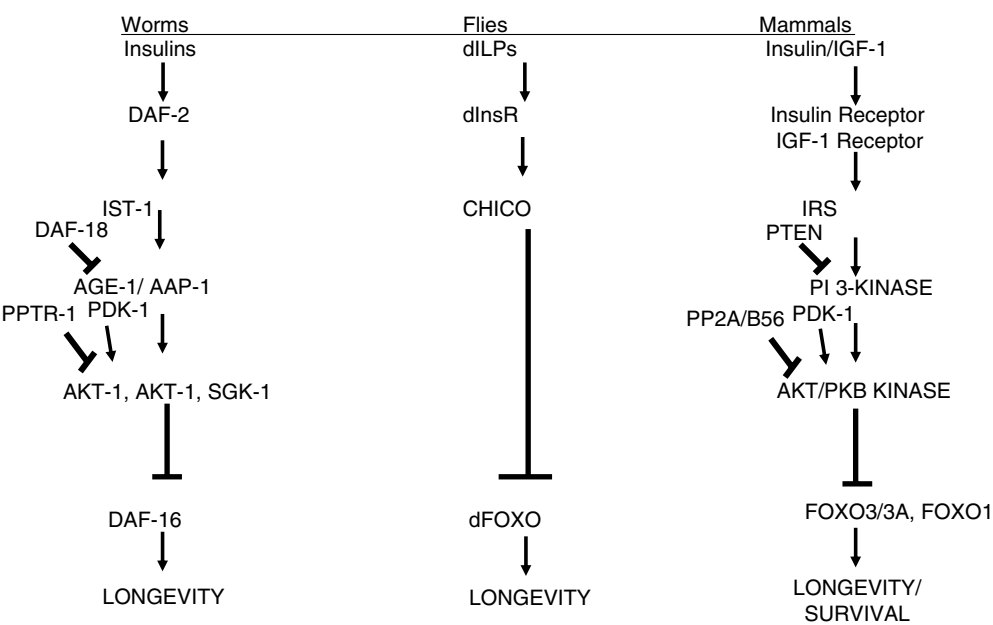


Fig. 2.1. Insulin/IGF-1 regulates longevity. Extremely conserved across species. *C. elegans* focus.

Flachsbart *et al.*, 2009; Li *et al.*, 2009; Soerensen *et al.*, 2010; Zeng *et al.*, 2010; Banasik *et al.*, 2011; Malovini *et al.*, 2011), with the strength of the association increasing with age (Flachsbart *et al.*, 2009). Therefore, to date, FOXO3 remains a candidate human longevity gene with the foundational work occurring in *C. elegans*.

Dauer Formation and Longevity

It had been known for some time that dauers were “ageless”, meaning that time spent as a dauer did not affect adult post-dauer survival (Golden and Riddle, 1982, 1984a,b). Therefore, when the initial study connecting the gene *daf-2* and longevity was revealed, some dismissed the longevity as merely activation of part of the dauer program manifested as long-lived adults. However, Kenyon *et al.* (1993) performed lifespan analyses on several other dauer constitutive mutants (later shown to be part of a transforming growth factor- β (TGF- β) signaling cascade) and reported that these dauer constitutive mutants did not affect lifespan. Many years later, however, Shaw *et al.* (2007) examined the TGF- β mutants and found that these same dauer constitutive mutants did indeed exhibit lifespan extension and the dauer connection remained. More recent studies have suggested that the IIS pathway and the TGF- β pathway intertwine yet again. Both the lifespan extension and the dauer formation phenotypes of *daf-7* TGF- β pathway mutants are dependent on the IIS *C. elegans* FOXO/*daf-16* (Vowels and Thomas, 1992; Thomas *et al.*, 1993; Narasimhan *et al.*, 2011).

In parallel with the IIS pathway, the DAF-7 TGF- β signaling pathway also regulates development, longevity, and dauer formation. In brief, this pathway includes the TGF- β ligand encoded by the gene *daf-7* which functions through the DAF-1/DAF-4 dimer TGF- β receptors to modulate the downstream pathway members: DAF-3, a SMAD family member and DAF-5, a member of the SKI/SNO family. It is suggested from genetic and molecular epistasis analysis that the IIS and TGF- β signaling pathways converge on the IIS/DAF-16 and DAF-12, a nuclear hormone receptor (reviewed in Fielenbach and Antebi, 2008).

Finally, multiple microarray analyses have suggested that *daf-2* mutants show similar

transcriptional profiles to dauers (McElwee *et al.*, 2003, 2004, 2006a,b). Therefore, the connection between longevity and dauer formation remains unsettled.

Why IIS?

Almost all studies on longevity or regulation/modulation of lifespan using *C. elegans* evaluate the dependency on *daf-16* and the IIS pathway. Thus far, IIS has emerged as the best characterized pathway that functions to regulate longevity across phylogeny. Genetic and molecular analysis identified additional components of the pathway downstream of the DAF-2 receptor. As shown in Fig. 2.1, the IIS pathway genes have homologs across evolution. However, what is absent is that lifespan involves multiple tissues, timing, and that all of this signaling is happening in the 3D context of an entire organism. There are no expression patterns or tissues revealed in the Fig. 2.1 diagram. Interestingly, more than two decades after identification of these genes, although many advances have been made, we still do not understand how IIS occurs within the context of the 3D animal.

Tissues Changing with Age

How do we measure health in the laboratory experimentally? A biomarker of aging would greatly have helped but never materialized. However, in *C. elegans* several studies have categorized changes that occur as the worm ages. These include changes at the tissue, physiological, and molecular genetic levels. Herndon *et al.* (2002) found that while the nervous system was intact in older animals, the muscle tissue declined with age. Moreover, this seemed to scale with longer-lived *age-1* mutants. Huang *et al.* (2004) revealed many physiological changes with age, including that both body movement and pharyngeal pumping exhibit a decline with age such that the age-related decline positively correlated with lifespan. Interestingly, these studies (Huang *et al.*, 2004) also revealed that measurements of these processes could be used to predict lifespan and detect premature aging in several mutants (both long-lived and short-lived

mutants). Using the mutants *clk-1* (mitochondrial function is an ortholog of human coenzyme Q7) (Jonassen *et al.*, 1998; Vajo *et al.*, 1999; Nakai *et al.*, 2001) and *eat-2* (a genetic model of caloric restriction) as well as components of the IIS pathway *daf-2*, *age-1*, *daf-16*, it was suggested that the physiological parameters could be used to predict lifespan and also detect whether the animals are aging prematurely (Huang *et al.*, 2004). Measurements were: (i) behavioral and physiological parameters, including body movement, pharyngeal pumping, egg-laying, and defecation frequency; (ii) tissue-level changes, including tissue integrity, yolk protein distribution, sperm number, body size, and accumulation of fluorescent material; and (iii) biochemical alterations, including DNA integrity (Huang *et al.*, 2004).

A more intensive description of how to measure age-related changes that can be examined and evaluated in the laboratory was established by Collins *et al.* (2008). Changes in aging *C. elegans* were evaluated, including behavior, tissue organization, and biochemical analyses. Moreover, genetic and environmental factors were shown to influence measurable age-related changes, including neuromuscular behaviors, reproduction, morphological changes, and biochemical changes (Collins *et al.*, 2008).

At the tissue level, a more detailed analysis of the intestine in aging worms used several different microscopy techniques combined with 3D reconstruction (McGee *et al.*, 2011). This analysis captured age-related differences in the intestinal morphology and revealed how the intestine is subject to substantial changes during the aging process.

Caloric Restriction (CR)

Apart from the IIS, the second major category for modulating the aging process is caloric restriction (CR) or dietary restriction (DR) where lifespan extension is tied to a more limited use of energy. DR leads to lifespan extension across phylogeny from yeast through humans. First observed in 1935 in rodents, CR where dietary calories are reduced, without malnutrition, results in extension of lifespan in all species tested (Masoro, 2005). CR refers to a reduction in calories whereas many different methods can limit

dietary intake for DR. In *C. elegans*, eight different methods have been used to model CR/DR, with somewhat different methods/genes but all result in lifespan extension. According to Greer and Brunet (2009), the eight CR/DR methods in worms are: (i) a genetic model, using an *eat-2* mutant since *eat-2* encodes for a pharyngeal nicotinic receptor and mutation reduces the pharyngeal pumping rate/feeding rate, which results in a decrease in food intake (Avery, 1993; Lakowski and Hekimi, 1998); (ii and iii) limiting food intake by two different methods that dilute the bacterial food/diet; (iv and v) two methods that involve liquid media to limit food intake (axenic and chemically defined) (Houthoofd *et al.*, 2002; Szweczyk *et al.*, 2003); (vi) reducing the ability of the bacterial diet to grow but only altering the amount of peptone in the agar media for the growth plates (Hosono *et al.*, 1989); (vii) dietary deprivation, which involves the complete removal of bacteria on the agar growth plates (Kaeberlein *et al.*, 2006; Lee *et al.*, 2006); and (viii) limiting food through serial dilution of the bacteria that is spread on agar growth plates (Greer *et al.*, 2007). These methods differ in the type of media, source of food, timing, genetic background, percentage lifespan extension, and effect on reproduction. Together, these different combinations result in requiring different genes to exert the lifespan extension effect of caloric restriction. Importantly, the genes discussed below have all been linked to the regulation of CR/DR lifespan extension.

Additional Inputs to the IIS

C. elegans SIRT1/*sir-2.1*

Silencing information regulator 2 (*sir-2.1*) is the *C. elegans* SIRT1 ortholog encoding for an NAD-dependent histone deacetylase and is a part of the larger Sir2 family that in *C. elegans* includes four members: *sir-2.1*, *sir-2.2*, *sir-2.3*, and *sir-2.4*. Initial studies revealed that overexpression of SIRT1 in several model organisms resulted in an extension of lifespan (Kaeberlein *et al.*, 1999; Tissenbaum and Guarente, 2001; Rogina and Helfand, 2004). With this unexpected result, evolutionary conservation and the identification of SIRT1 as an NAD-dependent

histone deacetylase (Imai *et al.*, 2000), the field of sirtuin biology and its connection to the aging process was undeniable. For *C. elegans* longevity, only *sir-2.1*, the closest relative to mammalian SIRT1, has been shown to modulate lifespan. Increased dosage of *sir-2.1* extends lifespan, while decreased dosage by mutation or RNAi reduces lifespan or has no effect (Tissenbaum and Guarente, 2001; Berdichevsky *et al.*, 2006; Wang and Tissenbaum, 2006; Viswanathan and Guarente, 2011; Viswanathan and Tissenbaum, 2013).

Even though the initial studies on *sir-2.1* lifespan helped launch the field of sirtuin biology, there has been controversy on the role of *sir-2.1* in lifespan regulation in model organisms, including both the overexpression analysis as well as the mutant (Viswanathan and Tissenbaum, 2013). Multiple studies on *sir-2.1* overexpression have shown an extension of lifespan; however, the extent of the lifespan extension remains controversial (Tissenbaum and Guarente, 2001; Viswanathan and Guarente, 2011; Viswanathan and Tissenbaum, 2013). Initial studies also showed that overexpression of *sir-2.1* enhanced dauer formation and positioned *sir-2.1* as part of the IIS pathway, although no other study arguing the effect of lifespan has examined dauer formation (Tissenbaum and Guarente, 2001). For *sir-2.1*, both a mutant and RNAi have been used to decrease gene dosage and have shown either a slight decrease or no change in lifespan. First, *sir-2.1(ok434)* mutant and *sir-2.1* RNAi in a wild-type background have either no effect or a reduction in lifespan (Wang and Tissenbaum, 2006). Second, dependent on the *eat-2* allele used to model caloric restriction, *sir-2.1* is or is not required for caloric restriction (Hansen *et al.*, 2007; Wang and Tissenbaum, 2006).

Molecular studies revealed that SIR-2.1 physically interacts with the 14-3-3 proteins, albeit somewhat temporarily. SIR-2.1 directly interacts with the two *C. elegans* 14-3-3 proteins, FTT-2 and PAR-5, which allows SIR-2.1 to interact with *C. elegans* FOXO/DAF-16. *C. elegans* 14-3-3 proteins, FTT-2 and PAR-5, interact with *C. elegans* FOXO/DAF-16 (Berdichevsky *et al.*, 2006; Wang *et al.*, 2006). Therefore, the molecular studies agree with the *sir-2.1* genetic analysis where lifespan extension by *sir-2.1* overexpression is dependent on *C. elegans* FOXO/*daf-16* (Tissenbaum and Guarente, 2001).

Since the original study in 2001, despite some controversies on the extent of lifespan increase by *sir-2.1* overexpression, mammalian sirtuin1 (SIRT1) has been shown to modulate mammalian cellular senescence and aging. Moreover, SIRT1, as an NAD-dependent histone deacetylase, has been shown to target and deacetylate signaling components including p53, PPAR δ , Foxo, NF- κ B, AMPK, and mTOR, and has been linked to modulation of processes such as DNA damage, oxidative stress, mitochondrial integrity, autophagy, and inflammation. Therefore, although there may be some disputes with regards to the extent of SIRT1 orthologs on lifespan regulation in model systems, the importance of the sirtuin family in lifespan is uncontroversial.

Adenosine 5' monophosphate-activated protein kinase (AMPK)

AMPK is a heterotrimeric complex comprising of a catalytic (α) and regulatory (β , δ) subunits. In *C. elegans*, this highly evolutionary conserved serine/threonine protein kinase containing one α , β , and γ subunits is regulated both by allosteric and by post-translational modifications under different physiological conditions. The two AMPK genes in *C. elegans*, AAK-1 and AAK-2, were found to be 52% for AAK-1 and 40% for AAK-2 identical to human AMPK (Apfeld *et al.*, 2004). Lifespan studies revealed that reducing the dosage of *aak-1* had no effect while *aak-2* mutant or RNAi resulted in about a 10% decrease in lifespan (Apfeld *et al.*, 2004). Moreover, higher dosage of *aak-2* resulted in an average of about 13% increase in lifespan; therefore, *aak-2* modulates lifespan in a dose-dependent manner.

Molecularly, AAK-2 acts as an energy sensor integrating energy needs with lifespan regulation. AAK-2 is a serine/threonine protein kinase that itself is normally activated by phosphorylation, which in mammals is well defined as phosphorylation at T172 of the α -subunit by AMP and/or adenosine diphosphate (ADP) binding to γ -subunit. Regulation of AAK-2 is as follows: when in conditions where the AMP/ATP and ADP/ATP ratios are low, as when there is little or no available energy, T172 of the AMPK α -subunit is not phosphorylated. However, at high levels of AMP and ADP, such as when energy is replete,

T172 of the AMPK α -subunit increases its phosphorylation. AMPK itself can act upstream of the IIS *C. elegans* FOXO/*daf-16* to extend lifespan and has been shown to directly phosphorylate *C. elegans* FOXO/*daf-16* on multiple sites *in vitro* (Greer *et al.*, 2007). Therefore, in addition to the upstream IIS kinases (AKT-1, AKT-2, SGK-1), AMPK also directly phosphorylates *C. elegans* FOXO/*daf-16*.

Target of Rapamycin (TOR)

Target of rapamycin, or TOR, is a serine/threonine kinase that has become a leading player in the search for genes that regulate lifespan and healthy aging across phylogeny. Much of the attention has come due to the anti-aging effects of the drug rapamycin. As such, there is a real push to identify the regulation upstream and downstream of TOR and the mechanism underlying the action of rapamycin in the aging process. First identified in yeast, TOR (*C. elegans* *let-363*), also known as mTOR in mammals, functions as part of two different complexes, each with different regulatory partners: TOR Complex 1 (TORC1) and TOR Complex 2 (TORC2). TOR interacts exclusively with raptor (*C. elegans* *daf-15*) in TORC1, while in TORC2 it interacts with rictor (*C. elegans* *ric-1*) (reviewed in Blackwell *et al.*, 2019).

TOR signaling functions as a sensor that can respond to changes in nutrients and energy and to connect upstream nutrient status and physiology. For *C. elegans*, reduction in *C. elegans* TOR, *let-363*, by RNAi or loss of one copy of *C. elegans* raptor, *daf-15*, results in lifespan extension. Moreover, this activity mediates the beneficial effects of dietary restriction and modulates healthspan (reviewed in Blackwell *et al.*, 2019). Studies in *C. elegans* elucidated lifespan regulation signals downstream of TOR as well as differences and similarities between TORC1 and TORC2 (reviewed in Blackwell *et al.*, 2019). TORC1 comprises *C. elegans* TOR/LET-363, *C. elegans* raptor/DAF-15, PRAS40, and LST8; while TORC2 comprises *C. elegans* TOR/LET-363, *C. elegans* rictor/RIC-1, LST8, SIN1, and PROCTOR.

As an energy sensor, TOR is central to growth and survival. Mechanistically, TOR is central to multiple cell regulatory processes including protein translation, autophagy, and transcription. Briefly, under conditions where nutrients are sufficient, TOR is activated stimulating pathways

such as autophagy. However, when TOR is reduced, such as TOR RNAi, lifespan in *C. elegans* is extended dependent on DAF-16. In fact, RIC-1, a member of the TORC2 complex, phosphorylates AKT-1 on SER 517 (mammalian SER 473) (Hresko and Mueckler, 2005; Sarbassov *et al.*, 2005; Blackwell *et al.*, 2019). Downstream TOR has also been linked to a separate pathway, which is more important for CR/DR, with the transcription factor PHA-4/FoxA and autophagy (Panowski *et al.*, 2007). Interestingly, although *ric-1* phosphorylates AKT-1, combining the inhibition of IIS receptor *daf-2* and inhibition of *C. elegans* S6 kinase, a TOR target, *rsks-1*, results in a synergistic effect on lifespan extension (Chen *et al.*, 2013). Therefore, the genetic epistasis data suggest that the IIS pathway and the TOR pathway act separately and together to modulate *C. elegans* lifespan (Narasimhan *et al.*, 2009; Uno and Nishida, 2016).

Adult RNAi knockdown of *let-363*/TOR or *rsks-1*/S6K and rapamycin treatment leads to a reduction in protein translation and lifespan extension dependent on both DAF-16/FOXO and SKN-1/Nrf (Hansen *et al.*, 2007; Robida-Stubbs *et al.*, 2012). In yeast and human cells, TORC1 directly phosphorylates RSKS-1/S6K for ribosome function and protein synthesis although not yet shown in *C. elegans* (reviewed in Blackwell *et al.*, 2019), placing RSKS-1/S6K downstream of TORC1. Taken together, these findings suggest that the TOR and IIS pathways separated and intertwined at several potential places (Narasimhan *et al.*, 2009).

Cellular Processes That Modulate Longevity

Over the past decade, studies on the modulation of lifespan regulation have revealed that interfering with normal cellular processes can extend or decrease lifespan. These processes include protein translation, autophagy, and mitophagy. We will briefly review these processes and the genes associated with them.

Autophagy

Autophagy, an evolutionarily conserved process, is often considered the cells' "garbage disposal". Autophagy is a process whereby a double-membrane

structure, called the autophagosome, engulfs a portion of the cytosol including damaged organelles and then delivers them to the lysosome for degradation. In *C. elegans*, autophagy, a catabolic process, occurs in many cell types and in many developmental stages. There are many methods to measure autophagy depending on the age of the animal. Often a combination of assays is used in *C. elegans* to measure autophagy and can include assessing levels of LGG-1, the ortholog of yeast autophagy gene *Atg8*, where often LGG-1::GFP is used, assessing levels of the SQSTM1/p62 ortholog with SQST-1::GFP, examining gene expression of autophagic genes as well as examining tissues with electron microscopy analysis of autophagic structures (Zhang *et al.*, 2015). Many long-lived mutants, including genes in the IIS, CR mutants such as *eat-2*, and *let-363/tor* (RNAi) mutants, show changes in multiple autophagy assays (ex. LGG-1::GFP puncta changes), expression of autophagy genes (e.g., *lgg-1* q-RT-PCR), and/or require autophagy genes (e.g., *daf-2*; *bec-1*) for lifespan extension (Melendez *et al.*, 2003). Most recently, the transcription factor HLH-30/TFEB has been identified as playing a central role in the regulation of autophagy (Lapierre *et al.*, 2013). Importantly, it should be noted that, thus far, autophagy mutants have issues with development and are short-lived. Therefore, many studies use “adult-only” RNAi for lifespan studies on autophagy regulation (reviewed in Zhang and Baehrecke, 2015).

Protein translation

There are three stages of protein translation: initiation, elongation, and termination (Syntichaki *et al.*, 2007). Protein translation was connected to longevity regulation in *C. elegans* when it was revealed that inhibition of mRNA translation could alter lifespan (Hansen *et al.*, 2007; Pan *et al.*, 2007). The lifespan of *C. elegans* is extended by reduction of function in the initiation factor gene, *ife-2*, thereby inhibiting mRNA translation (Hansen *et al.*, 2007). Moreover, reducing eukaryotic translation initiation factor 4G (eIF4G) during adulthood also extends lifespan (Henderson *et al.*, 2006; Curran and Ruvkun, 2007; Hansen *et al.*, 2007; Pan *et al.*, 2007; Syntichaki *et al.*, 2007). Therefore, it is possible that a decrease in mRNA translation leads to an overall reduction

of *C. elegans* protein synthesis that results in an increase in lifespan. Another possibility is that lower mRNA translation results in decreased synthesis of both normal and erroneous proteins, resulting in a net upregulation of chaperone activity and homeostasis (Hipkiss, 2007). Further, adult-only inhibition of eukaryotic translation initiation factor 4G (eIF4G) reducing overall translation results in changes in the balance of mRNA translation from growth and reproduction to genes that regulate stress, thereby extending lifespan (Rogers *et al.*, 2011; Howard and Rogers, 2014). Taken together, these findings show that interfering with protein translation can affect *C. elegans* lifespan.

Mitophagy

In eukaryotic cells, the organelle responsible for energy production is the mitochondrion. Recent studies have also revealed that mitochondria play a central role in processes including metabolism and apoptosis. Therefore, mitochondria malfunction could lead to loss of homeostasis. Indeed, mitochondria play a critical role in the regulation of lifespan: reduction of function in the gene *mev-1*, which encodes for a subunit of the succinate dehydrogenase cytochrome b enzyme, part of the mitochondrial electron transport chain complex II, shortens lifespan (Ishii *et al.*, 1998), while reduction of function in the gene *clk-1*, a mitochondrial polypeptide essential for ubiquinone (coenzyme Q) synthesis, extends lifespan (Ewbank *et al.*, 1997; Felkai *et al.*, 1999). Therefore, maintaining functional mitochondria is essential to normal lifespan. The importance of the mitochondria to modulation of lifespan has been addressed in several manuscripts (Lee *et al.*, 2003b; Anson and Hansford, 2004; Wolff and Dillin, 2006; Bratic *et al.*, 2009; Hur *et al.*, 2010; Rera *et al.*, 2010; Gruber *et al.*, 2011; Torgovnick *et al.*, 2013).

Mitophagy, or mitochondria-specific macroautophagy, a relatively newly identified cell regulatory process, helps to maintain mitochondria quality since it targets dysfunctional or harmful mitochondria for degradation in the lysosome. Mitophagy is a selective type of autophagy targeting mitochondria for degradation. Mitophagy also potentially interfaces with mitochondrial biogenesis which affects mitochondrial content

and longevity (Palikaras *et al.*, 2015). Therefore, mitophagy functions to keep mitochondria healthy for normal lifespan. Mitophagy can be monitored in *C. elegans* by several methods, including observing co-localization of mitochondria and autophagosomes (Charmpilas *et al.*, 2018). Importantly, mitophagy allows the mitochondrion to maintain its function as a regulator of homeostasis as animals age.

Reproductive System

In *C. elegans* longevity regulation, the reproductive system is divided into two parts: the germline and the somatic gonad. The germline includes the germ cells, whereas the somatic gonad includes the reproductive organs. Initially in early development, the reproductive system consists of four cells named Z1, Z2, Z3, and Z4. After cell divisions/development, Z1 and Z4 are the precursors that give rise to the somatic gonad, which in the adult hermaphrodite includes the gonadal sheath, spermatheca, and uterus. Z2 and Z3 give rise to the germline after continued proliferation throughout development. As adult hermaphrodites, the germline consists of both eggs and sperm and also includes germline stem cells (GSC).

The first clue to the regulation of lifespan by the reproductive system was revealed using a laser capable of ablating the individual gonadal cells Z1–Z4. Laser ablation of the gonadal cells individually and in combinations revealed that there were different genetic regulators of the somatic gonad versus the germline. Initial studies showed that removing the germ cells by laser ablation results in lifespan extension dependent on an intact somatic gonad (Hsin and Kenyon, 1999). Laser ablation of Z2 and Z3 resulted in a 60% increase in lifespan. However, when all four cells were ablated (Z1, Z2, Z3, Z4), lifespan was normal/unchanged (Hsin and Kenyon, 1999). Together, this suggests that under normal growth conditions, wild-type animals produce a signal from the germline that inhibits lifespan, and absence of a germline leads to enhanced longevity in *C. elegans*.

As discussed above, the IIS is a central regulator of longevity. Combining germline ablation with a mutation in the DAF-2 IIS receptor revealed that while the DAF-2 receptor also functions to

repress longevity, germline signaling acts in parallel with the IIS. Further genetic analysis found that loss of the germline cells promotes nuclear translocation of the *C. elegans* FOXO/DAF-16 transcription factor. When germline ablation is performed in a *daf-16* mutant background, there is no effect on lifespan. Therefore, removal of germline cells requires the *C. elegans* FOXO/DAF-16 transcription factor for lifespan extension (Yamawaki *et al.*, 2008).

Removal of GSCs promotes lifespan extension and can be recapitulated by mutations that result in germline elimination, such as mutations in the gene *glp-1* (Arantes-Oliveira *et al.*, 2002). An experimental advantage is provided by *glp-1* since there are temperature-sensitive alleles. Therefore, upon selective shift to the non-permissive temperature, selective elimination of germ cells occurs, which revealed that removal of the GSCs causes lifespan extension (Arantes-Oliveira *et al.*, 2002).

Ablation of the germline results in lifespan extension dependent on the expression of DAF-16 in the intestine. Given that these results indicate the possibility of communication or signaling between the germline and the intestine, a genetic screen was performed and it identified the gene *kri-1* (Berman and Kenyon, 2006). The gene *kri-1* encodes for a protein that is similar to human protein krev interaction trapped 1 (KRIT1), also known as cerebral cavernous malformation 1 (CCM1) (Popiel and Derry, 2020). In mice, loss of KRIT1 leads to problems with both arteries and veins, leading to vascular malformations. In humans, loss of KRIT1 leads to a heritable form of cerebral cavernous malformation (Popiel and Derry, 2020). In *C. elegans*, in long-lived germline-less animals (*glp-1* mutant), *kri-1* mutants suppress lifespan extension and KRI-1 is required for DAF-16 nuclear localization. This reveals that it is possible that the only function of KRI-1 could be the regulation of DAF-16 nuclear translocation (Berman and Kenyon, 2006). Consistent with these results, constitutive nuclear localization of DAF-16 can bypass the need for *kri-1*.

Signals from the germline involve a number of transcriptional signals in the intestine including DAF-16/FOXO, PHA-4/FOXA, nuclear hormone receptors, such as DAF-12, as well as a transcription elongation factor TCER-1/TCERG1, that modulate cellular processes and lifespan

(Ghazi *et al.*, 2009). There is a vast literature and there are beautiful experiments elucidating roles for genes/proteins when the germline is removed, as in germline-less animals. For more information, see: Hsin and Kenyon (1999); Libina *et al.* (2003); Ghazi *et al.* (2009); Ghazi (2013); Ratnappan *et al.* (2016); Zhang *et al.* (2018). The limitation of these studies is that for survival of a species, reproduction is necessary and therefore the germline is required.

Summary/Future Studies

In this chapter, the focus has been on the regulation of longevity, despite the title of the chapter being “Aging”. Overall, there is not a universal definition for aging and to date in the laboratory, lifespan has been used as a proxy for aging. The future of aging research relies heavily on extension of healthspan: extending the length of time of healthy aging, and deferring or preventing the onset of age-associated disease. Recent findings

on longevity regulation revealing the importance of epigenetics are another area that will move the field forward. Lee *et al.* (2019) found that increased levels of H3K9me2, an epigenetic marker, exhibit lifespan extension. Remarkably, the authors revealed that this lifespan extension could be passed down to future generations (Lee *et al.*, 2019; Emerson *et al.*, 2020). Therefore, regulation of lifespan through epigenetic marks is only the beginning of our understanding of transgenerational control of lifespan. Taken together, *C. elegans* has been a premier system for the study of the aging process.

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3 Entomopathogenic Nematodes as a Model for Probing the Insect Immune System

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Introduction

Nematodes are a diverse animal phylum inhabiting a broad range of environments, including various parasitic niches and several hosts such as plants, insects, and animals (Dillman and Sternberg, 2012; Eleftherianos *et al.*, 2017). Among the vast variety of parasitic nematodes, some have evolved an association with insect-pathogenic bacteria and became known as entomopathogenic nematodes (EPNs). These types of nematodes seek out and invade potential hosts, releasing their pathogenic symbionts into the host's open circulatory system (hemolymph). As a result of this lethal pairing, infected insect hosts die quickly, and the bacteria proliferate while the nematodes feed on the insect carcass and reproduce (Dillman and Sternberg, 2012). Since their discovery in the 1920s, EPNs have received increasing interest due to their potential as augmentative biological control agents for insect pests (Piedra Buena *et al.*, 2015). In particular, their role as mobile vectors for their symbiotic bacteria cargo has led to their rapid commercialization as biopesticides, accompanied by an exponential growth in research on their application, biology, and ecology. Subsequently, extensive research over the past three decades has demonstrated their successes

and failures as biological control agents in sustainable food production across a wide range of agricultural commodities, which was followed by EPN mass production and application technology, as well as studies of their interactions with other management tools (Georgis *et al.*, 2006). For instance, EPNs have been used in insect pest management in important food crops, including orchards (Shapiro-Ilan *et al.*, 2005), small fruits, vegetables (Dara, 2017), greenhouses (Wraight *et al.*, 2017), and mushrooms (Jess *et al.*, 2005).

The most studied EPNs are those that can be used in the biological control of harmful insects, members of the Steinernematidae and Heterorhabditidae (Ehlers, 2001; Ffrench-Constant *et al.*, 2007). This in turn has aided EPN mass production, leading to the commercial development of at least eight *Steinernema* species and five *Heterorhabditis* species (Ehlers, 2001). The selection of EPNs for control of a particular pest insect is based on several factors, including the nematode's host range and host foraging strategy, as well as environmental factors and their effects on survival and infection efficacy. Such environmental factors include temperature, moisture, soil type, exposure to ultraviolet light, salinity and organic content of soil, means of application, agrochemicals, and others (Lacey and Georgis,

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2012). *Steinernema feltiae*, for example, can be infective from 2°C to 30°C, whereas some heterorhabditids can infect host insects from 7°C to 35°C, and *Steinernema carpocapsae* is almost inactive at 10°C (Kaya, 1990; Grewal *et al.*, 2005; Georgis *et al.*, 2006). One of the confounding issues with using nematodes for crop protection is that they can be responsible for substantial damages within the agriculture industry. In fact, plant-infectious nematodes account for approximately 5% of crop yield loss by limiting root growth, plant size, and photosynthetic rate (Gheysen and Mitchum, 2011). Thus, nematodes present in a plant's environment may have adverse effects, making their involvement highly complex.

Due to both the intricate three-organism system and the assortment of variables present in a soil environment, it is imperative to understand and guide nematode interactions to a desired result. This may require a significant amount of tact and subtlety that relies on a thorough knowledge of plant and insect immunity in relation to nematode infections (Kenney and Eleftherianos, 2016). This chapter provides an overview of the significant progress made in the research and application of EPNs in various ways, including insect pest management and organic agriculture. Using this information will further improve the use of EPNs with respect to efficacy, reduced costs, and ease of use. Moreover, the ability of EPNs to persist within host populations provides the opportunity to pursue long-term pest suppression, improve soil quality, and develop a control system that is more comparable with a wide range of synthetic insecticides.

Entomopathogenic Nematode Life Cycle

EPNs are mobile, obligate parasites that infect a variety of arthropods. They use the infected host to complete their life cycle and replicate within the cadaver before they disseminate to search for new hosts. To date, at least 96 *Steinernema*, one *Neosteinernema*, and 21 *Heterorhabditis* species have been described (Shapiro-Ilan *et al.*, 2018). They have a life cycle that includes the egg stage, four juvenile stages, and adult stage (Fig. 3.1). The main feature of these parasites is the presence of symbiotic bacteria that play an important role in

host infection. These bacteria are found in the gut of the third-stage infective juveniles (IJs), the stress-tolerant, non-feeding, developmental stage that actively invades the insect host (Pérez *et al.*, 2003). Naturally present in soil environments, foraging IJs (dauer larvae, equivalent to the dauer juvenile stage of the model nematode *Caenorhabditis elegans*) are unable to feed but can exist outside the host to seek out and enter new insect hosts (Pérez *et al.*, 2003). They use environmental and host cues such as carbon dioxide, vibration, and other chemicals produced in waste products to locate and recognize potential hosts.

Penetration into the insect host is the first step of the EPN infection process. Upon entering the host through natural openings (mouth, anus, or spiracles) or direct penetration through the intersegmental membranes of the cuticle, IJs release their symbiotic bacteria into the host's hemolymph by regurgitation or defecation (Eleftherianos *et al.*, 2017). This release does not occur immediately, however, and is instead delayed, by 30 min in the case of *Heterorhabditis*, and 4–6 h for *Steinernema* (Li *et al.*, 2007). Subsequently, the susceptible host perishes within 24–48 h by toxemia and septicemia (Pérez *et al.*, 2003). EPNs continue to feed on the host tissue and the increasing number of bacteria, growing and molting to the fourth stage and then to the adult stage, eventually completing one to three generations in the dead host. IJs of *Steinernema*, except for *Steinernema hermaphroditum*, develop into male and female adults and, following generations in the host, also produce males and females. *Heterorhabditis* IJs, on the other hand, develop into self-fertilizing hermaphroditic adults in the first generation. Subsequent *Heterorhabditis* generations of males, females, and hermaphroditic individuals are then produced. When space and food resources are depleted, a new cohort of free-living IJs leaves the host cadaver and disperses into the soil, foraging for a new host to continue their life cycle. IJs may survive for several months in the absence of a suitable host (Hominick, 1990).

The association between nematodes and bacteria is highly specific. Both *Heterorhabditis* and *Steinernema* are associated with bacteria of the genera *Photorhabdus* and *Xenorhabdus*, respectively (Ferreira and Malan, 2014). Currently, there are 19 species of *Photorhabdus* and 26 species of *Xenorhabdus*. Together, EPNs and

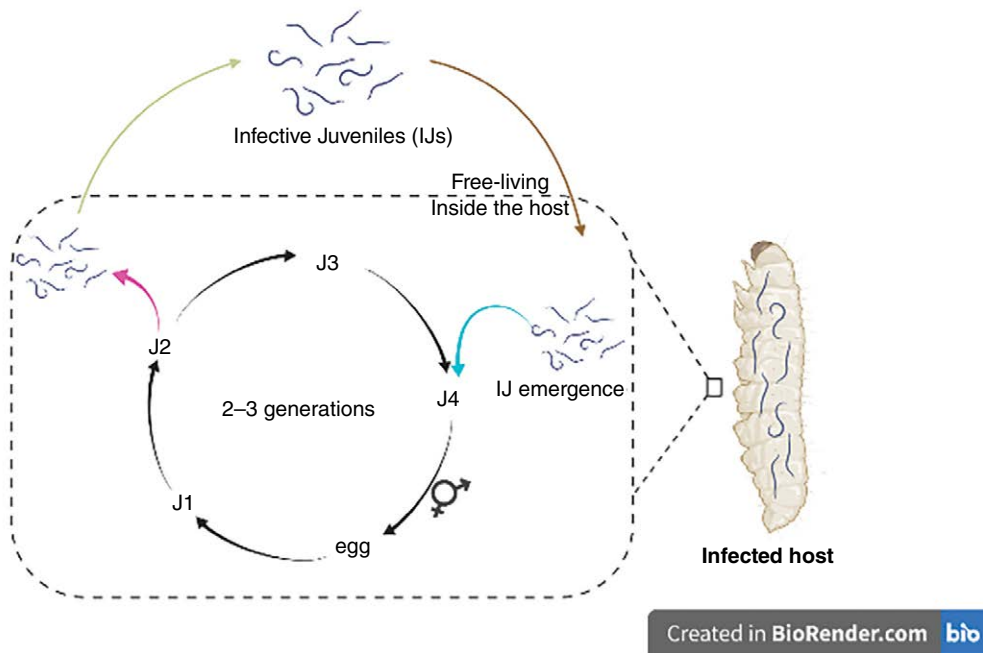


Fig. 3.1. Following the death of the insect host, the non-feeding, free-living infective juveniles (IJs) of entomopathogenic nematodes seek out new insect hosts and initiate infections. Once a suitable host has been located, the nematodes penetrate the insect body cavity or enter through natural openings and release their symbiotic bacteria, which multiply rapidly and together cause host death. The nematodes feed upon the bacteria and host carcass, eventually completing at least two cycles that include the egg stage, four juvenile stages, and adult stage. *Steinernema* IJs may become males or females, whereas *Heterorhabditis* develop into hermaphrodites that later produce males and females within the host.

their highly pathogenic bacteria kill their host within a few days, therefore distinguishing themselves from other insect parasites that develop longer associations with their host (Pérez *et al.*, 2003). Arguably, because EPNs always associate with pathogenic bacteria, the exact contribution of nematodes to this mutualism remains uncertain. One widely accepted assumption is that the nematodes serve mainly as vectors for the pathogenic bacteria and that it is the bacteria that suppresses host immunity and ultimately kills the host (Dillman and Sternberg, 2012; Lu *et al.*, 2017). *Photorhabdus luminescens*, for instance, has been well documented to produce a cocktail of toxins and immunosuppressive compounds that interfere with host defense responses and significantly contribute to mortality (Rodou *et al.*, 2010). However, while this is a good model for *Heterorhabditis* EPNs, there is evidence that EPNs in *Steinernema* contribute to host immune suppression and host killing (Han and Ehlers, 2000;

Sicard *et al.*, 2003). For example, axenic (lacking *Xenorhabdus nematophila*) IJs of *S. carpocapsae* are capable of killing their hosts. Thus, the success of EPN complexes may be based on the synergism between the nematode itself and its symbiont. More in-depth studies of the host–parasite relationship require a profound understanding of the processes and components of the insect immune system that are involved in the recognition and neutralization of invaders.

Mutualism regulators

Quantitative proteomic analyses have largely contributed to the identification and functional characterization of the main factors that regulate the symbiotic processes between EPNs and their associated bacteria. Noticeably, most of this information comes from studies using *Heterorhabditis* and *Steinernema* as tractable genetic

models, thus leaving a significant gap in our knowledge of nematode molecules that regulate their mutualistic interaction with their bacterial partners (Eleftherianos and Heryanto, 2020). Results from *Photorhabdus* studies show that they express several unique proteins that might be involved in governing host specificity between nematodes and their bacteria (Kumar *et al.*, 2016). These proteins determine host specificity in *Photorhabdus* subspecies by directly using outer membrane proteins, indirectly regulating bacterial secondary metabolism (malate dehydrogenase Mdh, pyruvate formate-lyase PflA, and flavo protein WrbA), or in a yet unknown manner (hypothetical proteins and transcription regulators). Furthermore, studies reveal that the colonization of *S. carpocapsae* IJs by *X. nematophila* is controlled by the bacterial genes *niID*, encoding a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) element, and *tdk*, encoding the enzyme deoxythymidine kinase (Orchard and Goodrich-Blair, 2005; Veesenmeyer *et al.*, 2014).

Recent efforts aim to develop whole-genome sequencing approaches and molecular procedures in various EPNs to genetically manipulate the vector nematode and determine the number and nature of genes and their products that act as regulators of mutualism in EPNs (Eleftherianos and Heryanto, 2020). These particular approaches have been significantly impeded due to the lack of availability of genetic and molecular tools in EPNs, which further complicates our understanding of the exact contribution of the parasites to their infection process. Prior work demonstrated the presence of species-specific satellite DNA motifs in *Heterorhabditis bacteriophora* and *Steinernema glaseri* nematodes (Grenier *et al.*, 1996, 1997), which were later expressed in *H. bacteriophora* and used in RNA-sequencing studies to confirm their involvement in various biological functions, including nematode parasitism (Sandhu *et al.*, 2006; Vadnal *et al.*, 2017). The complete sequencing of the *H. bacteriophora* genome is specifically a remarkable breakthrough in EPN research, as it presents a critical model organism for gene expression/silencing experiments that will largely uncover previously unknown players of the interrelationship between EPNs and their cognate bacteria (Bai *et al.*, 2013; McLean *et al.*, 2018; Vadnal *et al.*, 2018).

Insect Innate Immune Signaling and Function

Recent studies using model insects have unveiled the molecular components that nematodes and their bacteria employ to evade or defeat the insect immune system, as well as the hosts' immune responses to promote their survival and propagation (Castillo *et al.*, 2011; Kenney and Eleftherianos, 2016; Eleftherianos *et al.*, 2017). The conservation of primary components of innate immunity between vertebrates and invertebrates presents insects as compelling models for elucidating innate immune functions not only in arthropods but also in higher organisms (Riera Romo *et al.*, 2016). For instance, the mechanisms regulating innate immune responses in animals have been largely uncovered with the aid of genetic and molecular studies in the fruit fly *Drosophila melanogaster* (Lemaitre and Hoffmann, 2007). More specifically, the key role of the powerful *Drosophila* model for studying immunity was illustrated by the initial genetic identification of signaling pathways mediating antimicrobial peptide (AMP) gene expression. Therefore, investigation and characterization of antiparasitic mechanisms that regulate the insect immune response to EPNs will contribute significantly to the development of improved practices to control insects of agricultural, economic, and medical importance, and potentially nematode parasites that infect mammals (Campos-Herrera *et al.*, 2012).

Insects have developed an innate immune system that responds to challenges by various infectious agents and is controlled by a complex and diverse array of signaling pathways (Castillo *et al.*, 2011; Kenney and Eleftherianos, 2016; Riera Romo *et al.*, 2016). Generally, insect innate immunity exhibits physical and chemical barriers, humoral and cellular reactions, which are present in all jawed vertebrates (Riera Romo *et al.*, 2016). Physical barriers such as the larval cuticle, adult exoskeleton, chitinous lining of the tracheae, and peritrophic membrane of the midgut are the first line of defense against pathogens (Lemaitre and Hoffmann, 2007; Davis and Engström, 2012). Once the nematodes breach the host's external defenses and gain access into the insect hemocoel (body cavity), they are recognized as foreign by the insect immune system (Stokes *et al.*, 2015). Immune recognition consequently

leads to the induction of downstream signaling pathways (Fig. 3.2) that in turn result in the production of effector molecules such as AMPs and reactive oxygen species (ROS), and cellular functions involving phagocytosis, melanization, and encapsulation (Lemaitre and Hoffmann, 2007; Vlisidou and Wood, 2015).

Insect humoral immunity

Imd and Toll pathways

To combat infections and microbes, insects rely on multiple innate immune mechanisms, many of which are shared with vertebrates (Riera Romo *et al.*, 2016). The most investigated insect humoral responses, especially in *Drosophila*, are primarily mediated by two distinct nuclear

factor (NF- κ B) signaling pathways known as the Imd (immune deficiency) and Toll pathways (Lemaitre and Hoffmann, 2007; Castillo *et al.*, 2011; Kenney and Eleftherianos, 2016). These responses are characterized by the transient production and release of AMPs by the fat body, the major immune responsive tissue in insects and equivalent of the mammalian liver. There are seven classes of immune-inducible AMPs that have been extensively studied in *Drosophila*: dipterecin, attacin, drosocin, and cecropin are directed against Gram-negative bacteria; defensin acts against Gram-positive bacteria; drosomycin and metchnikowin have anti-fungal properties (Lemaitre and Hoffmann, 2007). Noticeably, attacin, drosocin, cecropin, and defensin are also found in other insects, whereas drosomycin, dipterecin, and metchnikowin are only found in fruit flies. Other immune effectors identified in lepidopteran insects (moths and

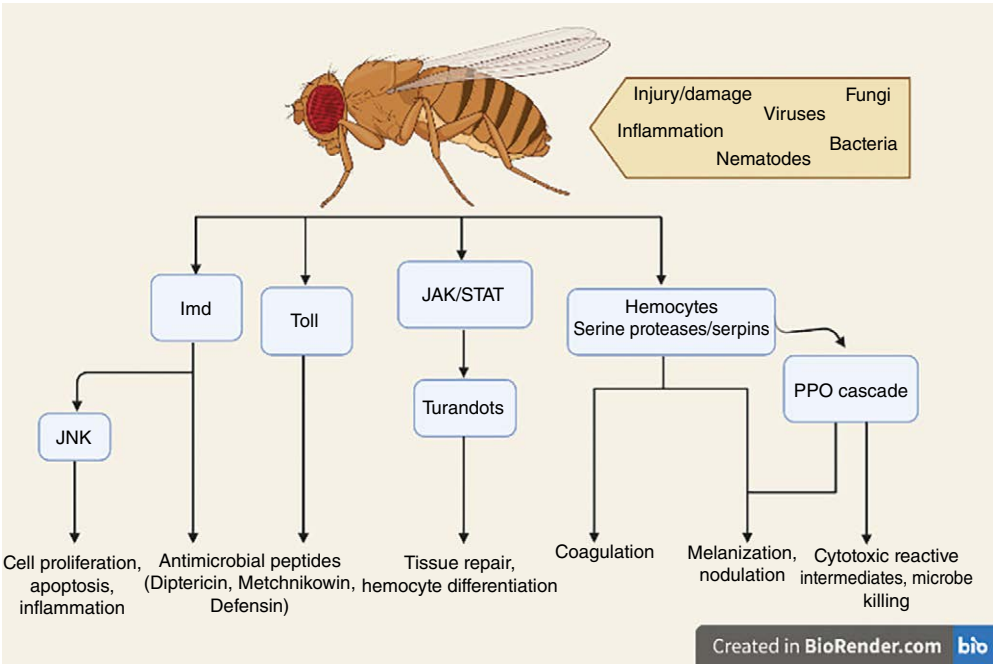


Fig. 3.2. To combat microbial infections, insect hosts rely on multiple innate immune mechanisms, many of which are classified as humoral and cellular responses. Humoral responses are mainly mediated by several signaling pathways: the Imd (Immune deficiency), Toll, JAK/STAT (Janus kinase/signal transducer and activator of transcription), and JNK (Jun-N-terminal kinase) pathways. The hallmark of these particular responses is the production and release of antimicrobial peptides (AMPs) and effector molecules. Cellular responses in insects include hemolymph coagulation, melanization, and encapsulation of larger objects such as parasitoid eggs and parasitic nematodes. PPO, prophenoloxidase.

butterflies) include C-type lectins, hemolin, serpins, and hemolymph proteases (Casanova-Torres and Goodrich-Blair, 2013).

The Imd signaling pathway is homologous to the mammalian tumor necrosis factor receptor 1 (TNFR1) signaling pathway (Kleino and Silverman, 2014). Imd mediates both systemic and local AMP expression by the fat body and gut epithelial cells, respectively, in response to Gram-negative bacteria. In particular, it has been suggested that members of the Imd pathway are targets of entomopathogenic bacteria such as *X. nematophila* and *P. luminescens* (Aymeric *et al.*, 2010). This pathway is activated via the detection of Dap-type peptidoglycan (PGN) by the recognition receptors PGRP-LC and PGRP-LE (Kleino and Silverman, 2014). Receptor binding leads to the intracellular activation of the adaptor molecule Imd (homolog of receptor interacting protein, RIP) and the downstream protein complexes of the signaling cascade. Transduction of the signal to the nucleus then leads to the expression of genes encoding antimicrobial peptides such as diptericin (Lemaître and Hoffmann, 2007; Kleino and Silverman, 2014).

The Toll pathway, which is homologous to the mammalian Toll/IL-1 receptor signaling, is an evolutionarily conserved signaling cascade that plays a key role in the establishment of the dorso-ventral axis of *Drosophila* embryos (Moussian and Roth, 2005). Additionally, Toll shares the cytoplasmic TIR (Toll/interleukin-1 receptor (IL-1R)) domain and leucine-rich repeats (LRRs) with mammalian Toll-like receptors (TLRs). This pathway regulates systemic expression of AMPs in response to Gram-positive bacteria and fungi (Lindsay and Wasserman, 2014). Toll is activated specifically via the detection of lysine-type PGN by the circulating pattern recognition receptors (PRRs) PGRP-SA and PGRP-SD. Microbial recognition triggers a common proteolytic cascade downstream of the PRRs and results in the activation of the cytokine spätzle, which binds to the cell transmembrane receptor Toll and induces the receptor's dimerization (Lindsay and Wasserman, 2014). This interaction culminates in the transcription of genes encoding the AMPs drosomycin, metchnikowin, and defensin. Interestingly, transcriptional analysis shows that *S. carpocapsae* strongly upregulates the Toll receptor-associated adaptor MyD88, indicating that this pathway participates in modulating

distinct aspects of the insect anti-nematode immune response (Yadav *et al.*, 2018).

Other immune pathways

The canonical Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway has an evolutionarily conserved immune function against a variety of invaders (Lemaître and Hoffmann, 2007; Myllymäki and Rämet, 2014). In addition to its association with several aspects of insect innate immunity and homeostasis, the JAK/STAT pathway has been implicated in the regulation of cell growth, differentiation, apoptosis, and inflammatory reactions (Myllymäki and Rämet, 2014; Bang, 2019). Loss-of-function mutation analysis and RNA interference-based gene silencing studies in *Drosophila*, *Aedes aegypti*, and *Anopheles gambiae*, as well as some hymenopteran (sawflies, wasps, bees, and ants) and lepidopteran species have tremendously contributed to the functional characterization of the JAK/STAT core components and regulators (Bang, 2019). For instance, in contrast to the multiple JAK/STAT pathway components in vertebrates, *Drosophila* possess only one JAK (encoded by the gene *hopscotch*), one STAT (encoded by *Stat92E*), three ligands, and one receptor, making the molecular events in this signaling pathway less redundant and simpler to analyze (Myllymäki and Rämet, 2014; Bang, 2019). Activation of the *Drosophila* JAK/STAT pathway is initiated by the extracellular binding of the unpaired ligand Upd to the transmembrane receptor Domeless (Dome, homolog of the vertebrate type I cytokine receptors), which undergoes a conformational transformation leading to the phosphorylation of the associated JAKs and subsequent recruitment of STATs. This reaction ultimately leads to the expression of target genes, including the stress-induced proteins known as turandots (Lemaître and Hoffmann, 2007; Myllymäki and Rämet, 2014). Remarkably, infection with *S. carpocapsae* axenic nematodes results in elevated transcript levels of Dome, which highlights the JAK/STAT pathway as one of the key components that can possibly serve to recognize molecular patterns of EPNs (Yadav *et al.*, 2018).

Among the other versatile and ubiquitous immune sensors in insects is the Jun-N-terminal kinase (JNK) signaling pathway (Tafesh-Edwards and Eleftherianos, 2020). JNK is an evolutionarily

conserved mitogen-activated protein kinase (MAPK) that branches out from the Imd pathway and participates in several physiological processes such as cell cycle regulation, apoptosis, and phagocytosis. Many of the vertebrate JNK pathway components have been identified in *Drosophila*: Hemipterous (hep), an ortholog of human MAP2K7, Bsk (DJNK), and DJun/DFos (Fos proto-oncogene), all of which function in dorso-ventral patterning and immunity (Sluss *et al.*, 1996). JNK signaling is activated by an extensive variety of intrinsic and extrinsic stress factors, including oxidative stress, UV radiation, protein synthesis inhibitors, chemotherapeutic agents, cell death ligand FasL, and proinflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-1 (Sluss *et al.*, 1996; Shen and Liu, 2006). Furthermore, JNK signaling participates in the *Drosophila* defense against axenic *S. carpocapsae*, thus conferring a survival advantage to the host during infection (Yadav *et al.*, 2018).

Insect cellular immunity

The body cavity of all arthropods is filled with a circulating hemolymph that contains blood cells known as hemocytes, which participate in the regulation of cellular immune mechanisms such as phagocytosis, encapsulation, and nodulation (Vlisidou and Wood, 2015; Rosales, 2017). Hemocytes are grouped according to their morphological and functional features into several types, including granulocytes, plasmatocytes, spherulocytes, and oenocytoids (Rosales, 2017). However, it is important to note that not all these hemocyte types are found in all insect species. Therefore, we will refer to *Drosophila* immune mechanisms as a general model that is commonly used for deciphering cellular responses in animals. *Drosophila* possesses several thousand hemocytes, which can be divided into three cell types: plasmatocytes, lamellocytes, and crystal cells (Vlisidou and Wood, 2015).

Plasmatocytes, or macrophage-like cells, comprise the majority of the hemocytes circulating in the hemolymph. They are responsible for phagocytosis, a vital component of innate immunity during which cells engulf and degrade microbes, foreign particles, or apoptotic cells (Gold and Brückner, 2015). The cell-surface proteins involved in this recognition process include, but are not limited to, scavenger receptors

CD36 homolog Croquemort (Crq), Draper, and NimC4/Simu, as well as the immunoglobulin superfamily protein DSCAM (Down syndrome cell adhesion molecule), which contains an RGD domain that may interact with integrins. In the larval and adult stages, recognition and rapid engulfment of invading microbes such as bacteria mainly rely on the cell surface receptors Eater and NimC1 (Chung and Kocks, 2011). Eater is generally known as the main phagocytic receptor for Gram-positive but not Gram-negative bacteria, and contributes to microbe tethering to hemocytes. NimC1 detects Gram-negative bacteria. However, more recent reports indicate that both receptors contribute synergistically to the phagocytosis of bacteria, but that Eater can bypass the requirement for NimC1 (Chung and Kocks, 2011).

Crystal cells, which constitute 5% of the larval hemocytes, are relatively large cells with crystalline inclusions. They are non-phagocytic cells involved in the melanization process, a major immune module found in arthropods but not in vertebrates (Dudzic *et al.*, 2015). Crystal cells mediate the rapid synthesis of melanin at the site of infection or injury in order to contain microbial pathogens and facilitate wound healing. A key enzyme in melanin synthesis is phenoloxidase (PO), which catalyzes the oxidation of phenols to quinones that subsequently polymerize into melanin. PO is usually synthesized as an inactive zymogen called prophenoloxidase (PPO), which is cleaved to generate active PO as a result of proteolytic cascade activation. The *Drosophila* genome encodes three PPOs. Two of them, PPO1 and PPO2, are produced by crystal cells, which are fragile, easily disrupted, and able to release their contents into the hemolymph upon activation. Melanin formation has been associated with several immune roles in *Drosophila* (Dudzic *et al.*, 2015). This reaction contributes to wound healing by forming a scab at the epithelial site of injury. In addition, by-products of PO activity include ROS, which are thought to contribute to the killing of pathogens. Melanization also participates in the encapsulation reaction against parasites (Dudzic *et al.*, 2015). Deposition of melanin on the parasite forms a physical barrier, allowing the localized and confined production of toxic compounds while ensuring the protection of the host.

Lamellocytes are relatively large, flat, and adherent cells that primarily function in the

encapsulation and neutralization of objects too large to be phagocytosed by plasmatocytes such as parasitoid eggs (Lemaitre and Hoffmann, 2007; Vlisidou and Wood, 2015). Lamellocytes are not found in embryos or adults, and are rarely observed in healthy larvae. Recognition of foreign objects by lamellocytes activates the host's hematopoietic organ (lymph gland) that rapidly increases the proliferation of lamellocytes in the hemocoel (Honti *et al.*, 2014; Satyavathi *et al.*, 2014). This leads to the formation of large nodules consisting of hemocyte aggregates (nodulation) and leading to the foreign object's entrapment (encapsulation). Inside the capsule the invading organism is killed by reactive cytotoxic products or by asphyxia (Carton *et al.*, 2009).

Coagulation

Because many invertebrates have an open circulatory system, effective coagulation mechanisms are in place to ensure fast and efficient closure of wounds, limit hemolymph loss, and, most importantly, prevent systemic infections (Loof *et al.*, 2011). Detailed analyses at the cellular level show that insect hemolymph coagulation promotes homeostasis and contributes significantly to the armor of host defense against several pathogens, including EPNs (Loof *et al.*, 2011; Dziedziech *et al.*, 2020). Much of our knowledge of hemolymph coagulation stems from several arthropod model systems such as *Drosophila* (Theopold *et al.*, 2014; Dziedziech *et al.*, 2020), horseshoe crab *Tachypleus tridentatus* (Osaki and Kawabata, 2004), and fall armyworm *Spodoptera frugiperda* (Huot *et al.*, 2019). The striking similarities of the coagulation systems between these models and vertebrates have been well characterized and can be observed in the family of transglutaminases (homolog of the vertebrate clotting factor XIIIa), with implications for a phylogenetic comparison of coagulation between divergent animal classes (Theopold *et al.*, 2014). Both insect and human transglutaminase activity accumulates on microbial surfaces, leading to their sequestration into the clot (Wang *et al.*, 2010). Once mobilized, pathogens can be either destroyed extracellularly by AMPs or eliminated intracellularly. Subsequent steps also include melanization and epithelial movements (Galko and Krasnow, 2004).

Studies on hemolymph clots from *Drosophila* show that the most abundant protein in a clot is hemolectin (homolog of vertebrate clotting factor, von Willebrand factor), which is expressed by plasmatocytes (Goto *et al.*, 2001). Blood coagulation led by plasmatocytes involves several clotting factors and is independent of both melanin production and phenoloxidase activity, which are also part of the wound healing process (Goto *et al.*, 2003). Among the best-characterized clotting factors are *Drosophila* transglutaminase (TG) and fondue (Galko and Krasnow, 2004; Wang *et al.*, 2010; Theopold *et al.*, 2014). Phylogenetically, TG is the sole component of clotting cascades that has been evolutionarily conserved. Together with fondue, TG primarily functions to crosslink clot fibers formed by hemolectin, thus reacting promptly to injury and infection. Interestingly, TG is found in hemocytes while fondue is expressed in the fat body under control of the Toll signaling pathway, which strongly suggests that both cellular and humoral factors are required in blood coagulation with indispensable contributions from hemocytes (Johansson *et al.*, 2005; Scherfer *et al.*, 2006). Hence, coagulation systems can be viewed as a primary link between humoral and cellular responses that mount a robust systemic immune response against various insults and pathogens, triggering sequential signaling events in cells and adjusting their transcriptional responses accordingly.

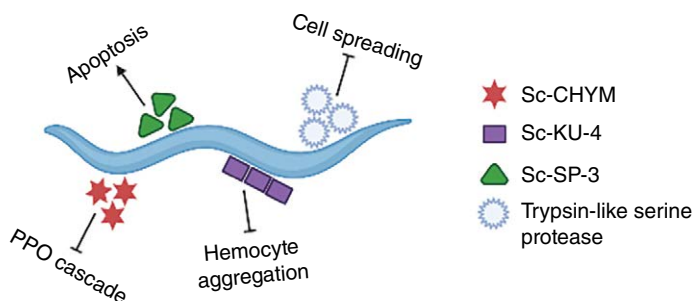
Nematode Immunomodulatory Strategies

Along with their pathogenic bacteria, EPNs stage a powerful infection that allows them to damage host tissue through migration and feeding while simultaneously evading the host immune system. Their successful parasitization is accomplished in part through the release of a varying set of molecules that contribute to pathogenicity and host immune suppression, a strategy known as immunomodulation (Cooper and Eleftherianos, 2016; Bobardt *et al.*, 2020). More specifically, this tactic promotes the survival of nematode parasites in the host by altering the activation of the host immune response during infection, which can be accomplished via three possible strategies: (i) the nematodes are recognized as

self and thus prevent the induction of immune responses from the host; (ii) the nematodes escape detection by the host immune system and are not recognized as non-self; and (iii) the nematodes employ mechanisms to suppress the host immune response after being recognized as non-self (Eleftherianos *et al.*, 2017). Accordingly, the mechanisms and molecules involved in nematode immunomodulation vary greatly depending on their mode of action and the type of host (Klei, 1997; Hartmann and Lucius, 2003; McSorley *et al.*, 2013). EPNs can migrate to remote tissues where they go undetected by the host immune system (anatomical seclusion), remove proteins from the hemolymph and sequester them to prevent immune detection (camouflage or mimicry), and produce molecules that cause the active suppression of host immune functions (interference) (Castillo *et al.*, 2011; Cooper and Eleftherianos, 2016; Eleftherianos *et al.*, 2017; Bobardt *et al.*, 2020). Understanding such mechanisms used by parasitic nematodes to modulate various aspects of the host innate immune response of insects and plants is crucial to devising novel approaches to protection against harmful parasitic nematodes, as well as the enhancement of beneficial nematode species that are commonly used for the biological control of insect pests.

Nematode immunomodulation in insects

The immunomodulatory and pathogenic properties of EPNs are largely attributed to the excretory/secretory (ES) products they release during infections in insects (Castillo *et al.*, 2011; Cooper and Eleftherianos, 2016; Eleftherianos *et al.*, 2017; Bobardt *et al.*, 2020). Proteases, in particular, have been identified as major components that *Heterorhabditis* and *Steinernema* nematodes exploit to downregulate the host immune responses (Fig. 3.3). These molecules can also aid in nematode invasion and digestion of proteins within the infected insect. *H. bacteriophora* nematodes have been reported to inhibit the production of cecropin, an AMP that constitutes a key part of the innate immune system of insects, using a specific protease that is synthesized and secreted by both *H. bacteriophora* nematodes and their mutualistic *P. luminescens* bacteria (Jarosz, 1998). This protease is able to suppress cecropin synthesis in *Galleria mellonella* (wax/honeycomb moth), as well as other lepidopteran and hymenopteran insects. In addition, *S. feltiae* body surface and its compounds, particularly epicuticular lipids, downregulate antibacterial peptide synthesis in *Galleria mellonella* (Brivio *et al.*, 2002). Following bacterial challenge, insects infected with purified *S. feltiae* cuticles exhibit a lack of bacterial



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Fig. 3.3. Entomopathogenic nematodes (EPNs) utilize numerous mechanisms to overcome their host's immune system. Proteases, for instance, have been identified as major components which EPNs exploit to significantly downregulate the host immune responses. Sc-SP-3, which belongs to the chymotrypsin-like serine protease family, induces apoptosis in insect cells. In addition, trypsin-like serine proteases block host cells from spreading and induce severe morphological changes in them. The BPTI–Kunitz family of inhibitors (Sc-KU-4) impairs the aggregation of hemocytes, and the chymotrypsin protease (Sc-CHYM) prevents activation of the PPO cascade in the hemolymph, subsequently suppressing melanization and encapsulation.

clearance from the hemocoel and reduced levels of AMPs due to the binding of certain hemolymph proteins by *S. feltiae* cuticular lipids (Brivio *et al.*, 2002). Further analysis shows that degradation of these cuticular lipids prior to injection results in the loss of their inhibitory activity, which complements rescue experiments in which simultaneous injection of purified hemolymph proteins with bacteria into insects that have been previously infected leads to the restoration of the host's antimicrobial activity (Brivio *et al.*, 2002; Eleftherianos *et al.*, 2017).

H. bacteriophora studies also reveal that their ES products are lethal to their insect hosts at high concentrations (Kenney *et al.*, 2019, 2021). Treatment of *Drosophila* adults, larvae, and isolated larval fat bodies with proteins extracted from activated *H. bacteriophora* consistently suppresses the expression of AMP-encoding gene *Diptericin* through the Imd pathway (Kenney *et al.*, 2021). This immunomodulatory mechanism propagates the infection of not only the EPN but also its associated bacterium *P. luminescens*, which would otherwise be killed by its insect host. In other words, by reducing the expression of diptericin, an AMP that acts against Gram-negative bacteria, the activated ES products allow a more rapid propagation of *P. luminescens* that corresponds to a quicker host mortality. Parallel experiments with *S. carpocapsae* and *X. nematophila* show that infection with nematodes, before the release of bacteria from their gut, leads to a significant reduction in total hemocytes, the core of the insect cellular immune response, thus suggesting that the nematode itself suppresses the host immune system, to the benefit of its endosymbiotic bacteria (Garriga *et al.*, 2020). Although this mechanism for immunomodulatory products from EPNs appears to be time sensitive, the molecular details remain undetermined. A similar study of infection in the lepidopteran *Mythimna unipuncta* (armyworm moth) with the *S. carpocapsae*–*X. nematophila* complex shows that EPNs produce factors that specifically target the host's hemocytes (Ribeiro *et al.*, 1999). When isolated, these factors show unstickling and cytotoxic properties towards plasmatocytes and granular cells, respectively, which likely disrupt the host's nodulation and encapsulation responses, in addition to reducing the number of hemocytes. Another study showed that *S. carpocapsae* IJs secrete products that prevent

the adhesion of plasmatocytes and granular cells during infection of *G. mellonella* and the forest tent caterpillar moth *Malacosoma disstria* (Walter *et al.*, 2008). This immunomodulatory mechanism leads to reduced phenoloxidase activity in the host's hemolymph and lower encapsulation of the nematodes (Toubarro *et al.*, 2009).

Other molecular studies using the *G. mellonella* model system reveal that *S. carpocapsae* IJs produce a protein, Sc-SP-3, that belongs to the chymotrypsin-like serine protease family (Toubarro *et al.*, 2009). Gene expression analysis shows that this protein is expressed only in IJs and that pre-invasive nematodes inside the midgut express higher amounts of Sc-SP-3 than those that already enter the hemocoel. Sc-SP-3 causes histolysis in the insect midgut, digests extracellular proteins, and induces apoptosis in insect cells, which suggests that this protein is a multifunctional chymotrypsin-like protease involved in the *S. carpocapsae* pathogenesis (Toubarro *et al.*, 2009). Another trypsin-like serine protease from *S. carpocapsae* displays potent activity against *G. mellonella* hemocytes by blocking them from spreading and inducing severe morphological changes to them (Balasubramanian *et al.*, 2010). Similarly, the BPTI–Kunitz family of inhibitors (Sc-KU-4), produced by the same nematode species, not only causes inhibition of encapsulation, but also impairs the aggregation of hemocytes (Toubarro *et al.*, 2013a). Evidence also shows that *S. feltiae* cuticle removes hemolymph proteins from the insect host, using them as a shield that enables the nematodes to suppress hemocyte aggregation and encapsulation, and therefore persist in the host (Mastore and Brivio, 2008).

Among the numerous mechanisms that EPNs utilize to overcome their host's immune system is their ability to interfere with the insect phenoloxidase response using their cuticle or ES products that actively suppress the melanization cascade. Isolation and purification analysis demonstrates that ES products released by *H. bacteriophora* significantly inhibit the PO cascade responsible for the melanization of the *G. mellonella* hemolymph (Eliáš *et al.*, 2020). In these products, six peptide sequences were identified, including proteins involved in ubiquitination and the regulation of a Toll pathway, for which a role in the regulation of insect innate immunity has been proposed in the aforementioned studies.

Additionally, infection with *S. feltiae* substantially reduces the enzymatic activity of the PPO cascade in the host *G. mellonella* (Brivio *et al.*, 2002). During this process, *S. feltiae* cuticle lipidic compounds interact with the host humoral components and remove them from the hemolymph (Brivio *et al.*, 2004). Thus, the depletion of these molecules seems to be linked to the drastic decrease in host PPO system and subsequent encapsulation activity. Biochemical and molecular examination of a chymotrypsin protease, Sc-CHYM, a novel virulence factor produced during the infective stage of *S. carpocapsae*, presents evidence that this compound prevents activation of the PPO cascade in the hemolymph of *G. mellonella* larvae (Balasubramanian *et al.*, 2009). It also suppresses the melanization and encapsulation responses against the parasitic nematodes. Sequence comparison and evolutionary marker evaluation demonstrate that Sc-CHYM is clustered with several insect chymotrypsins, forming an ancestral branch in the phylogenetic tree, which suggests that Sc-CHYM branches off at an early stage of a cluster divergence (Balasubramanian *et al.*, 2009). Although further expression pattern analysis and molecular characterization of Sc-CHYM are required for a complete delineation of the function of Sc-CHYM, these results contribute to the understanding of the pathogenesis strategies used by *S. carpocapsae* (Balasubramanian *et al.*, 2009).

Given these various transcriptomics studies, in combination with molecular and biochemical techniques, it is understood that EPNs exhibit potent strategies to disable the host immune system and create suitable conditions for themselves and their mutualistic bacteria. These mechanisms are highly specific to the nematode species and modulate both the humoral and cellular immune functions in the host. For instance, *G. mellonella* hemocytes can recognize IJs of *H. bacteriophora* but not *S. carpocapsae* or *S. glaseri* (Ebrahimi *et al.*, 2011). Inhibition of AMP and hemocyte functions results in a rapidly declining host immune response that promotes nematode migration in the host and enhances pathogenicity. The molecular effects of EPN infections are likely the product of a collection of different ES products fulfilling a variety of roles, each of which is important for understanding the host–parasite relationship. Remarkably, much of the available knowledge of these effectors is based

on studies in *S. carpocapsae*. In fact, it was only recently confirmed that *H. bacteriophora* can produce ES products that disrupt the host's innate immune responses (Kenney *et al.*, 2019, 2021). Therefore, little is known of the ES products from EPNs in general, and *H. bacteriophora* in particular. Identification of the specific EPN-derived molecules that target the innate immune pathways in insects, and whether they are conserved in mammalian parasitic nematodes, could allow the discovery of new immunotherapies and treatments of diseases associated with excessive inflammation.

Insect Immune Response to Entomopathogenic Nematodes

EPNs suppress the host immune system during the early stages of infection, causing it to tolerate not only nematode parasites but also their symbiotic bacteria, until the host succumbs to infection (Toubarro *et al.*, 2013b). Nonetheless, in response to nematodes that manage to enter the hemocoel, insects can mount a robust systemic immune response using distinct signaling pathways and effector molecules. The delayed release of symbiotic bacteria, which is variable depending on the species, allows the host insect a time window in which it may neutralize the parasite before being forced to compensate for the additional challenge of the bacterial infection (Li *et al.*, 2007). In general, nematode avirulence is primarily achieved by initiating hemolymph clotting, activating a melanization reaction, and encapsulating the nematode in layers of hemocytes. However, the exact insect immune response against EPNs correlates considerably with host and nematode specificity. Comparative analysis of the infectivity of *S. carpocapsae*, *S. glaseri*, *Steinernema scapterisci*, and *H. bacteriophora* to the Japanese beetle larva *Popillia japonica* indicates that *P. japonica* presents a strong encapsulation and melanization response to all species except *S. glaseri* (Wang *et al.*, 1994). Whereas *H. bacteriophora* and *S. carpocapsae* are able to overcome the immune response, *S. scapterisci* is eliminated by the host's immune responses. Moreover, infections of *S. scapterisci* and *H. bacteriophora* in house cricket *Acheta domesticus* adults result in the successful removal of the parasitic nematodes

without any significant differences in mortality (Wang *et al.*, 1994). Nematode inhibition via encapsulation is also evident in infections of the Colorado potato beetle *Leptinotarsa decemlineata* with *Heterorhabditis marelatus* nematodes (Armer *et al.*, 2004).

Similarly, later research confirmed that *S. glaseri* infections in *P. japonica* larvae cause reduced encapsulation and melanization activities in comparison with *H. bacteriophora*, thus explaining previous reports of *S. glaseri* persistence in this particular host (Li *et al.*, 2007). Despite this success in *P. japonica*, *S. glaseri* fails to establish an infection in *A. domesticus*. In addition, though *S. glaseri* FL strain, *Steinernema scarabaei*, *S. feltiae*, and *H. bacteriophora* are intensively melanized by hemocytes from the oriental beetle *Exomala orientalis*, tobacco hornworm *Manduca sexta*, and *P. japonica*, *S. glaseri* NC strain escapes from hemocyte encapsulation and suppresses the immune responses in the same hosts (Li *et al.*, 2007). This variation in immunity reveals that insect host defense mechanisms against EPNs are specific not only to the nematode species but also to its strain. These differences can also be attributed to the variation in the infectivity properties of the nematode used in infection. Thus, while host recognition underlies some of the differences between resistant and susceptible host species, escape from encapsulation following recognition can also allow successful infection in insect hosts.

Conclusions and Perspectives

Nematodes are a ubiquitous and vast phylum, consisting of many species that opportunistically inhabit a broad range of hosts, including plants, insects, and animals. Naturally, a nematode's success in terms of survival and parasitism is based on the type of host it infects and the context in which the infection is taking place, which can either be in line with, or sharply opposed to, human health, as well as therapeutic, agricultural, and economic interests. Most importantly, the life cycle of EPNs, which involves harboring mutualistic bacteria and infecting a suitable insect host, has gained considerable interest and support in the hope that these interactions can be mediated beneficially. EPNs and

their mutualistic bacteria are therefore widely used in biological control approaches and alternatives to chemical insecticides for the efficient and organic management of agricultural insect pests. EPNs also present powerful models for dissecting the molecular and mechanistic interactions between the host immune system and nematode virulence mechanisms, bacterial pathogenicity, and microbial mutualism.

The debate in relation to the specific contributions of EPNs and their bacteria to the complex's successful infection in insect hosts requires further investigation and identification of their core immunomodulatory components. However, evidence shows that the parasitic interaction with the infected hosts is primarily based on the synergism between the parasite itself and its symbiont. The high efficiency of EPNs is due to their ability to penetrate the insect cuticle and rapidly migrate in the host, as well as produce ES molecules that compromise the insect immune system or help them evade it. To complement this, bacterial symbionts synthesize and secrete several virulence factors that accelerate insect death by targeting major humoral signaling pathways, as well as the host's hemocytes.

During the initial stages of infection, EPNs may elicit a rapid cellular and humoral immune response in the insect host that could lead to the production of AMPs, encapsulation, melanization, and subsequent killing of the parasites. While several cellular responses to the nematode–bacterium complex have been documented, little is known about the specific molecules employed by the host defense mechanisms and how they relate to known functions of EPN-derived virulence and immunomodulatory products. Therefore, more targeted proteomics and molecular genetics studies can lead to the discovery of more genes that are potentially involved in parasitic processes of EPNs and confirm their mode of action and role as potent pathogenicity factors. Such discoveries will further characterize the genetic mechanisms that regulate the interactions between each nematode virulence component and the insect immune system and will identify novel host anti-nematode immune mechanisms, which will in turn lead to the implementation of improved strategies for the biological control of noxious insect pests and crop protection.

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4 The Use of the Root-knot Nematodes, *Meloidogyne* spp., for Studying Biotrophic Parasitic Interactions

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Introduction: Root-knot Nematode, *Meloidogyne* spp., a Global Pest

The world population is increasing at a faster rate than that of food production. Thus, increasing food availability has become challenging. Over 925 million people worldwide are reportedly facing extreme hunger, and 2 billion people do not have proper food security (FAO, 2010). To feed more than 9 billion people worldwide, global food supply must increase 70% by 2050 (Hassan *et al.*, 2013). Plant pathogens play a very important role in limiting food production. Among plant pathogens, plant-parasitic nematodes (PPNs) cause an average of 14.6% annual yield losses to the global food supply, which is projected to have a worth of US\$173 billion worldwide (Elling, 2013). Based on scientific and economic importance, root-knot nematodes (RKNs) are placed at the top among the list of damaging plant-parasitic nematodes (Jones *et al.*, 2013). RKNs parasitize nearly all vascular plant species, including both monocotyledons and dicotyledons (Blok *et al.*, 2008). RKNs belong to the genus *Meloidogyne*, which consists of approximately 100 species (Elling, 2013).

As far as infectious symptoms are concerned, RKNs may not develop any specific above-ground symptoms; heavily infected plants can experience

stunting and wilting or chlorosis (yellowing) of lower leaves, but these signs may also be associated with nutrition deprivation or other diseases. In contrast, the below-ground symptoms are apparent as galls or swellings that develop all over the roots. Moreover, nematode-infected plants become more susceptible to other pathogens, such as *Fusarium* wilt and root rot (Back *et al.*, 2002).

The mode of infection of RKNs is unique. During plant interaction, the infective stage pre-parasitic nematode juvenile (J2) penetrates behind the root cap, enters the vascular cylinder, injects various cell wall-degrading enzymes from its stylet, degrades the cell wall and eventually induces giant cells differentiation. Once giant cells (GCs) are formed, the J2s feed on them and develop into J3 and J4 stages and ultimately into adult females (Cabrera *et al.*, 2015). Cortical tissue surrounding GCs enlarges and develops into gall-like structures on the roots, a hallmark of RKN infection. Egg masses (collections of eggs in gelatin matrix) can be embedded in galls or plant tissue. RKNs usually take 3–6 weeks to complete their life cycle, largely depending on environmental conditions.

The vast majority of studies center on four species, *Meloidogyne incognita*, *M. javanica*, *M. arenaria* (tropical), and *M. hapla* (temperate),

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which cause major losses to agricultural production (Jones and Goto, 2011). Their global importance is generated by RKN's wide distribution and infectivity to a wide variety of major crops.

More recently, some of the less prominent *Meloidogyne* species have emerged as a serious problem to agriculture. For instance, *M. chitwoodi*, *M. enterolobii*, or *M. fallax* species are highly aggressive and have recently been listed as quarantine species in the European and Mediterranean Plant Protection Organization (EPPO) alert list (A2 EPPO quarantine pest list 2017); these species are able to infect several plant species, including potatoes, carrots, tomatoes, wheat, corn, alfalfa, and numerous weeds (EPPO, 2016). In 1980, *M. chitwoodi* was identified in the North-west Pacific region of the USA, and then it was observed in Mexico, Turkey, several countries in Europe, and South Africa. *M. fallax* was found in Belgium, France, Germany, the UK, Switzerland, New Zealand, Australia, and South Africa (EPPO, 2016). *M. chitwoodi* and *M. fallax* or *M. javanica* are big concerns for potato farmers, as the nematodes can reproduce on both potato roots and tubers (Wesemael *et al.*, 2011). Galls can be seen on the potato surface along with brown spots near the female, thereby causing quality defects, which are not accepted in fresh markets (Ingham *et al.*, 2007). Diagnosis of this nematode in seed potatoes prior to field sowing is very costly.

Emil August Göldi identified the RKN *Meloidogyne exigua* for the first time in 1887 (Göldi, 1887). This is also a growing pest, spread throughout Central and South America, India, and China, as well as several countries of southern Europe. *M. exigua* has a wide host range and causes huge yield losses to many crops. For example, in Brazil, 45% of coffee yield losses were reported to be due to *M. exigua* and a survey reported the existence of this nematode in 22% of coffee plantations (Barbosa *et al.*, 2004). A survey was conducted in Germany on organic farming for vegetable and cereal production, which revealed the presence of RKNs (*M. chitwoodi*, *M. hapla*, or *M. naasi*) in more than half (51%) of the samples, causing tremendous damage (Hallmann *et al.*, 2007).

A rice nematode, *M. graminicola*, is also a very damaging pathogen, which threatens upland (rain-fed), lowland (irrigated), and deep-water rice (Bridge *et al.*, 2005). Consequently, in many nations, this pest is included in quarantine lists.

M. graminicola is well adapted to flood conditions and can live for a long time in the form of an egg or juvenile (Mantelin *et al.*, 2017). A 70% loss of deep-water rice, infected with 4000 J2s per plant, was observed by Bridge and Page (1982). The short life cycle (19 days in Bangladesh, 23–27 days in the USA, and 26–51 days in India) and wide host range make this pest unstoppable (<https://www.cabi.org/isc/datasheet/33243>, accessed 1 December 2021); De Waele and Elsen, 2008). RKNs infect dozens of major crops growing in the greenhouse or in fields, such as potato, carrot, rice, sunflower, tomato, tobacco, corn, and sugar beet. Nonetheless, the potential harm caused by these nematodes is frequently underestimated, and there is little research on their economic effects on agriculture (Wesemael *et al.*, 2011).

This chapter is an overview of the latest literature on how RKN effectors (small peptide hormones, cell wall-degrading enzymes, etc.) are involved in establishing effective parasitism and how they exploit different pathways related to defense in plants to avoid incitation of resistance responses. We discuss how this literature would be useful for other PPNs to understand plant–nematode interactions. We propose RKN as a model organism because it is the most important plant-parasitic nematode; it is considered as a devastating nematode, and has been extensively studied in order to understand the mechanism of plant parasitism. The genome sequencing of several root-knot nematodes such as *M. incognita* and *M. hapla* (Abad *et al.*, 2008; Opperman *et al.*, 2008) has greatly improved our understanding of nematode genes and proteins involved in successful plant–nematode interactions. Among the many studies on plant–nematode interactions, a lot of information is available about the genes and proteins that determine nematode virulence (Bellafiore and Briggs, 2010).

Giant Cells, the Unique Feeding Structures of the Root-knot Nematodes

RKNs are biotrophic in nature and have an enormous ability to turn ordinary root cells into specialized feeding cells: giant cells that supply

the nutrients to the nematode (Caillaud *et al.*, 2008). The nematode releases a cocktail of effectors from its subventral oesophageal gland cells to form GCs.

The development of a giant cell is a complex process in which host cells undergo extensive re-programming. Within 24 h following nematode invasion, a number of modifications can be seen in the host cell around the nematode's head, such as mitotic division, binucleated cells, etc.; these are the first indicators of GC formation. Eventually in the developing GC, repeated karyokinesis (nuclear division) in the absence of cytokinesis (cytoplasm division) leads to the generation of many nuclei, and the cell becomes multinucleated. After this division, different chromosome numbers (polyploid) and more DNA content can be observed, presumably due to the endoreduplication cycles (de Almeida Engler *et al.*, 1999). DNA replication is important for primary GC establishment and it continues for approximately 14 days after infection. In young galls, upregulation of key cell cycle encoding genes (CDKA;1, CDKB1;1, CYCA2;1, and CYCB1;1) was observed and the same genes were down-regulated in late stages, indicating involvement of these genes in the early stage of GC development. These processes are involved in enlargement of nuclei and GC size to provide sufficient food for the developing nematode. Using electron microscopy, Jones and Payne (1978) recorded the GC initial process over 72 h of nematode invasion and observed that the nematodes are able to turn their heads and reinsert their stylets between various GCs during the interactions.

Histological analysis has shown that the nematode targets parenchymatic cells of the host vascular cylinder for GC formation. The anterior portion of the nematode head was located covering parenchymatic cells within the stele, which showed that the key molecules promoting the development of GC are esophageal gland secretions (Berg *et al.*, 2008). Nevertheless, knowledge of the exact type of cell preferred by the nematode and the GC ontogeny is elusive.

GCs remain metabolically very active and contain an excess number of organelles (such as endoplasmic reticulum, ribosome, mitochondria, and Golgi bodies), making the cytoplasm hugely dense (Berg *et al.*, 2008). One study showed that nematode-induced galls have high amounts of starch, glucose, sucrose, malate, and different

types of amino acids (Damiani *et al.*, 2012). Since GC formation is a long-term process, during cell expansion major reorganization occurs in cytoskeletal components such as in actin and microtubule cytoskeleton. In the absence of microtubule-associated protein (MAP65-3), GCs fail to fully differentiate and are eventually destroyed, suggesting the importance of MAP65-3 in GC development (Caillaud *et al.*, 2008). Earlier studies showed that cytoplasmic actin looks fragmented in GCs, and recently a report showed that the *MiPFN3* effector of *M. incognita* causes that fragmentation, which indicates the role of *MiPFN3* in plant cell reorganization to form GCs (Leelarasamee *et al.*, 2018). The availability of single-cell transcriptomics has enabled substantial strides in recognizing GC signatures (Fosu-Nyarko *et al.*, 2009; Barcala *et al.*, 2010; Portillo *et al.*, 2013; Ji *et al.*, 2013).

The Emergence of Plant Parasitism in Nematodes

To parasitize the host plant, nematodes use a stylet and a cocktail of effector proteins secreted from esophageal gland cells. The stylet is a needle-like structure localized near the head that punctures and delivers effector proteins into host-plant cells to turn them into a nematode feeding site (NFS), a permanent source of nutrition (Hussey, 1989; Davis *et al.*, 2008). RKN effectors are involved in functions such as host immunity suppression and formation and functioning of NFS. Emerging evidence suggests that during the process of parasitism these nematode effectors play an important role in avoiding defense responses in the host cells (Table 4.1) (Gardner *et al.*, 2019; Mejias *et al.*, 2019). In RKNs the majority of effectors are produced by two subventral esophageal gland cells and one dorsal gland cell and are a primary source of secreted effectors. A large number of RKN effectors secreted from these glands have been characterized in the past few years (Ali *et al.*, 2017; Siddique and Grundler, 2018; Vieira and Gleason, 2019; Kumar *et al.*, 2020). However, there remains a dearth of information on the mechanistic details of effector-manipulated host genes.

Various RKN parasitism genes encoding for putative effector protein have been identified

Table 4.1. Root-knot nematode effectors involved in plant immunity suppression (adapted and edited from Kumar *et al.*, 2020).

Nematode	Effector protein	Localization	Function	Reference
<i>Meloidogyne incognita</i>	<i>Mi-CRT</i>	Apoplast	Overproduction in plant cells increases plant resistance to RKNs	(Jaouannet <i>et al.</i> , 2013)
	<i>Msp40</i>	Cytoplasm and nucleus	Suppresses ETI-associated cell death	(Niu <i>et al.</i> , 2016)
	<i>Misp12</i>	Cytoplasm	Participates in the maintenance of giant cells during parasitism	(Xie <i>et al.</i> , 2016)
	<i>Mi-CM3</i>	Cytoplasm and nucleus	Suppresses plant immunity by manipulating the SA pathway at the early stage of nematode parasitism	(Wang <i>et al.</i> , 2018)
	<i>MiPFN3</i>	Unknown	Binds actin monomers to manipulate plant actin, which must undergo reorganization for giant cell formation	(Leelarasamee <i>et al.</i> , 2018)
	<i>MiSGCR1</i>	Cytoplasm and nucleus	Suppresses plant cell death	(Nguyen <i>et al.</i> , 2018)
	<i>MiISE5</i>	Cytoplasm	Interferes with various metabolic and signaling pathways, especially SA- and JA-mediated signaling pathways	(Shi <i>et al.</i> , 2018a)
	<i>MiISE6</i>	Nucleus	Interferes with various metabolic and signaling pathways, especially the JA signaling pathway	(Shi <i>et al.</i> , 2018b)
<i>M. javanica</i>	<i>MjTTL5</i>	Plastids	Encodes a transthyretin-like protein that may suppress host defenses	(Lin <i>et al.</i> , 2016)
<i>M. hapla</i>	<i>Mn265</i>	Cytoplasm	Suppresses host defenses	(Gleason <i>et al.</i> , 2017)
<i>M. enterolobii</i>	<i>MeTCTP</i>	Cytoplasm	Suppresses programmed cell death in host plants	(Zhuo <i>et al.</i> , 2017)
<i>M. graminicola</i>	<i>MgGPP</i>	Nucleus	Suppresses host defenses and enhances nematode parasitism	(Chen <i>et al.</i> , 2017)
	<i>MgMO237</i>	Cytoplasm and nucleus	Overexpression in plants increases plant susceptibility to RKNs by suppressing the host defense responses	(Chen <i>et al.</i> , 2018)
	<i>Mg16820</i>	Apoplast, cytoplasm and nucleus	Suppresses both the PTI and the ETI response	(Naalden <i>et al.</i> , 2018)
	<i>Mg01965</i>	Apoplast	Suppresses the PTI response	(Zhuo <i>et al.</i> , 2019)
	<i>Mg-Msp18</i>	Cytoplasm and nucleus	Suppresses defense-related programmed cell death	(Grossi-de-Sa <i>et al.</i> , 2019)

using gland-specific transcript data combined with bioinformatics (Davis *et al.*, 2000, 2008; Haegeman *et al.*, 2012; Hewezi and Baum, 2013; Mitchum *et al.*, 2013). Approximately 500 secreted proteins were identified from *M. incognita* pre-parasitic J2s and females (Bellafiore *et al.*, 2008; Wang *et al.*, 2012). Since the advent of advanced technologies and accessibility of RKN genomic data, identification of effectors has become relatively easy. The most widely used *in silico* approach for identifying the effector coding genes is based on three criteria: (i) presence of the N-terminal signal peptide for secretion; (ii) the lack of the transmembrane domain; and (iii) comparison with the other nematode effector sequence (Siddique and Grundler, 2018).

Recently a unique and reliable computer-based method for effector identification in cyst nematodes has been discovered, in which dorsal gland (DOG) boxes are found in the gene-encoding promoter region in dorsal gland cell effectors (Eves-van den Akker *et al.*, 2016a; Masonbrink *et al.*, 2019). The DOG box is a 6 bp (ATGCCA) sequence that is enriched in the 500 bp upstream region of effector gene promoters. Using this approach, the same box was also found in effector genes of *Bursaphelenchus xylophilus* and *Pratylenchus penetrans* nematodes (Espada *et al.*, 2018; Vieira *et al.*, 2018). Promoter motif prediction has therefore proven to be a reliable tool for the detection of effector genes and can be applied in RKNs and other evolving PPN species. Additional tools for classifying parasitic effectors are expected in the future.

Suppression, Manipulation and Induction of Host Defenses

The RKNs are biotrophic in nature; they spend most of the time within the host to complete their life cycle, and during that phase the nematodes are exposed to various levels of the plant's defense system. Therefore, to survive and further propagate, the nematode must suppress the host responses. Plants use several defense strategies to detect pathogens, including cell-localized plant pathogen recognition receptors (PPRs). These PPRs recognize pathogen-associated molecular patterns (PAMPs), which further activate the pattern-triggered immunity (PTI) in the host plant (Zipfel, 2014).

So far, only one nematode-associated molecular pattern (NAMP) (ascr#18) has been identified among the RKNs (Manosalva *et al.*, 2015) and recently the first identified pattern recognition receptor (PRR) for nematodes has been the *Arabidopsis* Nlir1 gene, which is widely conserved in the plant kingdom (Mendy *et al.*, 2017). PAMPs are important structural elements of the pathogens that cannot be altered to prevent their recognition. As a result, PAMPs are often conserved by taxa. Thus, PTI is successful in controlling a broad range of such pathogens. Damage-associated molecular patterns (DAMPs) are another level of plant protection. This form of immunity is triggered after nematode invasion and damage to the plant cell walls (Boller and Felix, 2009; Albert, 2013). To obtain entry into the host cell, nematodes use cell wall-degrading enzymes to break down the walls of the plant. Therefore, degradation products may act as DAMPs (Haegeman *et al.*, 2011). Successful pathogens deliver an effector protein for PTI suppression; this is known as effector-triggered susceptibility (ETS). In response, the plant uses resistance (R) genes to recognize the effectors, which contributes to the effector-triggered immunity (ETI) (Jones and Dangl, 2006). In addition, ETI results in a hypersensitive reaction (HR) at the site of infection. For example, during plant–RKN interactions, the *Mi* gene induces HR to inhibit the development of a nematode feeding site in tomatoes (Milligan *et al.*, 1998). Strikingly, RKNs are evolving to release diverse effector(s) to suppress ETI and allow nematodes to infect the host. The zigzag model illustrates how the pathogen effector and the plant R gene coevolved for their survival (Jones and Dangl, 2006).

Nematode NAMPS induce PTI in host plants

Plants are subject to a wide range of stress from PPNs. Several studies have demonstrated that plants can sense nematode infection, but the identities of the perceived nematode-derived signals are unclear. Nematode components, such as the surface coat perceived by the PPR plant, seem to be of great importance. Many researchers have shown the significance of the surface coat during nematode–host (plant and animal) interactions (Schmid-Hempel, 2008;

Patel *et al.*, 2009; Davies and Curtis, 2011). The surface coat is a cuticle made up of antigenic molecules (protein and glycoprotein) which are an obvious cause of NAMPs (Davies and Curtis, 2011). It is believed that, in order to prevent detection by host PPRs, nematodes undergo shedding and substitute with a new surface coat (De Veer *et al.*, 2007). Ascarosides (which are lipids) are the only NAMP that has been confirmed to exist. The compounds were originally found in a human parasite, *Ascaris lumbricoides*, and were later reported in various free-living as well as parasitic (plant, insect, and mammal) nematodes (Choe *et al.*, 2012). These glycolipids serve as dauer pheromones and are involved in the synchronization of nematode actions.

Ascaroside#18 is found most abundantly in the infective juvenile stage of RKNs (*M. incognita*, *M. hapla*, *M. javanica*) and cyst nematodes (*Heterodera glycines*) (Manosalva *et al.*, 2015). Exogenous treatment of Ascr#18 triggers host immune responses by activating MAPKs, apoplastic reactive oxygen species (ROS) bursts, defensive genes, and salicylic acid (SA) and jasmonic acid (JA) defense signaling pathways. As a result, the compound increases plant resistance to a broader range of pathogens, including root-knot nematodes, viruses, bacteria, fungi, and oomycetes. In addition, Ascr#18 is recognized by both monocot and dicot plant species such as *Arabidopsis*, tomato, potato, and barley, indicating that the recognition of Ascr#18 is well conserved. However, there is still a paucity of proof that Ascr#18 could be recognized by plant cell-surface receptors. Similar ascarosides are produced by most of the PPNs, which indicates that ascaroside biosynthesis is highly conserved among these nematodes.

For the analysis of early plant immune response, various sources of NAMP were used, such as NemaWater (Mendy *et al.*, 2017) and J2 nematode crude extract (Teixeira *et al.*, 2016). *Arabidopsis* seedlings treated with crude RKN (*M. incognita*) extract showed induction in the defense marker genes, *CYP71A12*, *MYB51*, and *WRKY11*. Roots treated with the same extract showed swollen structures similar to symptoms produced after RKN infection, suggesting the presence of elicitor(s).

Another study used NemaWater as a NAMP (Mendy *et al.*, 2017). This water is prepared by incubating the infective juvenile stage of *M. incognita*

and *Heterodera schachtii* nematodes in water for 24 h. *Arabidopsis* roots treated with this water developed apoplastic ROS burst and induced defense-related genes, signaling the presence of an immune elicitor(s) in the NemaWater. In addition, NemaWater treated with proteinase K or heat prior to inoculation in *Arabidopsis* roots has shown significantly decreased ROS burst and defense response, implying that the nature of this water is thermolabile and proteinaceous. Nematodes are supposed to have more than one distinct elicitor/NAMP, like PAMP, but how many elicitors are found in crude nematode extract or in the NemaWater is not certain.

In addition, NILR1 has been identified as a membrane localized pattern recognition receptor (PRR) in *Arabidopsis* that is induced upon exposure to nematode-derived molecules (Mendy *et al.*, 2017). Intriguingly, NILR1 mutants demonstrated greater susceptibility to both *H. schachtii* and *M. incognita*, indicating that NILR1 may be a common NAMP receptor for both nematodes (Kaloshian and Teixeira, 2019). This is the first PRR that recognizes NAMP, but more investigations are necessary to clarify NILR1's downstream signaling.

All of the above examples indicate the importance of RKN ascarosides (NAMPs) in inducing plant immunity systems, and similar studies may be carried out in other PPNs to understand the molecular level of plant–nematode interactions. Therefore, we are proposing RKN *M. incognita* as a model system.

RKN effectors involvement in suppressing plant immunity system

RKNs utilize an array of effector proteins to penetrate and establish feeding sites in the host plant (Jaouannet *et al.*, 2012). Many effectors are known to date, since the discovery of the first beta-1,4-endoglucanase effector expressed in the esophageal gland of cyst nematodes. For instance, 37 putative effector proteins of *M. incognita* have been identified that are expressed in the esophageal gland cells. Interestingly, all of the above proteins contain an N-terminal signal peptide and were found to be expressed in all the developmental stages (Huang *et al.*, 2003). In the past 10 years, several RKN effectors have been

characterized, showing an evolving trend in the suppression of plant defenses (Table 4.1). For example, calreticulin (*Mi-CRT*) is the first well-characterized gene expressed in the esophageal gland of *M. incognita*. Calreticulin (CRT) is a Ca²⁺ binding chaperone protein that is highly conserved in animals and plants. CRT plays an important role in parasitism of animal hosts, as evident in many studies (Khalife *et al.*, 1994; Kovacs *et al.*, 1998; Gao *et al.*, 2008). The *Mi-CRT* gene accumulates abundantly in giant cells during parasitism (Jaubert *et al.*, 2005). The knockdown of the *Mi-CRT* gene in pre-parasitic nematodes resulted in decreased gall formation (Dubreuil *et al.*, 2009), which displayed the significance of calreticulin to nematodes. In contrast, overexpression of this gene makes *Arabidopsis* more susceptible to nematodes and to pathogenic fungi. Further, *Mi-CRT* function in host defense modulation was investigated, and showed strong suppression of marker defense genes where the *Mi-CRT* gene was secreted to the apoplast by the nematodes (Jaouannet *et al.*, 2013). Another effector of *M. incognita*, *msp40*, manipulated plant immunity during plant parasitism by suppressing the programmed cell death (PCD) associated with activation of both PTI and ETI response (Niu *et al.*, 2016). It is assumed that *msp40* may be targeting typical components, such as MAPK cascade elements, or interfering with different components of PTI and ETI immunity pathways to suppress the plant defense system. Further identification of host target(s) and associated pathological cascades will help to elucidate the role of *msp40* in parasitism. More recently, a translationally controlled tumor protein (TCTP) family of proteins was identified in *M. enterolobii* and shown to be involved in calcium binding, apoptosis, stress protection, etc. (Lin *et al.*, 2016). The TCTP protein was previously identified in *M. incognita* secretions, but could not be validated functionally (Bellafiore *et al.*, 2008). The effector *MeTCTP* is localized in the cytoplasm of the plant cell and overexpression of this gene increased plant susceptibility to nematodes. Further experiments showed the suppression of plant immunity, indicating the importance of *MeTCTP* in parasitism. Similarly, the other RKN effectors, *MiSGCR1* (Nguyen *et al.*, 2018), *Mg01965* (Zhuo *et al.*, 2019) and *Mi-msp18* (Grosside-Sa *et al.*, 2019; Joshi *et al.*, 2020) have been shown as effectors manipulating plant immunity.

Many effectors are known in RKN that can interfere with salicylic acid (SA) and/or jasmonic acid (JA) mediated pathways for plant parasitism. In the RKN *M. javanica*, the suppression of the JA-mediated signaling by the fatty acid- and retinol-binding protein *FAR-1* in tomato may be the best example of how nematodes inhibit hormone-mediated signaling processes (Iberkleid *et al.*, 2013). *FAR-1* is generally expressed throughout the developmental stages of nematodes but the highest expression was observed in the infective juvenile stage. Immunocytochemical analysis revealed the accumulation of this protein across the *M. javanica* surface. Further, host-delivered RNAi of *FAR-1* decreases nematode infection whereas overexpression renders the tomato roots more susceptible to infection. The authors correlated the higher susceptibility of *Mj-FAR-1* overexpression lines with suppression of the JA pathway, as shown by downregulation of the two most important JA responsive genes. This research shows that *FAR-1* may regulate jasmonate-dependent defense responses to nematodes in order to enhance host susceptibility.

Another study focused on the chorismate mutase (CM) gene, *Mi-CM3*, of *M. incognita*, which manipulates the SA pathway and thereby enables parasitism (Wang *et al.*, 2018). CMs have also been identified in many PPNs and may have a major role in bacteria-like parasitism (Lambert *et al.*, 1999; Huang *et al.*, 2005b; Vanholme *et al.*, 2009). *Mi-CM3* is upregulated in the early stages of nematodes in the subventral gland. Expression of *Mi-CM3* disrupts SA biosynthesis and SA-mediated defense in *Nicotiana benthamiana* and increases susceptibility to the nematode. Altogether, *Mi-CM3* plays a significant role in suppressing plant immunity to facilitate parasitism, but the exact mechanism of association between the nematode, CM and the plant cell is yet unknown. In addition, another effector of *M. incognita*, *MiISE5*, is involved in manipulation of host hormones for both the JA- and SA-mediated signaling pathways (Shi *et al.*, 2018a). Similarly, *MiISE6* upregulates the jasmonate ZIM-domain, which eventually represses the JA signaling pathways (Shi *et al.*, 2018b). These reports have demonstrated that in nematode parasitism, *MiISEs* play an important role. Likewise, RKN effectors *Mh265* (Gleason *et al.*, 2017), *MjTTL5* (Lin *et al.*, 2016), *Mg16820* (Naalden *et al.*, 2018), *MgGPP* (Chen *et al.*, 2017), and

MgMO237 (Chen *et al.*, 2018) were also found to be involved in manipulating plant immunity.

Most of the known RKN effectors are synthesized in the esophageal glands. Recently the macrophage migration inhibitory factors (*MIF*-2) effector was described in *M. incognita*; it is secreted from the hypodermis into the plant apoplast and eventually targets the giant cell cytoplasm (Zhao *et al.*, 2019). Gene silencing of *MiMIF* decreases, and overexpression of *MiMIFs* increases *M. incognita* infection in the plant, which indicates the importance of *MiMIFs* in parasitism. Similarly, authors showed the ability of *MiMIF*-2 in PCD suppression. They investigated whether *MiMIFs* affect PTI in *Arabidopsis*, where overexpression lines showed the reduced levels of MPK3 and MPK6 phosphorylation, defense marker gene, and low or no cellulose deposition. MPK3 and MPK6 are considered to be key positive plant defense regulators; they are active in the biosynthesis of both ethylene (Meng and Zhang, 2013) and jasmonate (Schweighofer and Meskiene, 2008). In recent years, many PTI-suppressing effectors have been characterized in RKNs but knowledge of their mechanism of action is still lacking.

Peptides Mimicking Plant Hormones – a Special Class of Effector

Plant-parasitic nematodes have an advanced strategy to improve their ability to infect plants, such as small peptide hormones. These hormones are encoded by the PPNs, which can manipulate the plant cell while escaping the host defense response (Ronald and Joe, 2018). Several plant peptide hormones mimicking peptides (CLE-, CEP-, and IDA-like) have been reported in plant nematodes.

CLE-like peptide

One of the most intensively investigated secreted peptide families in plants is the CLE (CLAVATA3/Endosperm surrounding region-related) peptide family. So far, 1628 putative CLE peptides have been identified in 57 plant species (Goad *et al.*, 2017) and recently 104 CLEs have been found in wheat (Li *et al.*, 2019). The existence of CLE

genes has been recorded across the heritage of land plant species (Goad *et al.*, 2017) and even in PPNs (Yamaguchi *et al.*, 2016). CLE genes play an important role in plant development and plant–nematode interactions. Plant CLE encodes pre-propeptides containing the signal peptide at the N-terminal, a central variable domain (VD), followed by a 14-amino acid conserve (biologically active) domain at the C-terminus. Upon delivery via the secretory pathway to the apoplast, its signal peptide is removed. Subsequently, to generate the functional CLE peptide, 12–14 amino acids are cleaved by proteolytic processing; the domain is therefore referred to as the CLE domain/peptide (Kondo *et al.*, 2014; Yamaguchi *et al.*, 2016). These small, secreted peptides regulate many functions in the vascular plants and mediate cell-to-cell communication (Bird *et al.*, 2015). Similar CLE propeptides were identified in the dorsal gland cell of the cyst and reniform nematode but were lacking in the RKN nematode.

Several effectors of cyst and root-knot nematodes have shown some resemblance to plant peptides that play an important role in plant mimicry (Goverse and Smant, 2014). The first peptide-like CLE was found in the cyst nematode esophageal gland (Olsen and Skriver, 2003). Since that first discovery, numerous CLE-like peptides have been reported in several PPNs (Yamaguchi *et al.*, 2016; Gheysen and Mitchum, 2019). To facilitate feeding-site development in plants (Hewezi, 2015), nematode CLE signals have been shown to function via CLV1, CLV2, and RPK receptor complexes (Replogle *et al.*, 2011, 2013). Nevertheless, the underlying mechanism of these CLEs in NFS development is still elusive (Guo *et al.*, 2015). So far, most studies have demonstrated cyst nematode CLE-mediated mimicry in plants; however, the role of CLE in RKN has been less explored.

Similar to *Heterodera glycines* SYV-46 protein, a small secretory peptide, 16D10, was identified in RKNs (Huang *et al.*, 2003), expressing in the subventral gland cells. This protein releases a mature bioactive peptide of 13 aa, which is analogous to the plant CLE peptide. Despite the similarity of nematode peptide (GKKPSGPNP-GGNN) with the plant, its ectopic expression could not restore the wild-type phenotype of the *Arabidopsis* CLV3-1 mutant, unlike cyst nematode. Overexpression of 16D10 does not affect

shoot morphology but increases root growth. Interaction of 16D10 with the SAW domain of the tomato and *Arabidopsis* Scarecrow-like protein increases root growth, which indicates that the SCARECROW protein is a putative target for 16D10 functioning during nematode parasitism. However, to expand nematode feeding sites, 16D10 peptide may utilize an alternative mechanism from the CLE pathway (Huang *et al.*, 2006).

The secreted 16D10 peptide plays an essential role in the plant parasitism. *In vitro* and *in vivo* gene silencing of the 16D10 genes resulted in reduced nematode infestation in *Arabidopsis*, without changing plant morphology. Scarecrow-like protein plays a central role in the root meristem differentiation as well as in nodule formation (Hirsch *et al.*, 2009). Like the rhizobial nodule, similar processes (molecular and developmental) took place in RKN giant cell formation (Bird, 2004; Weerasinghe *et al.*, 2005). The somewhat shocking albeit robust discovery of 16D10 binding to the Scarecrow-like nuclear protein instead of the apoplastic transmembrane receptor tends to indicate that this particular gene likely does not encode the CLE (Mitchum *et al.*, 2008). Investigation of the collected genomic data showed eight CLE gene loci in *M. hapla* based on the presence of the signal peptide and the retained cleavage site upstream of the C-terminal (Bird *et al.*, 2015).

Rutter *et al.* (2014) identified CLE-like motifs in the RKN *Meloidogyne* avirulence proteins (MAPs) family; these motifs are very close to plant and cyst nematode CLE peptides. MAP-1 protein is secreted from *M. incognita* amphidial glands and its *in planta* secretion was observed along the wall of the giant cell and in the root apoplast (Semblat *et al.*, 2001; Vieira *et al.*, 2011). These CLE motifs are present in a single gene in multiple tandem arrays; however, it is unclear whether functional CLE peptides translated from complete MAP coding genes. It is presumed that the MAP family can act as CLE-mimics in some ability, maybe as a peptide processed *in planta*. However, the functional role of MAP CLE-like peptides and their target signaling pathways has the potential to enhance insight into the plant–RKN interactions (Haegeman *et al.*, 2011). Similar to RKN, potato cyst nematode (*Globodera rostochiensis*) also has CLE-like genes with multiple CLE patterns within the same gene, and these gene products are accurately

processed into functional CLE-like peptides that rescue *Arabidopsis* CLV3 mutant (Guo *et al.*, 2017). Taken together, the existence of CLE as a motif in MAP and conformation of their esophageal gland expression implies that RKN as well as potato cyst nematode has developed a strategy for co-opting CLE-dependent signaling pathways to parasitize the host.

C-terminally encoded peptides

In vascular plants, the C-terminally encoded peptide (CEP) family of regulatory peptide hormones governs plant growth and development. CEPs are processed into biologically active peptides of 15-amino acid in the apoplast and subsequently modified with hydroxyprolines at two different positions (Ohya *et al.*, 2008). These CEP peptides have been interacted with *Arabidopsis* CEPR1 and CEPR2 membrane-associated leucine-rich repeat receptor kinases (Tabata and Sawa, 2014). Like CLE, CEP is also post-translationally modified to generate mature peptides. To date, 15 CEP genes are known from *Arabidopsis*. Overexpression of some of these genes inhibits root growth and causes morphological shoot modification and loss of function, whereas mutants enhance root development (Ohya *et al.*, 2008; Delay *et al.*, 2013). Nevertheless, the function of CEPs is diminished by genetic redundancy. Apart from plants, the CEPs are a large and diverse family of effector mimic peptides that are released by some PPNs (Bobay *et al.*, 2013; Ogilvie *et al.*, 2014; Bird *et al.*, 2015; Eves-van den Akker *et al.*, 2016b). CEP-like peptide effectors are reported from the genomes of *Meloidogyne* and, lately, from reniform nematodes, but not from the genomes of other PPNs. When PPN genomes were explored for the CEP family, interestingly, 12 and eight CEP genes were found in root-knot nematodes *M. hapla* and *M. incognita*, respectively (Bird *et al.*, 2015). Similar to plant precursors, *Meloidogyne* species contain signal peptides (N-terminal) and a CEP motif (C-terminal) but lack a pro-protein domain. Eventually, proteolytic processing processes the pro-protein domain in the plant apoplast. This domain is absent in RKN CEPs, possibly enabling the secretion of matured peptide directly in the apoplast host cells in the formation of giant cells.

More recently, 12 *RrCEPs* have been found in another PPN, *Rotylenchulus reniformis* (*Rr*), which forms syncytium as a feeding site. These CEPs are expressed in pharyngeal gland cells and, during the parasitic interaction, the *RrCEP1* protein is probably secreted *in planta*. *RrCEPs* seem to have an independent origin as they contain one intron per domain sequence whilst RKN and plant CEPs harbor a single exon (Eves-van den Akker *et al.*, 2016b). Unlike RKN CEPs, the *RrCEPs* are secreted indirectly into the apoplast as they have a plant-like propeptides domain. The function of a CEP-like effector mimic remains an open question in nematode parasitism. RKN CEPs have sequence similarities with the other PPNs. Thus, the information gained from RKNs can be extended to other plant nematodes, such as *R. reniformis*, to understand a wide range of plant–nematode interactions. Therefore, we suggest RKN as a model organism.

The application of synthetic, active CEP peptides (15-amino acid), which correspond to domains of interest within genes, has made quick progress in the field (Bobay *et al.*, 2013; Delay *et al.*, 2013; Imin *et al.*, 2013; Tabata and Sawa, 2014). For example, Imin *et al.* (2013) reported an increase in CEP transcript in nitrogen (N_2) deficiency. The N_2 deficiency leads to the movement of active peptides from root xylem to the shoots through an unspecified signal activating the nitrate transporter (NRT2.1) in the root. Overexpression or exogenous application of CEP genes in *Arabidopsis* reduces root elongation (Delay *et al.*, 2013). The systemic upregulation of the nitrate transporters can be streamlined, possibly as an adaptive local response to decrease plant growth when nitrate is low, thereby upregulating CEPs.

CEPs are considered to be versatile, based on the modulation of plant architecture that was one of their first defined functions. For example, when *RrCEP1* peptide was applied on *Arabidopsis* exogenously, inhibition of the primary root and upregulation of NRT2.1 transporter were observed, which is similar to plant peptide (*AtCEP5*) applications. Furthermore, roots treated with *RrCEP1* showed smaller sizes of *Heterodera schachtii* feeding sites (syncytium), which indicates that regulation of the size of the feeding site may be one plausible function of nematode CEPs. It is hypothesized that nematode CEPs lead to a smaller syncytial size and increase the absorption of

host nitrate, thereby minimizing drain on the host throughout infection (Eves-van den Akker *et al.*, 2016b). However, further studies are needed to explain the specific role of CEP-like plant peptide hormone effector mimics during plant–nematode interactions and any possible role, like their plant counterparts, in host nitrate uptake (Gheysen and Mitchum, 2019).

IDA-like peptides

In addition to plant peptide hormones CLE and CEP effector mimics, several INFLORESCENCE DEFICIENT IN ABSCISSION-like (IDL) family members from several *Meloidogyne* species have also been identified. In the search for IDL peptides in cyst nematodes, a comprehensive data analysis was conducted; however, no IDL peptides were found from existing sequences, suggesting that IDL peptide is unique to the RKNs (Kim *et al.*, 2018). In plants, the IDA gene encodes a small, secreted protein that putatively binds to receptor-like kinases (RLKs) HAESA (HAE) and HAESA-like 2 (HSL2) (Santiago *et al.*, 2016). Then the gene activates a MAP kinase-signaling cascade that leads to the expression of a set of KNOX transcription factors, which subsequently modulate floral organ separation (Shi *et al.*, 2011). Some studies indicate that, during abscission, IDA controls the expression of cell wall modifying proteins, such as polygalacturonases, expansins, cellulases, etc. (Butenko *et al.*, 2003; Shi *et al.*, 2011). IDL peptides were shown to modulate plant stress and defense responses to pathogens (Wang *et al.*, 2017). Kim *et al.* (2018) showed how root-knot nematodes are capable of subverting signaling pathways to facilitate plant parasitism. In *M. incognita*, two putative IDA-like secretory peptides, *MiIDL1* and *MiIDL2*, have been identified, and they are similar to *AtIDA* at the sequence level. In *Arabidopsis*, an abscission mutation was found in which the floral organs do not abscise (namely, INFLORESCENCE DEFICIENT IN ABSCISSION (IDA)) (Butenko *et al.*, 2003). Interestingly, when *MiIDL1* synthetic peptides are used exogenously on the *Arabidopsis* IDA mutant, the abscission phenotype is significantly rescued, which provides initial evidence for the *MiIDL1* role in host-cell mimicry. Likewise, overexpression of the full-length *MiIDL1* gene in *Arabidopsis* *ida*

mutants showed wild-type floral abscission phenotype. Host-induced gene silencing of *MiIDL1* decreased the number (40%) and size of knots relative to control plant roots, showing the essential role of this gene in nematode parasitism. It appears that *MiIDL1* peptides imitate IDA function only in roots during infection and do not follow IDA-mediated signaling. However, direct binding of *MiIDL1* to HAE/HSL2 or other receptors is yet to be confirmed (Hu and Hewezi, 2018).

The Trend Toward Genome Reduction in Plant Parasites

Plant-parasitic nematodes are among the most abundant organisms and 4100 species have been reported to date (Nicol *et al.*, 2011). Van Megen *et al.* (2009) documented the existence of PPNs in four clades, including clade 1 (Triplonchida), clade 2 (Dorylaimida), clade 10b (Aphelenchoidea), and clade 12 (Tylenchida), concluding that they have evolved independently, as morphology, ontology, and nomenclature differ among all the clades. However, many nematodes share an analogous feeding device (stylet), probably due to converging evolution (Bird *et al.*, 2015).

Caenorhabditis elegans was the first animal whose genome was sequenced, in 1998, and this opened the door to new avenues in molecular biology (*C. elegans* Sequencing Consortium, 1998). Decades later, two plant parasitic nematodes, *Meloidogyne incognita* and *Meloidogyne hapla*, were also sequenced for the first time (Abad *et al.*, 2008; Opperman *et al.*, 2008). *Meloidogyne* species genomes provide an opportunity to be used as a model for other PPNs for comparative genomics. The draft genome of *M. floridensis* was also sequenced and compared with *M. incognita* and *M. hapla* genomes (Lunt *et al.*, 2014). Recently the *Bursaphelenchus xylophilus* (pinewood nematode) genome was sequenced using next-generation sequencing (Kikuchi *et al.*, 2011). The root-lesion nematode *Pratylenchus coffeae* genome was sequenced, which is believed to be the smallest sequenced genome so far known in the animal kingdom (Burke *et al.*, 2015). The genomes of two cyst nematodes were sequenced (Cotton *et al.*, 2014; Eves-van den Akker *et al.*, 2016a). Apart from these genomic data, transcriptomic

data for many nematodes are also available. These genome assets will promote substantial developments in the understanding of nematode plant parasitism. There is no doubt that the curated collection of gene and protein models, developed by the *Meloidogyne incognita* resources team (available at https://www6.inrae.fr/meloidogyne_incognita, accessed 30 December 2021) will be crucial in annotating the growing list of sequenced PPN genomes. Thus, we suggest RKN *M. incognita* as a model organism.

To get whole-genome sequencing of any nematode, the biggest hurdle until very recently had been the expense. However, with advancements in sequencing technologies, it is now possible to collect adequate raw sequencing data to assemble a complete genome of any PPN, normally costing less than a few thousand US dollars. Next-generation sequencing (NGS) has revolutionized plant nematology in terms of access information for scientists and methods used to study the biology of these species' invasion.

So far, genomic information is only known for two nematode clades, namely clade 12 and clade 10b. This information can further be used to study genomic adaptation in nematodes for their plant-parasitic behaviors. However, it is unlikely that the knowledge obtained from clades 12 and 10b is commonly applied to all PPNs. Flow cytometry is commonly used for precise calculation of the DNA content of cells (genome size) with high accuracy. The majority of nematode genome sizes between 50 and 250 Mb is determined by flow cytometry. One study used 90 nematode species and estimated the genome size as between 42 and 701 Mb (Leroy *et al.*, 2003), which indicates that genome size is far more dispersed than the above estimate.

Genome size analysis revealed that PPNs have a genome smaller than the free-living nematode *C. elegans* (100 Mb). Among RKNs, *M. hapla* has the smallest genome size (54 Mb). In addition, *M. hapla* codes for fewer genes (14,420) compared with *C. elegans* (20,000). On the contrary, *M. incognita* has a large genome size (86 Mb), probably due to the presence of multiple copies in the genome (Abad *et al.*, 2008). *M. incognita* has peculiar structures, where most of the genomic region is repeated several times with major modifications (Castagnone-Sereno *et al.*, 2013). Because of this structure, *M. incognita* contains two more copies of a gene while *M. hapla*

contains a single one. The genome of the root-lesion nematode *Pratylenchus coffeae* was determined by flow cytometry and is one of the smallest genomes (19.6 Mb) compared with any other nematode. In PPNs, reduced genome sets and gene numbers were found relative to free-living *C. elegans*. For example, *P. coffeae* and *M. hapla* have total gene numbers of 6712 and 14,040, respectively, which is fewer relative to *C. elegans* (21,000) (Burke *et al.*, 2015). The *M. hapla* genome and gene number are both smaller than those of a recently sequenced nematode, *Bursaphelenchus xylophilus* (assembly size 74 Mb; 18,040 genes). This reduction was found mainly in the gene families due to the reduction in repeat regions, introns, and copy numbers. This nematode may portray a minimal genome that might be valuable in the exploration of functional plasticity in nematode genomes, evolutionary mechanisms for increased specialization, and the specific sequence of genes required to make a multicellular organism (Burke *et al.*, 2015).

Five species of the *Bursaphelenchus* genus were sequenced with genome-assembled sizes ranging from 60 to 75 Mb, close to *B. xylophilus*. Similarly, *Globodera* species have been sequenced that are larger than other PPNs but fewer in number of genes (14,378) compared with free-living nematodes. Recently, the genome of the potato cyst nematode *G. rostochiensis* (95.9 Mb) has been sequenced, providing a precise representation of the *Globodera* species genome, since there is no pseudo-duplication. The above studies may show a genome reduction trend in PPNs compared with free-living nematodes. It can also be questioned whether a trend towards a genome reduction is present in animal-parasitic nematodes. The genome of mammal nematode *Trichinella spiralis* is about 64 Mb and contains about 16,000 genes (Mitreva *et al.*, 2011). However, the filarial nematodes (*Loa loa*, *Brugia malayi*, and *Wuchereria bancrofti*) all have genome sizes of about 90 Mb, comparable to *C. elegans*, but fewer genes predicted (about 15,000) (Ghedini *et al.*, 2007; Desjardins *et al.*, 2013). In contrast to this trend of reduction, the genomes of *Ascaris suum* and *Haemonchus contortus* are 273 and about 300 Mb, respectively (Jex *et al.*, 2011; Laing *et al.*, 2013), which is bigger than those of *C. elegans* and they do not encode substantially fewer genes (18,500 and about 22,000, respectively). Thus, although some examples can be

mentioned, there is no clear general trend toward genome reduction in parasitic nematodes. However, the general trend towards genome reduction is not apparent in PPNs.

Horizontal Acquisition of Parasitism Genes

Horizontal gene transfer (HGT) is the transmission of genetic material between two different species asexually, independent of evolutionary distance between them. This phenomenon has been commonly observed in prokaryotes, where most of the genetic material comprises horizontally transferred genes. For instance, bacterial antibiotic resistance genes have been horizontally passed to other bacteria independent of their genome or plasmid-based origin (Boucher *et al.*, 2003; Gogarten and Townsend, 2005). PPNs were the first animal species on earth where HGT was reported for cellulose genes. The most striking aspect was that these cellulases have no genomic similarity with any other animal but only with bacteria, which inferred that the enzymes had been transferred from bacteria through HGT (Smant *et al.*, 1998). Cellulose is the protective component of the plant cell wall, and cellulases degrade it. In addition to cellulase, other cell wall-degrading enzymes (CWDEs), such as xylanases, polygalacturonases, and pectin lyases, were also identified in root-knot nematodes (Jaubert *et al.*, 2002; Doyle and Lambert, 2003; Mitreva-Dautova *et al.*, 2006), which aid in invasion and migration through the plant cell (Rosso *et al.*, 1999; Ledger *et al.*, 2006). Perhaps the existence of this kind of enzyme coding genes in the nematode genome is itself a fascinating example of adaptation toward plant parasitism.

In addition to cell wall degradation and modification, these enzymes (cellulase, xylanases, pectin lyase, etc.) may also be involved in other functions in PPNs (Haegeman *et al.*, 2011). These functions are: (i) host defense suppression (for example, chorismate mutases and cyanate lyases found in RKN and cyst nematodes); (ii) processing of nutrients (for example, invertase and enzymes of salvage pathway); and (iii) feeding-site establishment (for example, the Nod factor, NodL, like in RKN). Paganini *et al.* (2012) reported that 3.34% of protein coding genes in

RKNs might have been received from non-animal donors. For identification of HGT candidates in various PPNs, genomes or ESTs were used with criteria such as a phylogenetic method, detailed analysis of distribution that indicates absence in related species, and habitat overlap between donor and receiver (Mitreva *et al.*, 2009).

HGT in eukaryotes, especially in animals, was previously thought to be a minor evolutionary occurrence. Nonetheless, several HGT cases have now been documented from prokaryotes to eukaryotes (Andersson, 2005; Dunning Hotopp, 2011). Thus, this phenomenon of horizontal gene transfer is more widespread than previously thought.

The most economically important PPNs come under clade 12, which includes the RKN and the cyst nematode as well as numerous genera of migratory endoparasite nematodes. Genomic and transcriptomic data are available for clade 12 and have been studied comprehensively (Van Megen *et al.*, 2009). The cell wall-degrading enzymes acquired through HGT were identified in *M. incognita*, and they belong to six different families of protein (Abad *et al.*, 2008). Some other putative HGT genes, such as chorismate mutase (Lambert *et al.*, 1999), a polyglutamate synthase (Veronico *et al.*, 2001), and genes similar to those present in *Rhizobium* species were also reported (Scholl *et al.*, 2003).

Expressed sequence tag (EST) analysis showed the presence of cellulase and an expansin-like protein in other PPNs, including *Xiphinema index*, *Bursaphelenchus xylophilus*, and *B. mucronatus* (Jones *et al.*, 2005; Kikuchi *et al.*, 2007). Several cell wall-modifying proteins have been characterized from these species, including cellulases (Kikuchi *et al.*, 2004), pectate lyases (Kikuchi *et al.*, 2006), and expansins (Kikuchi *et al.*, 2009). In addition, *B. xylophilus* and *B. mucronatus* have GH16 endo-1,3-beta-glucanases probably involved in degradation of the fungal cell wall and that could have been acquired by HGT from bacteria (Kikuchi *et al.*, 2005). Overall, the presence of HGT from bacteria in PPNs is well known. However, it is yet to be proven how these genes were acquired by the nematodes. All the above examples are evidence of the presence of CWDEs in RKNs as well as in other PPNs. Many of these genes have been characterized in RKN species. Together, these data show that RKN *M. incognita* can be used as a model system.

Genomic data is available for *M. incognita* and *M. hapla*, but it is undoubtedly a speculative approach to determine the mechanism underlying HGT occurrences using only genomic data. HGT has been reported in *Brugia malayi* (an animal-parasitic nematode) from *Wolbachia pipientis*, an intracellular symbiotic bacteria (Dunning Hotopp *et al.*, 2007). Some other PPNs also have endosymbionts in their reproductive tissue germ cells, providing a possible mechanism for HGT of heritable genomic information in nematodes (Haegeman *et al.*, 2009; Noel and Atibalentja, 2006). Strikingly, the symbiotic bacteria mentioned above do not have sequence similarity with CWDEs, which have been acquired by PPNs. It may also be likely that descendent PPNs had the bacterium from which the genes were inherited and eventually lost to this bacterium due to selection pressure and the capacity of the nematode to generate its own CWDEs.

It was also thought that perhaps PPN ancestors obtained these genes (cellulase, xylanases, pectin lyase, etc.) via feeding on bacteria or fungi after a parasitic or symbiotic association with plants was established. The transfer of genes from diets has already been identified in a sea slug that feeds on algae, but it needs to be verified by a larger-scale study (Rumpho *et al.*, 2011). Several species of PPNs feed on bacteria and complete their life cycle within the plant. It can be speculated that these PPNs may have inherited CWDE genes from nematodes that feed on either bacteria or fungi (Westcott, 1976) but, even if this speculation is correct, it would not provide a mechanism for HGT in nematodes.

Transposable elements offer a plausible explanation of how nematodes acquired bacterial genes. For example, numerous genes have been inherited by bdelloid rotifers from bacteria, fungi, and plants by HGT and these genes are said to be linked with transposable elements. In the *Meloidogyne* genome sequences, however, the correlation between HGT genes and transposable elements has yet to be established.

The Repertoire of Plant Cell Wall-degrading Enzymes in Root-knot Nematode Genomes

The composition of the plant cell wall is very complex and consists of a vast network of cellulose, microfibrils, hemicellulose, pectin, and several

types of protein that encounter many changes during plant development (Carpita and Gibeaut, 1993). The plant cell wall is the first barrier that plant-parasitic nematodes encounter during the phases of invasion, infection, and movement through the plant cells to create the feeding site (Ali *et al.*, 2017). To overcome these barriers, PPNs use a combination of approaches such as a stylet to physically puncture the cell wall, along with cell wall-degrading enzymes (Jones and Goto, 2011; Malinovsky *et al.*, 2014). Many CWDEs are secreted from esophageal glands (dorsal and subventral), such as cellulases or pectinases (Davis *et al.*, 2000; Jaubert *et al.*, 2002).

RKNs possess two stages throughout their life cycle: migratory and sedentary. During the migratory stage, CWDEs are secreted by nematodes to dissolve the cell wall, whereas in the sedentary stage they release cell wall-modifying enzymes to maintain the specialized feeding sites (Wieczorek, 2015). It was previously thought that PPNs only use the stylet and mechanical force to crack the cell wall during the migration process (Wyss and Zunke, 1986; Wyss *et al.*, 1992). Conversely, it was shown many years ago that CWDEs are also produced and secreted during nematode migration, and these enzymes help in cell wall degradation (Smant *et al.*, 1998). Several RKN CWDEs (such as β -1,4-endoglucanase, xylanase or pectate lyase) have been reported to be involved in plant cell wall degradation (Table 4.2) (Vanholme *et al.*, 2004; Huang *et al.*, 2005a; Mitreva-Dautova *et al.*, 2006).

In 2002, the first polygalacturonase (PG) enzyme was characterized in *M. incognita*; the enzyme is expressed in the subventral esophageal gland of infective juveniles (Jaubert *et al.*, 2002). This PG plays a key role in the breakdown of pectin, a major component of the plant cell wall. The function of this enzyme has not been validated but it is believed that the PG-1 protein could be secreted during nematode invasion to weaken or loosen cell walls. Phylogenetic analysis revealed the greatest resemblance between bacterial PG and *Mi*-PG-1, implying that the gene for nematode parasitism was possibly inherited from bacteria via HGT. *In silico* analysis indicated the presence of PG-like genes in only four RKN species (*M. javanica*, *M. hapla*, *M. chitwoodi*, and *M. arenaria*), suggesting a narrow range of distribution of the PG gene among PPN species.

Another enzyme, a beta-1,4-endoglucanase (*eng1*), is able to degrade cellulose, which is the

main component of the plant cell wall (Smant *et al.*, 1998). Since the discovery of this enzyme in RKNs, the presence of cellulases has been reported in several other PPN species, including *B. xylophilus*, *Ditylenchus africanus*, *H. glycines*, *Heterodera schachtii*, *Globodera tabacum*, *Globodera rostochiensis*, *Pratylenchus penetrans*, *P. coffeae*, *Rotylenchulus reniformis* and *Radopholus similis* (De Meutter *et al.*, 2001; De Boer *et al.*, 2002; Gao *et al.*, 2002; Abad *et al.*, 2008; Bellafiore *et al.*, 2008; Rai *et al.*, 2015). Twenty-one cellulases of the GH5 family have been identified in *M. incognita* (Abad *et al.*, 2008). The *Mi-eng1* enzyme coding gene was identified from *M. incognita* cDNA, and was shown to be expressed in subventral esophageal glands (Rosso *et al.*, 1999). The key function of the *Mi-eng1* enzyme is the breakdown of cellulose by hydrolysis of 1,4-glycosidic bonds. Therefore, *eng1* may participate in the softening of cell walls and allowing smooth entry of nematodes within roots.

A biochemical analysis of *Mi-eng1* indicated that *Mi-eng1* shares many properties with well-known plant and microbe endoglucanases (Béra-Maillet *et al.*, 2000). These proteins are composed of a catalytic domain and a cellulose-binding domain (CBD), followed by a linker domain. Such domain structures are also present in starch- and chitin-degrading enzymes. Despite the existence of CBD in *Mi-eng1*, it could not bind to plant cellulose or xylan under laboratory conditions, and thus did not degrade them. Altogether, these findings suggest that either the binding of the *Mi-eng1* to cellulose is weak or the conditions were not suitable. In contrast, another gene, *Mi-eng2*, was identified from the same family but it did have CBD and linker domains. Occasionally gain or loss of CBD and the linker domain could be due to duplication during evolution (Ledger *et al.*, 2006).

Thirty pectate lyases from the PL3 family were identified in *M. incognita* (Abad *et al.*, 2008; Bellafiore *et al.*, 2008). Pectate lyases are produced by diverse groups of organisms, including bacteria, fungi, and nematodes (Herron *et al.*, 2000), and are involved in maceration and soft rotting of the plant tissue during nematode invasion (Huang *et al.*, 2005a). For instance, *Pel-1* and *Pel-2* genes were identified from *M. incognita* and expressed in the esophageal gland, which suggests that these enzymes could be secreted into plant tissues to facilitate penetration during the early stages of plant parasitism. *Pel* homologs

Table 4.2. Nematode cell wall-degrading proteins acting on plant cell walls during the migration through the host root invasion and migration.

Gene/protein	Nematode	Class	Function	Reference
<i>Mi-pg-1</i>	<i>M. incognita</i>	Polygalacturonase	Cell wall breakdown	Jaubert <i>et al.</i> (2002)
<i>Mi-xyl-1</i>	<i>M. incognita</i>	Endo-1,4-β xylanase	Degrade hemicellulose and pectin networks in plant cell walls.	Mitreva-Dautova <i>et al.</i> (2006)
<i>Mi-eng-2 & 3</i>	<i>M. incognita</i>	1,4-β-endoglucanase	Cell wall softening	Ledger <i>et al.</i> (2006)
<i>Mi-CBP-1</i>	<i>M. incognita</i>	Cellulose-binding protein	Cell wall softening	Ding <i>et al.</i> (1998)
<i>Mi-Pel-1 & 2</i>	<i>M. incognita</i>	Pectate lyase	Weakening the cell wall	Huang <i>et al.</i> (2005a)
<i>Mj-Pel-1</i>	<i>M. javanica</i>	Pectate lyase	Role in early stage of infection	Doyle and Lambert (2002)

were also found in *R. similis*, *B. xylophilus*, *G. rostochiensis*, *H. glycines* and *H. schachtii* nematodes (Rai *et al.*, 2015). These enzymes degrade the plant cell wall by cleavage of α-1,4-linked galacturonosyl component (Yoder *et al.*, 1993), and thus function as a pathogenicity factor for nematode establishment.

Eighty-one putative CWDEs have been identified in *M. incognita* and 33 are common in *M. hapla* (Opperman *et al.*, 2008). Similar enzymes were identified in other PPN species, *B. xylophilus* and *Globodera pallida* (Rai *et al.* 2015). Many CWDE genes are annotated in RKN and can be assessed to other PPNs to generate nematode-resistant crops. Therefore, we propose *Meloidogyne* spp. root-knot nematodes as a model organism.

Apart from the very well-known CWDEs, such as cellulases, xylanases, pectate lyases and polygalacturonases, some novel carbohydrate active enzymes (CAZymes) (Cantarel *et al.*, 2009), arabinases, and invertases have also been identified in the RKN genome. Invertases are not CWDEs, yet they have an essential role in converting plant sucrose to glucose and fructose, which can easily be digested by the nematode. The role of CAZymes in cell wall alteration and host–pathogen interactions has been confirmed (Cantarel *et al.*, 2009). In order to infect, the endoparasite (migratory as well as sedentary) degrades the plant cell, and in addition endoparasitic nematodes further modify the plant cell wall

(Bird *et al.*, 2015). However, RKN genes coding for cell wall-degrading protein remain largely uncharacterized.

A significant number of expansins are known in PPN genomes (Qin *et al.*, 2004; Kudla *et al.*, 2005) that loosen the plant cell wall by non-enzymatic action. However, earlier these proteins were thought to be unique to the kingdom of plants. In RKNs, a family of *Meloidogyne* *Avirulence protein-1* (MAP-1) genes coding for expansin-like proteins has been identified (Sembelat *et al.*, 2001). These proteins are secreted during the early stages of plant–nematode interactions and may serve as an effector (plant-mimic peptide) to promote successful infection. The emergence of the MAP-1 family was only noticed in *Meloidogyne* species, claiming to be a taxonomically restricted genes family (Tomalova *et al.*, 2012).

Most of the cell wall-degrading enzymes identified in RKNs are expressed in the esophageal gland cells. Since these enzymes are identical to those found in fungi or bacteria, they appear to have been transmitted through HGT (Haegeman *et al.*, 2011; Danchin *et al.*, 2010). A nematode CWDE database has been developed (Rai *et al.*, 2015), in which extensive information on these enzymes is available. However, based on sequence similarity, correct function of the enzymes cannot be predicted and therefore wet laboratory analysis must be pursued.

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5 Nematodes as Models for Symbiosis

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Introduction to Symbiosis

Microbes are ubiquitous, being present throughout earth's oceans, in the soil, on and in plants and animals, in ice present at both poles, and even in the atmosphere (Giovannoni, 2017; Stolz, 2017). All multicellular eukaryotes therefore live in association with microbes. Some of these interactions are symbioses, defined as unlike organisms living closely together in long-term relationships, often with intertwined life cycles and evolutionarily derived structures and signals that promote specific associations (Stubbendieck *et al.*, 2019). Symbioses can span a range of interaction outcomes, from mutualism, in which all parties receive a net benefit from the interaction, to parasitism, in which at least one party receives a net benefit while another incurs a net cost (Fig. 5.1). Common types of microbial mutualisms are nutritional, in which a partner provides the other with essential metabolites not present in the diet, and defensive, in which a partner provides the other with protection against competitors, predators, and parasites (Douglas, 2010). Microbial symbionts can be passed directly from parent to offspring in vertical transmission events, or horizontally acquired from the environment, and symbioses can exhibit mixed modalities that combine these two transmission modalities to different degrees (Bright and Bulgheresi, 2010;

Ebert, 2013; Stoy *et al.*, 2020). Symbioses can be further categorized by the level of interdependence of the participants and the specificity of engagement with potential partners (Fig. 5.1). In obligate associations, one or more of the partners cannot exist without the other(s); while in facultative symbioses, interdependence is context dependent (Chomicki *et al.*, 2020). In generalist associations, symbionts can associate with a broad range of partners; while in specialist associations the partner number is limited to one or two, though the taxonomic level of the partners (for example, a type of organism, one species, or one subspecies) can vary across symbioses (Chomicki *et al.*, 2020). Whether antagonistic or mutualistic, obligatory or facultative, or generalist or specialist, bacterial interactions with hosts occur through conserved mechanisms. This conservation means that the study of experimentally tractable organisms can yield insights into processes of host-microbe associations all along the continuum. However, because such diverse types of host-microbe symbioses exist, it is important to study many different examples of such associations to understand the full range of parameters that modulate the specificity and outcomes of these associations.

Nematoda is a diverse phylum whose members participate in many types of symbiosis with different bacterial taxa, making them ideal models

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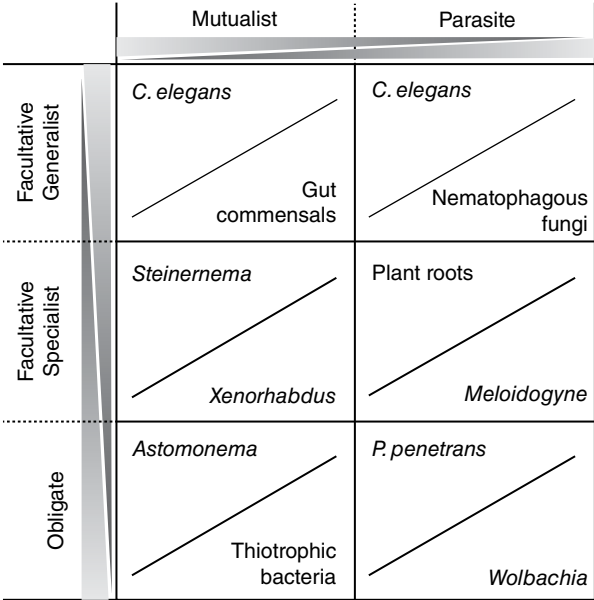


Fig. 5.1. Different types of symbioses and examples discussed in this chapter. Symbiotic interactions can be classified from mutualistic to parasitic and from facultative to obligate. Symbioses can move along the depicted gradients based on changes in environmental or evolutionary pressures. Several of the interactions discussed in this chapter are categorized as examples of the symbiosis types depicted.

for understanding host–microbe interactions. Symbioses can result in the participants gaining complex traits through their partnership that would take millennia to evolve *de novo*. This means that symbioses are a particularly potent biological force, because they allow organisms to break the evolutionary “rule” of descent with modification (Douglas, 2010). This review focuses primarily on bacterial symbionts, which vary greatly in their genomic potential, even among members of a single genus or species. Such variation among mutualistic symbiotic partners can result in variation in symbiotic traits and the ability to contribute to host fitness. This raises the question of what “goods and services” the symbionts provide to each other and at what costs. The corollary to this question is if, and how, hosts evaluate potential partners for their symbiotic potential, recruit and provide their own inducements, and transmit to new generations those symbionts that are most likely to provide optimal fitness to offspring. Potential bacterial symbionts also may compete with each other for colonization of specific host tissues and likely have evolved mechanisms to gain advantage over both distant and closely related competitors.

The molecular foundations of symbiont partner recognition and selection are not well understood, particularly in complex systems, in which many players influence the outcomes. Finally, how do microbes that are facultatively associated with a host, or that occupy more than one host over their life cycle, adapt to these changing environments for optimal fitness? How do hosts defend themselves from harmful associations while recruiting beneficial partners? In this chapter, we outline how nematodes are useful as biological models of animal–microbe symbioses, and how they have the potential to expand our understanding of fundamental principles of host–microbe interactions, including community recruitment and formation, transmission between individuals and generations, nutrient sharing and metabolic cooperation, and the molecular and cellular adaptations by both host and microbe that facilitate these interactions.

Diverse Nematode Models of Symbiosis

On the surface, the members of the phylum Nematoda look very similar. They are thread-like

roundworms with a relatively simple, cylindrical body and digestive system surrounded by a protective cuticle. Many, but not all, are small and transparent, facilitating the observation of symbionts by light microscopy. However, that is largely where the commonalities among members of this phylum end. Although difficult to quantify, nematodes are one of the most abundant multicellular organisms on earth, and they have been found in nearly every ecological niche that has been explored (Shah and Mahamood, 2017). These animals thrive in environments as different as soil, human microcapillaries, insects, the sea floor, and plant roots, and they have the trophic strategies to match. Their diets include other invertebrates, bacteria, blood, and plant fluids and tissue. Based on what we know about this diversity of nematode habitats and diets, it is perhaps unsurprising that there is an equivalent level of diversity in their interactions with microbes.

Bacterial taxa represented in the nematode–bacterium symbioses explored in this review span three different bacterial phyla (Table 5.1). Even within bacterial species that form symbioses with closely related nematodes, there can be a remarkable amount of subspecies- or strain-level genomic diversity and differences in genetic capacity (Scott *et al.*, 2012; Murfin *et al.*, 2015a,b; Brown *et al.*, 2016; Maher *et al.*, 2020). Further, there is variability in nematode dependence on symbiosis: even considering two closely related nematode species, one of them can require a bacterial symbiont to live and reproduce, while a sister species has no association with a symbiont (Bouchery *et al.*, 2013). It is clear that symbionts can be lost, gained, and changed even within closely related nematodes that occupy similar ecological niches (Murfin *et al.*, 2015b; Bouchery *et al.*, 2013; Wasala *et al.*, 2019). There are specialist species (such as entomopathogenic nematodes, filarial nematodes, and marine nematodes with ectosymbionts) that have a dominant interaction with a single major symbiont, and generalists (such as *Caenorhabditis elegans* and other free-living nematodes) harboring a community of associated symbionts, many of which are dispensable or interchangeable with other taxa, more similar to the human gut microbiome. The location and nature of the host association can vary dramatically, from endosymbionts that are within the body, both within (intracellular) and

between (extracellular) nematode cells, to ectosymbionts that are associated on the surface of the nematode.

Overall, this rich diversity in the types and features of symbioses makes nematode–bacterium associations useful for modeling a similarly diverse set of scientific questions. Nematode–bacterium symbioses provide important opportunities for comparative study of community assembly, the impact of environmental factors such as stress and diet on the association, how reproductive mode relates to symbiont transmission, and host–microbe coevolution (Morran *et al.*, 2016). Further, studying a broad range of nematode symbioses will reveal universal commonalities such as genomic features and metabolites that facilitate interactions, and patterns of adaptation in both host and microbe. This concept was the foundation for the 2009–2013 National Science Foundation Research Coordination Network “NemaSym”, led by Dr S. Patricia Stock, which fostered discourse and collaboration amongst scientists with interests in bacterial symbiosis with nematodes that had been historically siloed by their relevance to medicine, agriculture, ecology, and basic research areas (Stock and Goodrich-Blair, 2008; Stock *et al.*, 2011). These cross-disciplinary perspectives spurred new insights into diverse ways that microbes can contribute to nematode lifestyle and the common and distinctive genetic traits among nematodes that enable mutualistic interactions with nematodes (e.g., Dillman *et al.*, 2012; Murfin *et al.*, 2012). NemaSym also highlighted that each system of nematode–bacterium symbiosis offers distinctive technical features and intellectual opportunities to understand broad concepts of symbiosis biology. Since 2013 there has been an explosion of new literature that has revealed previously unrecognized microbial associations with diverse nematodes, described readily adaptable technologies that expand the number of experimentally tractable systems, and addressed pressing questions about the ecology, evolution, molecular mechanisms, and consequences of symbiotic relationships. In the following sections we discuss these advances, focusing on the best-studied examples of nematode–bacterium interactions and how they have contributed to our understanding of animal–microbe symbioses (Fig. 5.2) (Table 5.1) (Box 5.1).

Table 5.1. Resources available for nematode–bacterial symbiosis models.

Nematode	Culturable	Genome (Accession and BioProjectID)	Genetics	Symbionts identified (strain sequenced)	Phylum and Class	Culturable	Genomes (Accession)	Genetics
Entomopathogenic nematodes with endosymbionts								
<i>Steinernema carpocapsae</i>	Yes In insects or on plates with symbiont as food source	PRJNA202318 Dillman <i>et al.</i> , 2015 (Draft) Rougon-Cardoso <i>et al.</i> , 2016 (Draft) Serra <i>et al.</i> , 2019 (PacBio [more complete])	RNAi Morris <i>et al.</i> , 2017	<i>Xenorhabdus nematophila</i> ATCC 19061	Proteobacteria, Gammaproteobacteria	Yes	FN667742 (chromosome) FN667743 (plasmid) Chaston <i>et al.</i> , 2011	Yes
<i>Steinernema feltiae</i>	Yes In insects or on plates with symbiont as food source	PRJNA204661 PRJNA353610 Dillman <i>et al.</i> , 2015 Fu <i>et al.</i> , 2020	None found	<i>Xenorhabdus bovienii</i> Xb-Sf-FL Xb-Sf-FR Xb-Sf-MD Xb-Si Xb-Sj Xb-Sj-2000 Xb-Sk-BU Xb-Sk-CA Xb-So Xb-Sp Xb CS03	Proteobacteria, Gammaproteobacteria	Yes	Many strain genomes published Murfin <i>et al.</i> , 2015a Bisch <i>et al.</i> , 2016	Yes
<i>Heterorhabditis bacteriophora</i>	Yes In insects or on plates with symbiont as food source	PRJNA13977 Bai <i>et al.</i> , 2013	Transgenesis Hashmi <i>et al.</i> , 1995 RNAi Ciche and Sternberg, 2007 Ratnappan <i>et al.</i> , 2016	<i>Photorhabdus luminescens</i> TT01	Proteobacteria, Gammaproteobacteria	Yes	BX470251 Duchaud <i>et al.</i> , 2003	Yes
Insect parasitic nematodes with endosymbionts								
<i>Howardula aoronymphium</i>	Raised on <i>Drosophila falleni</i>	None found	None found	<i>Symbiopecto- bacterium</i>	Proteobacteria, Gammaproteobacteria	No	PRJNA415854 Martinson <i>et al.</i> , 2020	No

Continued

Table 5.1. Continued.

Nematode	Culturable	Genome (Accession and BioProjectID)	Genetics	Symbionts identified (strain sequenced)	Phylum and Class	Culturable	Genomes (Accession)	Genetics
Free-living nematodes with an endosymbiont community								
<i>Caenorhabditis elegans</i>	Yes On plates, with <i>E. coli</i> OP50 strain as food source	PRJNA13758 <i>C. elegans</i> Sequencing Consortium, 1998	RNAi, transgenesis, CRISPR-Cas9 Reviewed in: Ahringer, 2006 Nance and Frøkjær- Jensen, 2019 Dickinson and Goldstein, 2016	Over 500 isolates to date. CeMbio provides a representative 12-member microbiome: CEent1 JUb66 BIGb0393 MYb10 JUb19 MYb11 MSPm1 BIGb0172 MYb71 JUb134 BIGb0170 JUb44	Proteobacteria: Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria Bacteroidetes: Sphingobacteriia, Flavobacteriia	Yes for the CeMbio community – variable otherwise, particularly those identified by amplicon sequencing	PRJNA624308 PRJNA400855 GCA_004341505.1 SAMN13190037 GCF_003752235.1	Tractability unknown in most of 12-member microbiome CEent1 – plasmid introduced by triparental mating Berg, 2019 Can be fed tractable bacteria such as <i>E. coli</i> .
Filarial parasitic nematodes with intracellular endosymbionts								
<i>Brugia malayi</i>	Yes – in jirds (gerbils)	PRJNA10729 Ghedini <i>et al.</i> , 2007	RNAi Misra <i>et al.</i> , 2017 In mosquito Song <i>et al.</i> , 2010 Transgenesis Liu <i>et al.</i> , 2018 CRISPR Liu <i>et al.</i> , 2020b	<i>Wolbachia</i> <i>pipientis</i> wBm	Proteobacteria, Alphaproteobacteria	No	AE017321 Foster <i>et al.</i> , 2005	No
Plant-parasitic nematodes with intracellular endosymbionts								
<i>Globodera rostochiensis</i>	Yes – on potato plants	PRJEB13504 Eves-van den Akker <i>et al.</i> , 2016	RNAi Chen <i>et al.</i> , 2005	<i>Candidatus</i> <i>Cardinium hertigii</i>	Bacteroidetes, Sphingobacteriia	No	None found	No
<i>Heterodera avenae</i>	Yes – on wheat	E-MTAB-2221 Kumar <i>et al.</i> , 2014 (transcriptome only)	RNAi Cui <i>et al.</i> , 2018 <i>in planta</i> Blyuss <i>et al.</i> , 2019	<i>Candidatus</i> <i>Cardinium hertigii</i>	Bacteroidetes, Sphingobacteriia	No	None found	No

Continued

Table 5.1. Continued.

Nematode	Culturable	Genome (Accession and BioProjectID)	Genetics	Symbionts identified (strain sequenced)	Phylum and Class	Culturable	Genomes (Accession)	Genetics
<i>Heterodera glycines</i>	Yes – on soybean	PRJNA381081 Masonbrink <i>et al.</i> , 2019	RNAi Urwin <i>et al.</i> , 2002	<i>Candidatus</i> <i>Cardinium hertigii</i> cHgTN10	Bacteroidetes, Sphingobacteriia	No	CP029619 Showmaker <i>et al.</i> , 2018	No
<i>Heterodera goettingiana</i>	Yes – on pea plants	None found	None found	<i>Candidatus</i> <i>Cardinium hertigii</i>	Bacteroidetes, Sphingobacteriia	No	None found	No
<i>Pratylenchus penetrans</i>	Yes – on cucumber and raspberry plant roots	SRP068315 Denver <i>et al.</i> , 2016 (sequenced by genome skipping – shallow sequencing)	RNAi <i>in planta</i> Vieira <i>et al.</i> , 2015	<i>Candidatus</i> <i>Cardinium hertigii</i> cPpe <i>Wolbachia pipientis</i> wPpe	Bacteroidetes, Sphingobacteriia (cPpe) Proteobacteria, Alphaproteobacteria (wPpe)	No	SRR3097580 Brown <i>et al.</i> , 2018 (cPpe) MJMG000000000 Brown <i>et al.</i> , 2016 (wPpe)	No
<i>Radopholus similis</i>	Yes – on carrot discs	PRJNA522283 Mathew and Opperman, 2019	RNAi Li <i>et al.</i> , 2015 Roderick <i>et al.</i> , 2018 <i>in planta</i> Li, 2017	<i>Wolbachia pipientis</i> wRad	Proteobacteria, Alphaproteobacteria	No	None found	No
<i>Xiphinema americanum</i>	Yes – on sorghum- sudangrass	NC_005928 He <i>et al.</i> , 2005 (mitochondrial only)	None found	<i>Candidatus</i> <i>Xiphinematobacter</i>	Verrucomicrobia, Spartobacteria	No	CP012665 Brown <i>et al.</i> , 2015	No
Marine nematodes with chemosynthetic endosymbionts								
<i>Astomonema southwardorum</i>	No	None found	No	<i>Astomonema</i> sp. Symbiont – not named	Proteobacteria, Gammaproteobacteria	No	None found	No
<i>Astomonema jenneri</i>	No	None found	No	<i>Astomonema</i> sp. Symbiont – not named	Proteobacteria, Gammaproteobacteria	No	None found	No
Marine nematodes with chemosynthetic ectosymbionts								
<i>Laxus oneistus</i>	No	None found	No	<i>Candidatus</i> <i>Thiosymbion</i> <i>oneisti</i>	Proteobacteria, Gammaproteobacteria	No	FLUZ000000000 FLUY000000000 Peterson <i>et al.</i> , 2016 Paredes <i>et al.</i> , 2020	No
<i>Robbea hypermnestra</i>	No	None found	No	<i>Candidatus</i> <i>Thiosymbion</i> <i>hypermnestrae</i>	Proteobacteria, Gammaproteobacteria	No	In progress Viehboeck, 2019	No

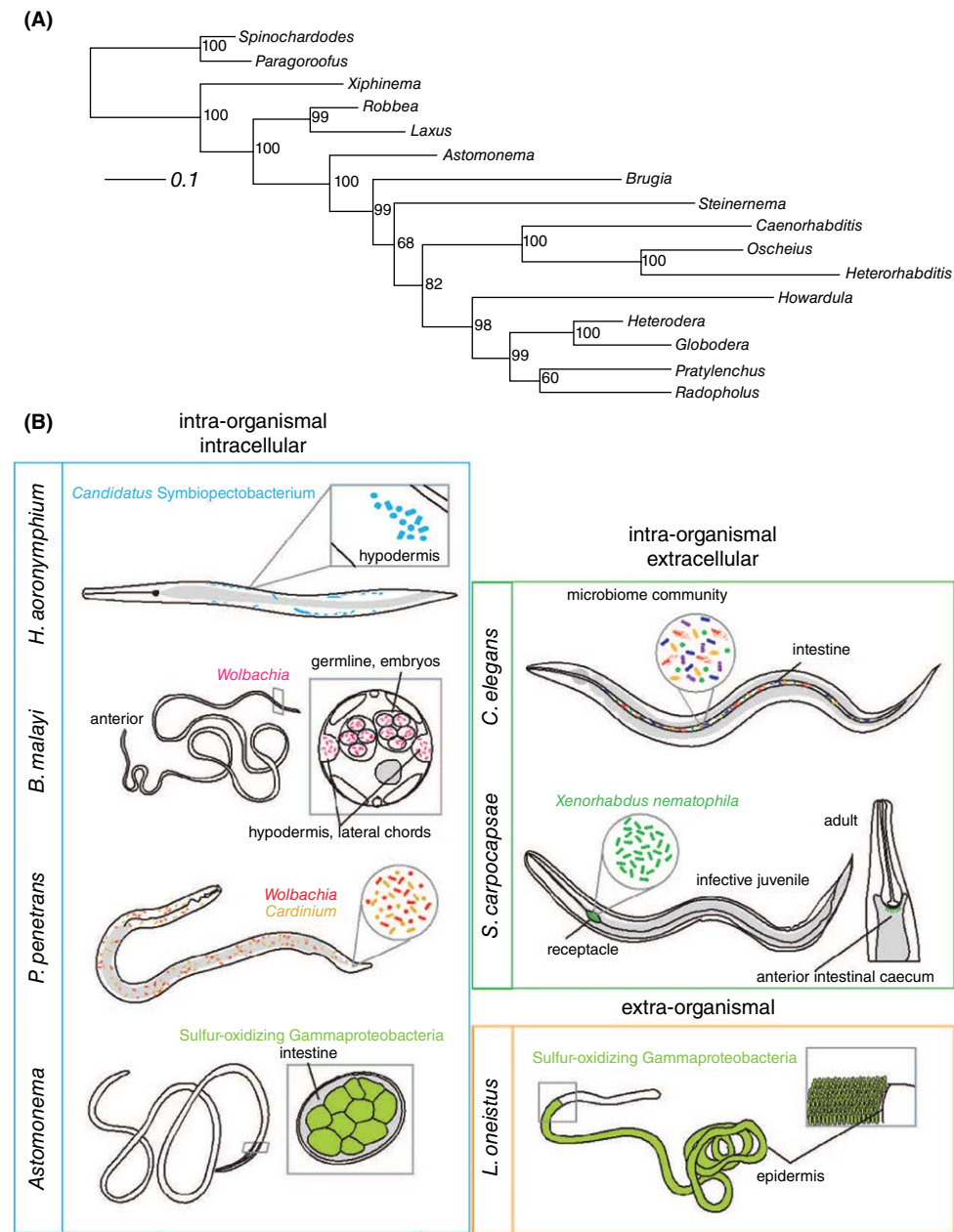


Fig. 5.2. Illustrated representation of the main types of nematode–bacterium symbiosis represented in this review. **(A)** Phylogeny of select nematode genera discussed in this chapter based on ITS, 18S, COI sequences as available from GenBank. Evolutionary relationships were estimated using a GTR+ γ +I substitution model and bootstrapped 1000 times. Percentage support values are indicated on the phylogeny. Not all discussed genera had sufficient sequence data to be included. **(B)** Representation of nematodes and bacterial symbionts. Symbiont niche tissues are specified when known. Bacterial symbionts are depicted and named in color. Insets show magnified views of symbiont colonized tissues. Nematode names are listed horizontally beside each organism, and intestine is gray where pictured.

Box 5.1. What makes a good symbiosis model system?

When choosing a model system to explore symbiosis, it is important to remember that what makes a good *genetic* model system is not necessarily or entirely what makes a good *symbiosis* model system. Model systems have historically been chosen for their ubiquity, ease of propagation, and ease of genetic manipulation. The same strategies that made common household pests such as mice, vinegar flies, and compost nematodes globally ubiquitous also made them ideal laboratory tools, with high fecundity and uncomplicated husbandry. Once a model system is established, it grows more powerful as researchers develop, borrow, and adapt the tools developed to answer a series of biological questions. This culminates in community-curated genomic and transcriptomic datasets, techniques, and strain collections which can represent, in some cases, genetic control of entire genomes (Kamath and Ahringer, 2003). As genetic engineering techniques and next-generation sequencing have improved, scientists have brought more and more species into the fold as model systems. The process is even speeding up over time; the progression from transgenesis to binary gene expression systems in *Caenorhabditis elegans* took 45 years (Brenner, 2009; Wei *et al.*, 2012), while that same progression took only 13 years in the more recent model system of *Aedes aegypti* (Matthews and Voss hall, 2020). Establishing a symbiosis model is even more complex, since it requires finding two (or more) organisms that live in close association with each other but that can be propagated readily and, ideally, separately in the laboratory. It also requires that both can be made genetically tractable. What is more, not every pair of symbionts is going to provide the same opportunities for learning; some symbioses are obligate and lifelong, while others are facultative and transient. Some symbioses are mutualistic, while others are pathogenic. Fortunately, we do not need to find a single pair of organisms to explore all of these interactions. The phylum Nematoda is diverse, possessing multiple convergent evolutions of each type of symbiotic relationship discussed here (generalist, specialist, obligate). Some of the nematodes described in this chapter are well-developed models with extensive genetic tools, while others are fledgling models that have not yet been cultured but nonetheless offer insights into specific biological questions, due to their niches and life histories. This breadth of possibility is precisely what makes nematodes such an exciting system to examine microbiomes and symbiosis.

Entomopathogenic Nematodes: a Versatile Platform for Symbiosis Research

Entomopathogenic nematodes (EPNs) infect and kill insects in cooperation with intergenerationally transmitted bacterial symbionts (Dillman *et al.*, 2012). This tripartite relationship between the insect, the nematode, and the bacterium has allowed researchers to explore the range of symbiosis outcomes, as the partners in the system exhibit activities from mutualism to antagonism at various points in the life cycle. EPNs can be reared with their symbionts outside of insects, and *Steinernema*, though not *Heterorhabditis*, can be cultivated on specialized media without bacteria, enabling the establishment of a range of tools for bacterial manipulation and an ever-expanding toolset for nematodes. These tools are used to answer questions about the molecular basis of colonization and host recognition, the necessity of metabolites and nutrient environments for symbiosis, and the evolution and maintenance of precise, strain-level host–microbe interactions

relevant to a range of fields, from the human microbiome to agricultural pest control.

Discovery of entomopathogenic nematodes

EPNs were first discovered in 1923 (Poinar and Grewal, 2012). Because up to one-fourth of crops are destroyed by insects worldwide (Kergunteuil *et al.*, 2016; Clarke, 2020), early research focused on utilizing EPNs for agricultural pest control. Although difficulties in producing EPNs on an industrial scale initially limited enthusiasm about their use in biocontrol of insect pests (Ehlers, 2001), EPN research was revived in the mid-20th century when, in Europe and North America, scientists independently isolated EPN populations from codling moth larvae (Weiser, 1955; Dutky and Hough, 1955). These nematodes became a cornerstone of the EPN research field and were later classified as *Steinernema carpocapsae* (Poinar, 1967). Today, two EPN families, Heterorhabditidae and Steinernematidae, are commercially produced

for use against insect pests in agricultural products such as NemAttack™, which consists of *Steinernema feltiae* nematodes (Shapiro-Ilan *et al.*, 2014; Lacey *et al.*, 2015). Most research on EPN–microbe symbiosis to date focuses on *Heterorhabditis* and *Steinernema* nematodes because of their development for use as entomopathogens throughout the 20th century.

Although researchers had observed the presence of bacteria inside the nematodes as early as 1937 (Bovien, 1937), the roles of the symbionts in the life cycles of EPNs would not be discovered for nearly 30 years (Poinar and Thomas, 1966, 1967). Symbiont laboratory isolation and cultivation required the development of media enabling propagation of the nematode–bacterium pairs outside of insects, as well as separately from each other (Stoll, 1953; House *et al.*, 1965). Poinar and Thomas's isolation of the *S. carpocapsae* bacterial symbiont *Xenorhabdus nematophila* in 1965 paved the way for the development of EPNs as a symbiosis model system (Poinar and Thomas, 1965). We now know that all members of both EPN families are associated with and dependent on a microbial symbiont: *Heterorhabditidae* with *Photorhabdus* bacteria and *Steinernematidae* with *Xenorhabdus* bacteria (Poinar, 1990).

Advancement of EPNs as a model for host–microbe interaction

The value of EPN nematodes as a model system for symbiosis lies partly in their life cycle, which includes measurable and reproducible stages of reproduction and development as well as discrete and observable interactions with the bacterium (Stock, 2005, 2019; Goodrich-Blair, 2007; Richards and Goodrich-Blair, 2009; Clarke, 2014). Early research showed that the non-feeding, soil-dwelling infective juvenile stage of an EPN nematode is intestinally colonized by the bacterial symbiont (Poinar, 1966; Poinar and Leutenegger, 1968; Poinar *et al.*, 1977). Poinar discovered that *Xenorhabdus* symbionts colonize the anterior intestinal lumen of infective juveniles in a specialized compartment referred to as the receptacle (previously termed the vesicle) (Poinar, 1966; Kim *et al.*, 2012), and that *Photorhabdus* symbionts colonize the lumen of the pharynx and intestine of infective juveniles (Poinar *et al.*,

1977). After finding prey, the nematode–bacterium pair infects the insect through the mouth, anus, spiracles, or cuticle (Peters and Ehlers, 1994; Lacey and Kaya, 2007). The nematode releases its symbiont into the insect hemolymph, where the bacteria replicate, kill the insect, and degrade the cadaver for nutrients. The nematode farms the bacteria for its own food until nutrients are depleted (Stock, 2019; Clarke, 2020; Mucci *et al.*, 2021). At that point, a new generation of infective juveniles reassociates with its bacterial symbiont and exits the cadaver in search of a new insect host to repeat the cycle.

The relationship between EPN hosts and their bacterial symbionts is most likely to be obligate in nature, as to date neither has been isolated without the other in field samples. This statement is supported by the observations that the bacteria depend on the nematode for transport between insect prey (Poinar and Thomas, 1966) and, in turn, serve as the major EPN food source (Stock, 2019; Clarke, 2020; Mucci *et al.*, 2021). Further, *Xenorhabdus* bacteria are auxotrophic for nicotinic acid dinucleotide (NAD), an essential coenzyme for cellular metabolism, and must derive this molecule from host environments (Chaston *et al.*, 2011). However, in the laboratory when nutrients are supplemented abiotically, the symbiosis can be made facultative as the bacteria can be cultured independently, and, at least for *Steinernema*, the nematodes can be raised axenically on specialized media. The technical advances in culturing the *Photorhabdus* and *Xenorhabdus* symbionts independently in the laboratory (Stoll, 1953; Poinar and Thomas, 1965) and in genetically manipulating them (Xu *et al.*, 1989, 1991) paved the way for breakthroughs like the expression of the green fluorescent protein (GFP) in the bacterial symbionts. This enabled investigation of processes such as symbiont intergenerational transmission and laid the foundation for EPNs as a model system.

The bacterial symbionts of EPN are transmitted to progeny infective juveniles (and therefore to new insect hosts) through nematode tissue-specific localization and symbiont bottlenecks that select for one or a few individual cells (Martens *et al.*, 2003a; Ciche *et al.*, 2008). In both *Heterorhabditis bacteriophora* and *S. carpocapsae*, as the nematodes develop into infective juveniles a few bacterial cells localize to the pharyngeal-intestinal valve (Ciche *et al.*, 2008; Chaston

et al., 2013). During this stage, the *S. carpocapsae* intestine fully constricts before the anterior intestinal region expands so that the lumen forms the receptacle, which is colonized by 1–2 *X. nematophila* cells that are presumed to derive from those that colonized the pharyngeal-intestinal valve. These few receptacle-colonizing *X. nematophila* then replicate to fill the receptacle (Bird and Akhurst, 1983; Martens *et al.*, 2003a; Snyder *et al.*, 2007; Chaston *et al.*, 2013). In *H. bacteriophora*, the transmission process by which *Photobacterium luminescens* cells colonize the infective juvenile pharyngeal-intestinal valve has been elucidated. In adult female *H. bacteriophora* nematodes, *P. luminescens* adheres to and invades posterior intestinal cells, and it is these maternally colonizing cells that are transferred to progeny infective juveniles that develop inside the mother. As in the *X. nematophila*–*S. carpocapsae* symbiosis, these *P. luminescens* cells colonize the pharyngeal-intestinal valve of the developing infective juveniles (Ciche *et al.*, 2008). Curiously, in *S. carpocapsae* adult nematodes, *X. nematophila* bacteria localize to the anterior intestinal caecum, and posterior colonization has not been observed (Chaston *et al.*, 2013). The role, if any, of anterior intestinal colonization in transmission has not been experimentally established. Overall, these findings suggest that while details of the transmission process vary, there is a bottleneck in both systems that limits the diversity of symbionts that are transmitted to the next generation. These observations added to a growing number of examples across diverse symbiotic systems demonstrating that symbiont populations become genetically bottlenecked at specific life-cycle stages (Chaston *et al.*, 2013). The concept that symbiont bottlenecks are common among horizontally transmitted systems contradicts the prevailing theory that such bottlenecks will not occur because they are expected to result in accumulation of deleterious mutations in the symbiont that will negatively impact symbiotic fitness. They also lend support for the evolutionary concept that high population densities of host and symbiont can balance the negative effects of bottlenecks (Muller, 1964; Pettersson and Berg, 2007; Chaston and Goodrich-Blair, 2010).

The findings summarized above have implications for how hosts select and transmit specific symbiotic partners. Colonization and transmission by only one or a few bacterial cells necessarily

indicate that other cells were restricted access to these niches. These observations further bolstered the idea that selection and partner recognition mechanisms play a role in EPN symbiosis. Such selective processes appear to include physical (for example, the colonization site, such as the pharyngeal-intestinal valve, is spatially limited and only accommodates a few cells) and molecular (for example, interactions between surface molecules present on both the nematode and bacterial cell surfaces). For instance, within the *S. carpocapsae* infective juvenile receptacle, *X. nematophila* associates with a mucosal layer surrounding a cluster of non-cellular (i.e., lacking a nucleus) spherical bodies, termed the intravesicular structure (IVS). Similarly, both *P. luminescens* and *X. nematophila* appear to be embedded in a polysaccharide material within their respective infective juvenile host's intestine (French-Constant *et al.*, 2003; Martens and Goodrich-Blair, 2005). Bacterial attachment to host mucus could indicate a lectin–glycan mechanism of attachment and specificity, with a bacterial protein acting as the lectin (Hooper and Gordon, 2001; Martens and Goodrich-Blair, 2005; Chaston *et al.*, 2013).

EPNs as a model for host–microbe interaction

One of the major contributions of the EPN–bacterium model to the symbiosis field is the expansion of our understanding of the bacterial proteins and molecular mechanisms of animal host colonization. The development of new experimental tools for genetic manipulation of the symbiont (Xu *et al.*, 1989, 1991) enabled identification of loci necessary to establish and maintain symbiosis (Ciche *et al.*, 2001; Heungens *et al.*, 2002). These included both site-directed and transposon mutagenesis followed by assessment of host colonization phenotypes (Ciche *et al.*, 2001; Vivas and Goodrich-Blair, 2001; Heungens *et al.*, 2002; Martens *et al.*, 2003a,b; Easom *et al.*, 2010; Somvanshi *et al.*, 2010). The development of a signature-tagged mutagenesis approach, the precursor to currently used techniques such as RbTnSeq (Shields and Jensen, 2019), allowed higher-throughput pooled screening of *X. nematophila* mutants for a colonization-defective phenotype (Heungens *et al.*, 2002).

Further, the availability of sequenced bacterial genomes enabled the rapid identification of transposon-disrupted loci (Wilkinson *et al.*, 2009; Chaston *et al.*, 2011).

The discovery of colonization-deficient bacterial mutants in both *Xenorhabdus* and *Photorhabdus* allowed researchers to delve into the molecular mechanisms that enable bacterial colonization of animal hosts, using EPNs as a model system. In particular, unbiased screens have revealed novel factors not previously implicated in symbioses. For instance, the screen for colonization-defective *X. nematophila* signature-tagged mutants revealed a locus dubbed “Symbiosis Region 2”, or SR2, in which the transposon had inserted into a sequence (*nilD*) with palindromic repeats that were also present at other locations in the genome and with sequence identity to the mysterious “iap” repeats found in *Escherichia coli* (Nakata *et al.*, 1989; Heungens *et al.*, 2002). In the same year that the repeat sequence *nilD* of SR2 was reported as being necessary for colonization, the term CRISPR was coined to describe “clustered regularly interspaced palindromic repeats”, including the *E. coli* iap repeats (Jansen *et al.*, 2002). Subsequent analyses confirmed that NilD is a CRISPR RNA, the first, and perhaps only, known example of a CRISPR RNA necessary for mutualistic colonization of a host, broadening the scope of CRISPR biological function beyond bacterial defense (Veesenmeyer *et al.*, 2014).

Bacterial surface proteins mediating host interactions

Symbiotic partners often physically interact at a cellular level where surface-exposed molecules of one species come in contact with surface-exposed molecules from the other in order to elicit some response. In both *X. nematophila* and *P. luminescens*, transposon mutagenesis screens revealed surface-localized proteins necessary for interactions with host nematodes (Heungens *et al.*, 2002; Somvanshi *et al.*, 2010). In a screen for *X. nematophila* mutants defective in colonizing the infective juvenile intestine, five independent mutants each had the transposon insertion located in one region, termed Symbiosis Region 1 (SR1) (Heungens *et al.*, 2002). Each of the transposons were within one of three genes in the SR1 locus, and these were named *nilA*, *nilB*,

and *nilC* to denote their role in nematode intestinal localization. The absence of SR1 from other *Xenorhabdus* genomes prompted investigations into its role in determining *X. nematophila* specificity for associating with *S. carpocapsae*. Non-native *Xenorhabdus* species that lacked the SR1 locus were unable to colonize *S. carpocapsae* infective juveniles. The SR1 locus was sufficient to confer upon these non-native *Xenorhabdus* species the ability to colonize *S. carpocapsae* infective juveniles, demonstrating that the SR1 locus is a specificity determinant that determines the nematode host range of the symbiont (Cowles and Goodrich-Blair, 2008; Chaston *et al.*, 2013). Furthermore, the SR1 locus also is necessary for species-specific colonization of the anterior intestinal caecum of developing juvenile and adult nematodes (Chaston *et al.*, 2013). SR1 was the first discovered example of a single locus capable of expanding host range in an animal–bacterium mutualism. Each SR1 *nil* gene is individually important for achieving robust colonization of infective juvenile nematodes (Heungens *et al.*, 2002; Cowles and Goodrich-Blair, 2008), but at the time they were discovered, none had homologs of known function in public databases (Heungens *et al.*, 2002). As such, elucidating their roles in promoting bacterial symbiont colonization required extensive subsequent studies.

Bioinformatics and structure/function analyses of NilB, a member of a large family of proteins typified by a DUF560 domain, revealed that it is an integral outer membrane protein with homologs in pathogens with discrete host range (Cowles and Goodrich-Blair, 2008; Bhasin *et al.*, 2012). Several *Neisseria* and *Haemophilus* DUF560 homologs were described as Slams (surface lipoprotein assembly modulators), which facilitate surface exposure of target lipoproteins, while another *Xenorhabdus* DUF560 homolog, HrpB, secretes a soluble cargo protein HrpC across the outer membrane (Grossman *et al.*, 2021). Based on analogy, NilB is predicted to secrete substrate proteins across the outer membrane (Mauer *et al.*, 2020). NilC is a likely candidate substrate for NilB, based on the close genomic proximity of *nilB* and *nilC*, and that NilC is an outer membrane-associated lipoprotein (Cowles and Goodrich-Blair, 2004). How these proteins function in symbiont colonization is still not entirely clear, but the homology of NilB with Slams important for surface localization of iron-binding molecules

may indicate a role in metal homeostasis (Mauer *et al.*, 2020). The idea that metals are important in EPN symbioses is supported by studies in *P. luminescens* (reviewed in Clarke, 2020). Exploration of this possibility not only will yield insights into the function of NilB in colonization of a nematode host, but also has implications for our understanding of the molecular function of this broad DUF560 family of outer-membrane proteins present throughout the proteobacteria (Mauer *et al.*, 2020).

An alternative hypothesis that has been posed for NilB or NilC function is that they may mediate adherence to nematode tissues through direct interactions with host cell-surface molecules (Chaston *et al.*, 2013). A common theme for both mutualistic and pathogenic symbionts is an interaction between lectins of one organism and glycans of another for attachment and recognition (Hooper and Gordon, 2001; Chaston and Goodrich-Blair, 2010; Etzold and Juge, 2014; Tailford *et al.*, 2015). Surface proteins of the bacteria or host can act as lectins or adhesins that adhere to specific polysaccharides on the surface of the other organism. NilB-mediated colonization of the anterior intestinal caecum (AIC) is a potential site of adhesion and glycan involvement, as it involves an intimate association of symbiont with a nematode tissue (Chaston *et al.*, 2013). In addition, within the infective juvenile receptacle, bacteria associate with a wheat-germ agglutinin-reactive substance, indicating the presence of *N*-acetyl glucosamine and/or *N*-acetyl neuraminic acid (Martens and Goodrich-Blair, 2005). Therefore, it is tempting to speculate that the Nil proteins are involved in some sort of host recognition process or adhesion to molecules presented at these colonization sites. Future work on further understanding the roles of these molecular players, in particular how they function in specificity between the nematode and bacterium partner pairs and with which, if any, host molecules they interact, is an exciting direction of EPN symbiosis research.

Transposon screens also led to the discovery of “Mad” (maternal adhesion defective) surface fimbriae essential for the attachment of *P. luminescens* to the posterior intestinal cells of adult females, a necessary step in transmission to infective juveniles (Somvanshi *et al.*, 2010). Of 30 mutants with defects in transmission to progeny infective juveniles, 12 occurred in a single locus

encoding 11 *mad* fimbrial genes. Expression of the Mad fimbriae varies between ON and OFF states controlled by an invertible promoter, in a phenomenon called phase variation (Somvanshi *et al.*, 2010, 2012). Fimbriae are encoded throughout bacteria and mediate attachment to surfaces. While they had long been known for their role in pathogenic associations, their established function in *P. luminescens* host colonization was among the first reports of a similar role in mutualism (Somvanshi *et al.*, 2010). One of the striking aspects of this study is that the control of fimbrial expression through phase variation led to the discovery that clonal populations of *P. luminescens* are heterogeneous, made up of so-called “P-form” and “M-form” cells, and that only the latter is capable of attaching to maternal tissues and therefore being transmitted to progeny, while the former is essential for pathogenic behaviors (Somvanshi *et al.*, 2010, 2012). This heterogeneity and division of labor is similarly displayed in *X. nematophila* symbionts (Park *et al.*, 2007), though in this bacterium heterogeneity is controlled by the global transcriptional regulator Lrp. *X. nematophila* *lrp* mutants have defects in virulence against insects, supporting nematode reproduction, and colonizing infective juveniles (Cowles *et al.*, 2007). Wild-type populations of *X. nematophila* are heterogeneous with respect to steady-state Lrp levels and host-interaction phenotypes. In turn, the levels of Lrp dictate whether *X. nematophila* exhibits traits associated with pathogenesis or mutualism (Hussa *et al.*, 2015; Cao *et al.*, 2017). Within infective juvenile nematodes, the proportion of low and high Lrp-expressing cells in a population changed as time passed after infective juvenile emergence (Cao and Goodrich-Blair, 2020).

Taken together, the independent analyses of *P. luminescens* and *X. nematophila* colonization processes establish that EPNs have evolved distinct molecular processes that result in the same outcome: heterogeneous bacterial populations in which individual cells express genes that contribute to either mutualism or virulence. These findings may indicate that the commonalities of EPN symbiotic lifestyles have selected for phase-variable regulatory pathways to ensure optimal and controlled gene expression of bacterial symbiont genes appropriate for each life stage (Cao and Goodrich-Blair, 2017). As more associations with similar stage-dependent

reliance on specific traits are investigated, it will be important to consider the role that phenotypic variation plays in the symbiosis.

Nutrition and secondary metabolism

Lrp is a member of the feast-or-famine regulator family (Yokoyama *et al.*, 2006). In *X. nematophila*, this regulator of population heterogeneity and symbiotic behaviors is likely sensing and responding to the prevailing metabolic conditions which may indicate its symbiotic state. Similarly, specific metabolites control the DNA-binding activity of LysR-type regulators of which the *P. luminescens* transcription factor HexA is a member (Joyce and Clarke, 2003). HexA controls numerous symbiotic activities in *P. luminescens*, and, like Lrp, may be important for controlling gene expression temporally over the symbiotic life cycle (Joyce and Clarke, 2003; Clarke, 2020). An important aspect of metabolism controlled by each of these regulators in their respective bacteria is the production of complex small molecules that fall outside primary conserved metabolism and are therefore known as secondary metabolites (Engel *et al.*, 2017). The importance of bacterial secondary metabolites in *Steinernema* and *Heterorhabditis* symbiosis is suggested by the fact that in both *X. nematophila* and *P. luminescens*, *ngrA* mutants lacking 4'-phosphopantetheine (Ppant) transferase, a key enzyme in the non-ribosomal-peptide-synthetase (NRPS)-dependent biosynthesis of secondary metabolites, do not support the reproduction and development of their respective nematode partners (Ciche *et al.*, 2001; Singh *et al.*, 2015).

Secondary metabolites produced by *Photorhabdus* may have garnered curiosity over a century ago in the form of the bioluminescence it produces. In fact, *Photorhabdus* is the only terrestrial bacterium known to date with bioluminescent properties. Insects infected with *Heterorhabditis* nematodes associated with their symbiont will glow in the dark (Poinar and Grewal, 2012). Legends from the battlefields of the American Civil War describe soldiers with glowing wounds (Nealson and Hastings, 1979). Although the cause of the luminescence was not discovered at the time and there is no way to investigate the reports, modern microbiologists have speculated that the luminescence was due to opportunistic *Photorhabdus* infections. Physicians in the Civil

War era believed that the presence of the bacterium would lead to good patient outcomes (Nealson and Hastings, 1979). The discovery of antibiotic properties of *Xenorhabdus* and *Photorhabdus* means that this belief could have some credibility (Dutky, 1959; Webster *et al.*, 2002), although there is at least one bioluminescent species of *Photorhabdus* that is pathogenic to humans (Gerrard *et al.*, 2003, 2004; Hapeshi and Waterfield, 2016). To date, little is known about the functionality of the luminescence to the nematode or the symbiont, but it has been suggested to help protect an insect cadaver from predation (Maher *et al.*, 2020).

Prior work on the nutritional demands of *X. nematophila* elucidated some of the components of the host environment needed for colonization and symbiosis, including that *X. nematophila* is a nicotinamide adenine dinucleotide (NAD) auxotroph. Using *X. nematophila* mutants that were found to be auxotrophic for various nutrients in culture, Martens *et al.* (2005) examined the colonization levels of *S. carpocapsae* to probe the nutrient environment of the nematode. *X. nematophila* mutants unable to produce select amino acids, vitamins, and nutrients were able to colonize *S. carpocapsae* infective juveniles to normal wild-type levels, suggesting that the colonization environment of the nematode receptacle is able to provide some nutrients to the symbiont (Martens *et al.*, 2005). However, colonization levels were decreased among *X. nematophila* with mutations in the para-aminobenzoate, pyridoxine, and L-threonine biosynthesis pathways (Martens *et al.*, 2005), leading to the conclusion that the nematode environment lacks sufficient quantities of one or more essential metabolites related to these pathways, with one-carbon metabolism representing a common thread. Therefore, at least at the infective juvenile stage, the symbiont appears to be dependent on some host-provisioned nutrients.

EPN–symbiont pairs as models of symbiont specificity and host switching

Strain variation can play a part in the fitness outcomes of one or more of the partners in a symbiosis, and the *Steinernema*–*Xenorhabdus* model represented an excellent opportunity to investigate

this concept. A *Steinernema* nematode exhibits the greatest fitness when associated with its native symbiont strain (Chapuis *et al.*, 2009; Murfin *et al.*, 2015a,b) and cannot be well colonized by non-native symbionts (Bird and Akhurst, 1983; Sicard *et al.*, 2004; Cowles and Goodrich-Blair, 2008). Further, *Xenorhabdus bovienii* provides an opportunity to assess strain-level specificity, because the bacterial strains have a 96% average nucleotide identity with each other yet associate with *Steinernema* nematodes that segregate phylogenetically into different clades within the *Steinernema* genus (Murfin *et al.*, 2015a,b; McMullen *et al.*, 2017). Within the *X. bovienii* species, there is variation in genomic coding regions between strains, especially in genes predicted to be involved in host interactions, indicating the potential for distinctive symbiotic traits expressed within the species (Murfin *et al.*, 2015b).

The effect of strain variation on host fitness was tested using *X. bovienii* strains and the *Steinernema* nematodes that they colonize. When pairs of *X. bovienii* strains and *Steinernema* nematodes were combined and examined for fitness using several different parameters (including percentage of productive infections, number of progeny produced, and progeny infective potential), the strains that were genetically less related to the native symbiont of the nematode species resulted in lowered fitness for the host (Murfin *et al.*, 2015a; McMullen *et al.*, 2017). In addition, *X. bovienii* strains isolated from different strains of *Steinernema feltiae* nematodes produce bacteriocins (tailocins) that are active against other *X. bovienii* strains capable of colonizing the same *S. feltiae*, indicating the potential for interspecies competition during colonization (Ciezki *et al.*, 2017). In some cases, an association with the *X. bovienii* bacterial symbiont can even lead to death of the non-native nematode host but is not harmful to the native nematode host (Murfin *et al.*, 2015a, 2019; McMullen *et al.*, 2017). This indicates that nematodes and bacterial symbionts might compete for the resources of an insect cadaver during co-infection with closely related nematode–bacterium partners.

A similar study in heterorhabditids found differential fitness in *Photorhabdus* symbiont species that colonize a common host nematode, *Heterorhabditis downesi*, and prey on a common insect population. Traits important for both

virulence and mutualism were compared in *Photorhabdus cinerea* and *Photorhabdus temperata* (Maher *et al.*, 2020). *P. cinerea* had increased antibiotic activities against non-*Photorhabdus* bacteria and deterred insect cadaver invasion by other nematodes at a higher rate than *P. temperata* (Maher *et al.*, 2020). In plate choice experiments, *P. cinerea* was preferred by *H. downesi* nematodes, and in local head-to-head competition between the two strains in insects, *P. cinerea* displayed a significant colonization advantage in infective juveniles and colonized to a greater level in infective juveniles co-colonized by both species (Maher *et al.*, 2020). Based on these studies, it is unclear what fitness advantages might be conveyed by *P. temperata* that allow it to persist in competition with *P. cinerea*, but it may be linked to the role of bioluminescence, as *P. temperata* was shown to be brighter than *P. cinerea* (Maher *et al.*, 2020). The evolution of bacterial symbiont traits that impact their virulence and mutualism phenotypes, and how those traits might be selected for by hosts, is still an area of active exploration. However, it appears that selection for the maintenance of mutualism is stronger than other selection pressures (Morran *et al.*, 2016). The number of culturable nematode and bacterial symbiont pairs, existing genomes, and high-confidence predictions of their phylogenetic relationships put EPNs and their bacterial symbionts in an extremely favorable position for studying the evolution of symbiont specificity down to the strain level.

Role of non-obligate symbionts in EPN life cycles

Advances in next-generation sequencing have expanded our knowledge of the nematode-associated microbial communities, including in EPNs. Early investigations of EPN colonizing bacteria focused primarily on the cognate bacterial symbiont, though there were reports using culture-dependent techniques which suggested that there may be additional members of the *Steinernema* microbiome (Bonifassi *et al.*, 1999; Gouge and Snyder, 2006). Recently, a study using metabarcoding of the V3V4 region of the 16S rRNA gene and housekeeping *rpoB* markers showed that both laboratory and wild populations of *Steinernema* species frequently are associated

with a dozen additional Proteobacteria species in addition to their obligate *Xenorhabdus* symbiont (Ogier *et al.*, 2020). Several species of *Pseudomonas* were predicted to play a role in the insect-killing “pathobiome” of *Steinernema* due to their entomopathogenic nature, but, in general, the function of the recently discovered “frequently associated microbiome” is unknown (Ogier *et al.*, 2020). *Xenorhabdus* is well equipped to compete with other bacterial species (Thaler *et al.*, 1995; Ciezki *et al.*, 2017; Dreyer *et al.*, 2018; Thappeta *et al.*, 2020), so it will be interesting to explore in the future how these additional members of the EPN microbiome survive within the insect cadaver and how they are transmitted by nematodes between insects. With the increasing availability of sequencing tools, our understanding of the traditional EPN tripartite insect–nematode–symbiont life cycle will expand to include these additional non-obligate symbionts.

Advantages of EPNs as a model system for symbiosis research

As noted in previous sections, EPNs present several distinct advantages as nematode model systems for understanding symbiosis. Unlike other models of beneficial animal–microbe interactions, where complexity necessitates artificial simplification of the microbiota, this is a naturally occurring, highly specific animal–microbe association. Both the nematode host and the bacterial symbiont can be cultured and manipulated independently or together in the laboratory and are amenable to experimental evolution studies (Morran *et al.*, 2016). High-quality genomes and multiple transcriptomes exist for multiple nematode–microbe species pairs (to date, most notably: *S. carpocapsae*–*X. nematophila*, *S. feltiae*–*X. bovienii*, and *H. bacteriophora*–*P. luminescens*), providing a strong foundation for future evolutionary comparisons (Duchaud *et al.*, 2003; Chaston *et al.*, 2011; Bai *et al.*, 2013; Dillman *et al.*, 2015; Murfin *et al.*, 2015b; Rougon-Cardoso *et al.*, 2016; Lu *et al.*, 2017; Chang *et al.*, 2019; Serra *et al.*, 2019; Fu *et al.*, 2020). Evidence is accumulating that pheromone signaling between EPNs, both within and amongst species, via conserved signaling molecules called ascarosides, is likely an important regulator of nematode behavior (Choe *et al.*, 2012; Kaplan *et al.*, 2012;

Hartley *et al.*, 2019). Future experiments will reveal if and how these signaling molecules are involved in aspects of symbiosis and whether symbionts can sense and respond to these important host-generated cues. A full suite of genetic tools has been developed for modifying the *Xenorhabdus* and *Photorhabdus* bacteria. The development of the nematodes as genetic model systems has lagged behind their bacterial counterparts, with relatively scarce reports of successful gene knockdown by RNAi and transgenic gene expression (Hashmi *et al.*, 1995; Ciche and Sternberg, 2007; Ratnappan *et al.*, 2016; Morris *et al.*, 2017). New advances in genome manipulation, including CRISPR-Cas9 gene editing, have been successfully applied in other “non-model” nematodes and are likely to work in EPNs as well (Ward, 2018; Castelletto *et al.*, 2020). These efforts are well worth the investment of time and resources, as EPNs have a wealth of interesting biology to offer, including diverse host-seeking behaviors, reproductive patterns such as mating and egg-laying, a nutrient responsive life-cycle transition, insect virulence, and questions surrounding aging, all of which may be influenced by their symbiotic partnership.

Nematode–microbiome models of insect and gastropod pathogenesis

While *Steinernema* and *Heterorhabditis* are accepted as the “true” entomopathogenic nematodes, the family *Rhabditidae* contains other organisms that have adapted this lifestyle as a function of their microbiomes. Most notably, the nematode genera *Heterorhabditidoides* (not to be confused with *Heterorhabditis*) and *Oscheius* include members that are facultatively entomopathogenic in insects of multiple orders (Torres-Barragan *et al.*, 2011; Zhang *et al.*, 2012; Dillman *et al.*, 2012). These nematodes can exist as free-living bacterivores but gain insect-killing potential when their external cuticle is colonized by specific strains of *Serratia marcescens* or *Serratia nematodiphila* (Zhang *et al.*, 2009). This flexible microbiome is facultatively generalist and could represent an intermediate step towards the evolution of a more intimate host–microbe association, like that seen in *Steinernema*. Studying the mechanisms of microbiome assembly in

these facultative EPNs and comparing them with the obligate EPNs would provide insight into the evolution of parasitism as well as demonstrating the evolutionary trade-offs of carrying a surface-associated symbiont compared with a gut-associated symbiont.

The genus *Phasmarhabditis* has been extensively studied as a biological control for slugs and snails that act as agricultural pests (Wilson and Rae, 2015; El-Danasoury and Iglesias-Piñeiro, 2017). In fact, *Phasmarhabditis hermaphrodita* is commercially available under the trade name Nemaslug® throughout Europe. Like the aforementioned *Heterorhabditidoides*, *Phasmarhabditis* spp. can exist as free-living bacterivores or as facultative parasites. *Phasmarhabditis* spp. were initially assumed to utilize highly specific microbial associations to promote gastropod pathogenicity, supported by evidence that specific symbionts such as *Moraxella osloensis* displayed toxicity to slugs (Tan and Grewal, 2001). However, later discoveries showed that *Phasmarhabditis* can associate with variable communities and that no specific bacterium is uniquely necessary for pathogenesis (Rae *et al.*, 2010). Sequencing-based studies could be utilized in the future to identify a core microbiome for this economically relevant nematode. Nematodes have independently evolved to become invertebrate parasites on many occasions and these groups each provide unique snapshots of host–microbe specificity, symbiont acquisition, and inter-kingdom communication which can be reconstructed readily in the laboratory and contextualized across evolutionary space. Few biological systems provide so many examples of convergent evolution that can be phylogenetically linked.

C. elegans as a Model for Symbiosis

The microbial community of the *Caenorhabditis elegans* intestine was characterized for the first time relatively recently. *C. elegans* is a useful model of facultative generalist symbiotic interactions where microbes from the environment colonize host tissue, and therefore the symbiotic community is diverse and variable. This raises interesting and important questions about host mechanisms of selection or restriction, if and how benefits are gained from symbionts, and community interactions between symbionts,

that can be addressed using *C. elegans* as a model of symbiosis. These questions are also fundamental to understanding human microbial communities that have a parallel symbiotic structure. The established genetic tools, behavioral assays, and developmental progression of *C. elegans* position it particularly well for studies of host biology.

History of microbial community research in C. elegans

C. elegans has a long and storied history as a genetically tractable model organism. In the early 1960s, the field of molecular biology coalesced around the discoveries of mRNA (Brenner *et al.*, 1961) and the triplet genetic code (Crick *et al.*, 1961). The discoveries of this time period revealed for the first time the basic unit of biological information: the gene. However, in order to determine how minute genes could build complex organisms of flesh and blood required incorporating an animal into the molecular biologist's toolset (Goldstein, 2016). In a 1963 letter, Sydney Brenner, a scientist whose name would become synonymous with *C. elegans* genetics, wrote that he “would like to tame a small metazoan organism to study development directly” (Reeve, 1988). *C. elegans* turned out to be an ideal tool, propagating readily and autogamously in the lab (Brenner, 1974). Over the decades these tiny worms facilitated the discovery of programmed cell death (Ellis and Horvitz, 1986) and RNA interference (Fire *et al.*, 1998) alongside advancing almost every aspect of developmental biology (Packer *et al.*, 2019). They even bear the honor of being the first multicellular organism to have its whole genome sequenced (Table 5.1) (*C. elegans* Sequencing Consortium, 1998). Despite the pivotal role played by *C. elegans* and its experimental value as a genetically tractable organism, this nematode has only recently been explored as a model system for symbiosis.

C. elegans as a model for host–microbe interaction

Researchers spent years overlooking the ways in which microbes and *C. elegans* interact, choosing instead to sterilize nematodes and raise them

axenically with the allopatric bacterium *E. coli* (Brenner, 1974; Stiernagle, 2006). The earliest forays into adapting *C. elegans* into a model for microbial symbiosis did not begin until 1999 and focused on pathogenesis. Human pathogens such as *Pseudomonas aeruginosa* (Tan *et al.*, 1999) and *Salmonella typhimurium* (Aballay *et al.*, 2000) were introduced to nematodes in order to discover cellular mechanisms of infection and resistance. While profoundly valuable in developing our understanding of innate immune signaling (Irazoqui *et al.*, 2010), these studies failed to recreate the microbial communities with which *C. elegans* would interact in their natural habitat amongst decomposing plant matter. Mammalian pathogens frequently display adaptations specific for growth in a mammal (such as thermal tolerance, halotolerance, immune evasion strategies, etc.), which are unlikely to reconstruct the patterns of infection natively encountered by wild worms (Granato *et al.*, 2017). Observing the impact of *C. elegans* on soil communities has suggested a potential application of *C. elegans* in soil remediation. *Bacillus anthracis* is a potent human pathogen that can be difficult to deplete from natural reservoirs or contaminated soil, due to spore formation, but application of nematodes alongside spore-germinating compounds significantly depletes spores (Schelkle *et al.*, 2018). While more recent studies have looked at ecologically relevant pathogens such as the microsporidia *Nematocida parisii* (Troemel *et al.*, 2008), the bacterium *Microbacterium nematophilum* (Hodgkin *et al.*, 2000), the fungus *Drechmeria coniospora* (Zhang *et al.*, 2016), and the virus OrV (Frézal *et al.*, 2019), pathogenic interactions also have revealed heritable mechanisms of sensing and avoiding specific bacteria (Moore *et al.*, 2019). However, it remains to be seen if similar mechanisms are involved in sensing and recruiting commensal community members, and fewer studies in general have looked beyond pathogenesis into the commensal and mutualistic communities associated with *C. elegans*.

Early attempts to study commensal communities in *C. elegans* followed in the footsteps of pathogen research by focusing on commensals native to humans. Worms that were fed hypothetical human probiotic species of *Lactobacillus* and *Bifidobacterium* displayed extended lifespans and increased resistance to infection by *Salmonella* (Ikeda *et al.*, 2007). Strains of *Lactobacillus*

acidophilus have protective effects against infection by Gram-positive bacteria, *Enterococcus faecalis* and *Staphylococcus aureus* (Kim and Mylonakis, 2012). The common interbacterial signaling molecule indole endows worms with relative resistance to colonization by the opportunistic fungal pathogen *Candida albicans* (Oh *et al.*, 2012). Additionally, recent work revealed that the probiotic strain *Bacillus subtilis* PXN21, or metabolites derived from it, can prevent the aggregation of α -synuclein in a DAF-16-dependent manner. Such aggregation is associated with disease severity in Parkinson's disease (Goya *et al.*, 2020).

To provide a more natural context, later investigations focused on the impacts of nematode-derived microbes on *C. elegans* biology. *C. elegans* transcriptomics, enabled by the nematode's well-annotated genome, revealed that exposure to soil bacteria in general altered expression of genes involved in metabolism, innate immunity, and cuticle biosynthesis (Coolon *et al.*, 2009). More specifically, nematodes that were exposed to an artificial community of *C. elegans* microbiome isolates displayed upregulation of immune genes and hydrolases known to be expressed in the intestine (Berg *et al.*, 2019). In another study, culture-based methods isolated two soil bacteria, *Bacillus megaterium* and *Pseudomonas mendocina*, which were protective against *Pseudomonas aeruginosa* infection. The protective effects of *B. megaterium* were mediated by a behavioral change in nematodes that resulted in diversion of energy resources away from egg-laying and toward immunity. The protective effects of *P. mendocina* were dependent on stimulating the innate immune response via the p38 MAPK pathway (Montalvo-Katz *et al.*, 2013). Collectively, strains isolated from nematodes suggest that bacterial exposure is important for establishing gene expression, priming the innate immune system against pathogen exposure, and potentially modulating nematode behavior.

Characterization of native microbiome of *Caenorhabditis*

In 2016, three independent studies (Berg *et al.*, 2016; Dirksen *et al.*, 2016; Samuel *et al.*, 2016) utilized amplicon sequencing of the V4 region of the 16S ribosomal subunit gene to characterize the microbiome of *C. elegans*. Samuel *et al.* (2016)

holistically examined bacterial communities from rotting vegetative matter inhabited by *C. elegans* and revealed 564 culturable bacteria and about 2400 operational taxonomic units (OTUs). Of the culturable isolates, 80% enhanced nematode growth compared with *E. coli*. The remaining 20% impaired growth and/or upregulated expression of one or more stress genes. Berg *et al.* (2016) allowed aposymbiotic laboratory-strain nematodes to recruit bacteria from environmental soil samples prior to amplicon sequencing. Of the approximately 2400 OTUs found within the worm intestine, members of nine bacterial families were actively recruited and were proposed to constitute a “core microbiome”. Additionally, spatial distribution and soil temperature impacted the composition of the *C. elegans* gut community. Finally, Dirksen *et al.* (2016) sequenced amplicons from wild isolates of *C. elegans* as well as the nematodes *Caenorhabditis briggsae* and *Caenorhabditis remanei*. This analysis demonstrated that gut microbiomes differ according to nematode species, genotype, and developmental stage. Using individual isolates alongside an artificial community of 14 bacteria, the authors demonstrated that commensal isolates enhanced nematode growth compared with *E. coli* and increased resistance to pathogenic fungal infection by *D. coniospora*. A meta-analysis of all three datasets used principal components analysis to show that while bacterial communities vary a great deal by soil microenvironments (for example compost, fruit, microcosms), nematode gut communities across all studies cluster closely together and are dominated by bacteria from the phyla *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* (Zhang *et al.*, 2017). Furthermore, certain bacterial isolates, such as *Ochrobactrum* MYb71 and *Stenotrophomonas* MYb57, are enriched in worms compared with the environment. This enrichment supports the hypothesis that these isolates are colonizing the intestines and actively reproducing (Dirksen *et al.*, 2016). Like the human gut microbiome, the *C. elegans* microbiome is selected from environmental reservoirs for specific representatives that support increased growth, prime the immune system, and increase resistance to colonization by pathogens.

Tools for microbiome research

The above meta-analysis resulted in the generation of the *Caenorhabditis elegans* Microbiome Resource,

henceforth CeMbio (Dirksen *et al.*, 2020). CeMbio is a collection of 12 bacterial isolates that represent the most common OTUs found in *C. elegans* collected from the wild. These 12 isolates represent all nine of the bacterial families identified as being constituents of the *C. elegans* core microbiome. Each of these representatives has a fully sequenced genome and accompanying metabolic network to facilitate microbiome studies (Table 5.1). These strains can be identified and enumerated via quantitative polymerase chain reaction (qPCR) amplification with diagnostic primers, enabling the construction of defined artificial communities (Dirksen *et al.*, 2020). This toolset complements the already robust strain collections available for *C. elegans* and paves the way for future studies to determine mechanisms of host–microbe nutrient exchange, immune activation, and protection from pathogens. Resources on the nematode side of the symbiosis include the *Caenorhabditis* Genetics Center (CGC), which offers an extensive collection of *C. elegans* mutants, and the *Caenorhabditis elegans* Natural Diversity Resource (CeNDR), which offers fully sequenced wild isolates of *C. elegans* with detailed geographic metadata (Cook *et al.*, 2017). These resources make *C. elegans* a uniquely valuable tool for a broad range of investigators to examine genetic determinants for microbiome acquisition, maintenance, and specificity.

Responding to bacteria: *C. elegans* innate immunity

C. elegans is uniquely qualified for exploring the role of host innate immunity in establishing and maintaining a resident microbiome. *C. elegans* entirely depends on humoral immune signaling to respond to microbial challenge, since nematodes have no adaptive immune system. Genetic immune determinants can be examined without the confounding variables of acquired immunity (Shapira, 2009). Also, the array of genetic tools available for *C. elegans* enables measuring the impact of immune pathway disruption on microbiome and nematode health/fitness/behavior. Forward genetic screens can be achieved via mutagenesis, while targeted reverse genetic manipulations can be accomplished by RNAi exposure (Shapira and Tan, 2008) or CRISPR-Cas9 editing (Dickinson and Goldstein, 2016). Finally, the *C. elegans*

microbiome can be flexibly populated with a range of members from a single monoclonal isolate to thousands of OTUs from the wild. The core microbiome, as represented by CeMbio, facilitates well-controlled studies on the role played by innate immunity in community recruitment, while human pathogens that also infect *C. elegans* are used to discover molecular mechanisms by which innate immunity combats pathogenic infection.

C. elegans has already been essential to a number of discoveries in innate immunity. The process of RNAi, now used experimentally to knock down gene expression in a wide range of organisms, was originally discovered in *C. elegans* where it naturally serves as a protective measure against RNA viruses (Fire *et al.*, 1998). This process is conserved in many eukaryotes, including plants and animals, where, in addition to defending against viral infection, it acts as a regulatory element of endogenous genes. Investigation of the *C. elegans* RNAi system yielded knowledge of viral defense against its native pathogens, such as the OrV virus (Frézal *et al.*, 2019), as well as diverse mammalian viruses such as Indiana vesiculovirus and human immunodeficiency virus-1 (Diogo and Bratanich, 2014).

C. elegans does not appear to have the canonical immune deficiency (IMD) pathway found in insects, or its mammalian equivalent, the tumor necrosis factor (TNF) receptor pathway. However, *C. elegans* does have a single Toll-like receptor (TLR) protein whose role in development expands the functional range of the TLR family (Irazoqui *et al.*, 2010). The Toll protein was first discovered in *Drosophila* where, in addition to its involvement in dorso-ventral patterning, it has roles in detecting microbe-associated molecular patterns (MAMPs) and initiating cellular development. TLRs in humans and mice serve similar roles. The *C. elegans* TLR is essential for pathogen avoidance behaviors but this activity does not involve detection of MAMPs. Instead it serves a strictly developmental role in maturing the BAG neurons, which are responsible for CO₂ detection (Brandt and Ringstad, 2015). As such, reducing TLR function leaves worms susceptible to infection by some microbes (*Salmonella marcescens* and *Salmonella enterica*) but has no effect on others (*Salmonella aureus*, *P. aeruginosa*, and *D. conio-spora*). Potentially, this disparity could be caused by pathogenic microbes having different CO₂

signatures in the environment. This finding highlights that the roles of TLR in development and immune signaling may be more tightly integrated than currently appreciated.

As in humans, the p38 immune signaling pathway is integral to innate immunity in *C. elegans*. *C. elegans* has been used to demonstrate that neuronal tissues are responsible for coordinating responses to infection via stimulation of the p38 MAPK pathway (Styer *et al.*, 2008). As such, neurons need to be able to differentiate commensal bacterial signals from pathogenic bacterial signals in order to respond appropriately to microbiome recruitment. As mentioned previously, the p38 pathway is essential for *C. elegans* to benefit from the protective effects of priming by the gut-dwelling commensal *P. mendocina*. *P. mendocina* activates the p38 pathway in a manner similar to *P. aeruginosa*, causing the worm to pre-emptively adapt for potential infection. The MAPK cascade initiated within *C. elegans* is homologous to that seen in humans; however, the transcription factors activated by this cascade have yet to be identified (Montalvo-Katz *et al.*, 2013). Despite the knowledge gaps in this immune pathway, it is hypothesized to drive production of antimicrobial peptides and programmed cell death based on its responses to challenge with *P. aeruginosa* (Irazoqui *et al.*, 2010). The similarities and differences in p38 pathways between *C. elegans* and mammals help to bridge the gap between vertebrate and invertebrate immunology.

C. elegans possesses a transforming growth factor- β (TGF β) signaling pathway which is activated by the neuronally derived growth factor DBL-1 (Irazoqui *et al.*, 2010). This pathway has been implicated in defense against diverse microbes and across multiple tissue types. The TGF β pathway facilitates resistance against epithelial infection by the fungus *D. conio-spora* by detecting paracrine signals from neuronal tissue and inducing expression of the antimicrobial peptide caenacin (Zugasti and Ewbank, 2009). Meanwhile in the gut, TGF β pathway mutants lacking the gene encoding DBL-1 show an altered microbiome, in which total bacterial abundance is only modestly increased, but Enterobacteriaceae abundance doubled. This community disruption occurred in a tissue-specific manner centered on the anterior gut and enabled a normally commensal *Enterobacter* to reach a pathogenic abundance

(Berg *et al.*, 2019). The homologous TGF β signaling seen in humans also shapes microbial communities in a number of ways, including regulation of inflammatory response and priming lymphocytes to be tolerogenic of commensal bacteria (Zeuthen *et al.*, 2008). These observations demonstrate the vital role played by neuroendocrine signaling in innate immunity and establishes *C. elegans* as a valuable system for exploring neuroendocrine signaling's effects on microbial communities, and vice versa.

The insulin-like signaling pathway, represented by the insulin receptor DAF-2 and the transcription factor DAF-16, is also capable of mediating microbiome response in *C. elegans* (Irazoqui *et al.*, 2010). Insulin-like signaling contributes independently to nematode longevity and bacterial resistance (Miyata *et al.*, 2008). DAF-16 is responsible for transcription of many genes implicated in bacterial defense, but only specific infections result in activation of this pathway. For example, *P. aeruginosa* infection repressed expression of DAF-16 and enteropathogenic *E. coli* (EPEC) infection induced DAF-16 expression resulting in resistance to subsequent infection (Anyanful *et al.*, 2009). Encoding multiple signaling pathways, like *C. elegans* does, is essential for organisms to finetune immune responses against distinct microbes. It should be noted that some bacteria, such as *S. aureus*, induce a response in nematodes that is independent of the previously mentioned pathways (Irazoqui *et al.*, 2010). This suggests that *C. elegans* has novel signaling pathways yet to be characterized, leaving the field open for growth. Additionally, future research could utilize more commensal communities instead of allopatric pathogens to determine how the innate immune system avoids unnecessary activation against its own microbiome.

Advantages of *C. elegans* as a model system for symbiosis research

C. elegans has been a staple model organism for decades and its value has not yet been exhausted, especially as it relates to host–microbe interactions. *C. elegans* offers an experimental sandbox to explore facultative generalist interactions in a simplified microbiome. *C. elegans* can reproduce by self-fertilization, rapidly, and in the laboratory.

It has one of the most extensive genetic toolkits of any metazoan and the developmental history of every cell in its body has been carefully mapped (Sulston *et al.*, 1983). Protocols and automation pipelines have already been developed to describe life stages and behavioral outputs, such as egg-laying, activity, and microbial prey-seeking (Collins *et al.*, 2016; Pitt *et al.*, 2019). Thanks to the extensive mapping of the *C. elegans* nervous system, exposure to the commensal *Providencia rettgeri* has already been demonstrated to impact the worm's olfactory responses and feeding preferences via the production of the neurotransmitter tyramine (O'Donnell *et al.*, 2020). Finally, isolated microbiome constituents and host nematodes have been collected from around the globe and can be reared together or in isolation to parse apart genotypic determinants of symbiosis. The combination of host genetic tools, fate mapping, neurobiology, and established behavioral assays makes *C. elegans* an especially powerful model for understanding how animal hosts assemble beneficial microbial communities. The genetics of the recently identified microbial community members are just beginning to come online, with full genome sequences adding power to possibility (Dirksen *et al.*, 2020). The lives of *C. elegans* nematodes are spent seeking out bacteria in their own terrain, where being able to separate commensal from pathogen can be the difference between dinner and death. In a sense, they are some of the original microbiologists and, if we listen closely enough, they might just tell us their secrets.

Nematode Models of Intracellular Endosymbiosis

In contrast to the EPNs and free-living *C. elegans* discussed above, where endosymbionts are housed within the host body but outside cells in niches or within the intestine, intracellular endosymbiosis occurs when microbes are housed directly within host cells. Intracellular symbioses between eukaryotes and bacteria are found throughout the tree of life, including in diverse nematode lineages (Ott *et al.*, 1982; Taylor *et al.*, 2012; Brown, 2018; Martinson *et al.*, 2020). Here we discuss an insect–parasitic nematode, filarial parasitic nematodes, plant-parasitic nematodes

(PPNs), and marine nematode models of intracellular endosymbiosis. Interestingly, many of the endosymbionts discussed in this section (*Symbiopectobacterium*, *Wolbachia*, *Cardinium*, and *Burkholderia*) are also intracellular endosymbionts of arthropods (Martinson *et al.*, 2020), suggesting that the flexibility required for endosymbionts to switch between arthropod hosts and nematode hosts may be a relatively common characteristic of these symbionts. Nematode models of intracellular endosymbiosis may be useful for understanding these interactions across host families, how intracellular endosymbioses evolve or devolve through genome erosion and horizontal gene transfer with their hosts, and where a given intracellular endosymbiont resides on the mutualist–pathogen continuum.

Most intracellular endosymbiotic interactions are obligate, and the microbe cannot survive or be cultured outside of host cytoplasm. Therefore, studying intracellular endosymbionts has historically presented some challenges, especially in cases where free-living relatives have not been identified. However, current sequencing and -omics approaches have increased their tractability. Hosts can also become dependent on their intracellular microbial symbionts. This dependence can present opportunities for targeting detrimental host nematodes through their symbionts, such as using antibiotics to combat filarial nematodes (Bouchery *et al.*, 2013). In addition to being experimental models of intracellular endosymbiosis, a greater understanding of symbiosis in filarial nematodes and PPN models may play a role in mitigating their negative impacts on human health and agriculture.

The intracellular endosymbionts of an insect-parasitic nematode

The insect-parasitic nematode *Howardula aoronymphium* (Hexatylini) is an obligate parasite of *Drosophila neotestacea*. *Howardula* spp. nematodes reproduce on mushrooms where individual inseminated adult females infect the larva of mushroom-eating (mycophagous) *Drosophila*, such as *D. neotestacea* (Jaenike and Perlman, 2002). The adult female nematode, or motherworm, grows within the developing fly larva and, after the emergence of the adult fly, begins

producing juvenile nematodes, which are deposited on mushrooms through the fly ovipositor or anus. A strong correlation between *Howardula* nematode-infected flies and the presence of *Candidatus Symbiopectobacterium* (hereafter *Symbiopectobacterium*), followed by visual confirmation by fluorescence *in situ* hybridization (FISH), led researchers to identify *Symbiopectobacterium* as an intracellular endosymbiont of several *Howardula* spp. (Martinson *et al.*, 2020).

The presence of the *Symbiopectobacterium* intracellularly within nematode hypodermal cells of motherworms and developing juveniles, found both within and outside of motherworms, is highly suggestive of vertical symbiont transmission (Martinson *et al.*, 2020). So far, attempts to cure *H. aoronymphium* nematodes of their endosymbionts using antibiotics have not been successful, but antibiotic treatment greatly reduced the success of nematode infections in *Drosophila*, suggesting obligate dependence (Martinson *et al.*, 2020). Phylogenetic comparison of *Symbiopectobacterium* with other bacteria found a close relation to *Pectobacterium*, a group of plant pathogens known as “soft rot Enterobacteriaceae”. *Symbiopectobacterium* was found to segregate within a clade of arthropod-associated intracellular endosymbionts with diverse life histories, and comparison of these related species revealed a reduction in genome size and many pseudogenized loci, leading the authors to conclude that the endosymbionts and host came together relatively recently (Martinson *et al.*, 2020). Unlike the EPN extracellular endosymbionts *Xenorhabdus* or *Photorhabdus*, it appears unlikely that these endosymbionts play any direct role in insect killing, but they may play a role in the nematode’s ability to colonize and reproduce within *Drosophila* (for example, by nutrient supplementation, immune avoidance, defense, etc.).

Filarial parasitic nematodes

Wolbachia are obligate intracellular endosymbionts found in diverse host species, including arthropods and nematodes (Scholz *et al.*, 2020). All *Wolbachia* classified to date form a monophyletic group within the Rickettsiales order, with 18 clades known as “supergroups” currently described (Landmann, 2019; Scholz *et al.*, 2020).

These symbionts are prolific and best studied in arthropods (over 60% of insect species are estimated to carry these microbes), where they have strong impacts on host biology (Werren *et al.*, 2008). *Wolbachia* are scientifically famous manipulators of host reproduction by inducing changes in sex or sex ratios, or by inducing sperm–egg cytoplasmic incompatibility. These strategies increase *Wolbachia* prevalence in the next generation through vertical transmission of the endosymbiont in the germlines of infected females (Russell, 2019).

The filarial nematodes that carry *Wolbachia* endosymbionts are pathogens of terrestrial vertebrates and are vectored between hosts by arthropods. Two such groups of human filarial pathogens (family Onchocercidae) are *Onchocerca volvulus*, which is vectored by biting blackflies and causes a disease known as river blindness or onchocerciasis, and *Brugia malayi*, *Brugia timori*, and *Wuchereria bancrofti*, which are vectored by mosquitoes and cause lymphatic filariasis (Werren *et al.*, 2008). These neglected tropical diseases are non-lethal, but cause significant human debilitation and suffering across the globe. Filarial pathogens of veterinary concern, including *Dirofilaria immitis*, which causes heartworm disease, or *dirofilariasis*, in canines, also harbor *Wolbachia* endosymbionts. The endosymbiotic relationship between Onchocercidae pathogens and *Wolbachia* is of great interest, not only because these systems are effective models for understanding one of the most prolific intracellular endosymbionts but also because of the therapeutic potential of targeting these pathogens through their symbionts (Slatko *et al.*, 2010).

Transmission mechanisms of Wolbachia in Brugia malayi

The filarial nematode *B. malayi* has proven to be a fruitful model system for understanding the molecular and cellular details of intracellular endosymbiosis from both the host and microbial perspectives. In contrast to arthropods where *Wolbachia* is facultative and a reproductive parasite, in *B. malayi*, *Wolbachia* (wBm) is essential for nematode development, fertility, and survival, leading to the conclusion that there is an obligate dependency on this symbiont, at least for some nematode species (Landmann, 2019). *Wolbachia* resides in the cytoplasm of the lateral hypodermal

chords in both males and females, and in the ovaries, oocytes, and embryos of reproductive females (Kozek, 1977). However, at the time of discovery, the trajectory of these intracellular endosymbionts during development was unknown.

Highly stereotyped early cell divisions and a remarkable degree of developmental similarity between the genetic model *C. elegans* and *B. malayi* allowed landmark studies using high-resolution microscopy and immunostaining to map the location of *Wolbachia* cells throughout the *B. malayi* life cycle and revealed the transmission mechanisms of the symbiont (Landmann *et al.*, 2010, 2012). Beginning in the newly fertilized one-cell-stage embryo, *Wolbachia* cells segregate to the posterior of the embryo, then over subsequent cell divisions become enriched in the precursor cell that gives rise to the hypodermal tissue later in development (Landmann *et al.*, 2010). Segregation of *Wolbachia* to the posterior of the embryo at the first mitotic division required the *B. malayi* host-cell microtubules, dynein motor protein, and polarity proteins (Landmann *et al.*, 2014). In female L4-stage larva, *Wolbachia* migrates from the hypodermal chords to the germline and invades the tissue near the distal tip (Landmann *et al.*, 2012). This invasion appears to involve disruption or manipulation of the actin cytoskeleton (Landmann *et al.*, 2012), consistent with putative *Wolbachia* surface protein–actin interactions that have been identified (Melnikow *et al.*, 2013; Carpinone *et al.*, 2018). *Wolbachia* is associated with nuclei in the syncytial germline and spreads along the rachis to other parts of the germline, ensuring infection and transmission in developing oocytes (Landmann *et al.*, 2012).

Multiple instances of *Wolbachia* endosymbionts impacting the development of *B. malayi* nematodes have been observed. The presence of *Wolbachia* endosymbionts is important for proper polarization of the first cell division, suggesting integration of the endosymbiont into the anterior–posterior patterning and proper *B. malayi* host development (Landmann *et al.*, 2014). *Wolbachia* also influences mitotic proliferation and stem cell fate and organization in the female germline of *B. malayi* (Foray *et al.*, 2018). Transcriptomic studies in *B. malayi* and *D. immitis*, profiling both nematode and *Wolbachia* gene expression simultaneously in females, suggest conserved upregulation of genes involved in DNA replication and repair, ribosomes, and the

purine biosynthesis pathway in both filarial species (Luck *et al.*, 2015; Grote *et al.*, 2017).

Nutrient exchange may be another piece of the filarial nematode–*Wolbachia* endosymbiont interaction puzzle. Unlike free-living nematodes such as *C. elegans*, in which the majority of nutrients are taken up by the intestinal cells after ingested food is digested, some filarial nematodes such as *Brugia pahangi* absorb nutrients such as amino acids from their animal hosts through their cuticles (Howells *et al.*, 1983). This means that the hypodermal compartment just below the cuticle that houses *Wolbachia* is ideally positioned to function in nutrient uptake. *B. malayi* is auxotrophic for riboflavin and heme (Ghedini *et al.*, 2007; Scott and Ghedin, 2009). It is hypothesized that *Wolbachia* may provision heme to its nematode host. This idea is supported by findings that chemical inhibition of heme biosynthesis in *Wolbachia* endosymbionts has adverse effects on nematode hosts and that *B. malayi* can transport exogenous heme (Lentz *et al.*, 2013; Luck *et al.*, 2016). Also, there is emerging evidence that wBm lacks key glycolytic enzymes, and that glucose metabolism may be a shared metabolic pathway between *B. malayi* and wBm (Voronin *et al.*, 2016). However, direct evidence of nutrient provisioning between *Wolbachia* and its host, *B. malayi*, is lacking.

Brugia malayi and wBm *Wolbachia* genomes

More clues about the relationship between host and endosymbiont can be gleaned from genomic analysis of both species. The original sequencing and comparison of the wBm genome to the arthropod wMel (*Wolbachia* from *Drosophila melanogaster*) revealed that the wBm genome was smaller, with fewer protein coding genes and a lack of repetitive DNA, mobile genetic elements, and prophages (Foster *et al.*, 2005). wBm contains complete biosynthesis pathways for purines and pyrimidines, but lacks complete pathways for many other cofactors such as NAD, biotin, and folate, suggesting that these pathways may be a part of metabolic exchange with the host. A conserved type IV secretion system (T4SS), a syringe-like protein complex used to secrete proteins across the bacterial cell periplasm and outer membrane, and putative effectors may aid *Wolbachia* in its molecular interactions with the

host cytoplasm (Foster *et al.*, 2005; Li and Carlow, 2012; Carpinone *et al.*, 2018). Although many lines of evidence point to a role for *Wolbachia* in *B. malayi* and other filarial nematodes as obligate mutualists, the situation is more complicated than it first appears. A broad survey of *Wolbachia* across filarial nematodes revealed that not all filarial nematodes have *Wolbachia* endosymbionts, and there have been multiple acquisition and loss events across these species, suggesting that the filarial parasitic lifestyle does not require these endosymbionts (Ferri *et al.*, 2011). This includes early branching filarial species, leaving open the possibility that symbiosis with *Wolbachia* may not have been the ancestral state (Ferri *et al.*, 2011; Bouchery *et al.*, 2013). However, it appears that, in *B. malayi* at least, there has been a significant amount of lateral gene transfer between symbiont and host, and that there are over 200 wBm genes in the host genome, some of which exhibit stage-specific transcription (Ioannidis *et al.*, 2013). Further studies will reveal the significance of these transfers and whether filarial species that have lost *Wolbachia* endosymbionts still contain nuclear signatures of past symbioses.

Wolbachia endosymbionts as a therapeutic target

Antifilarial drugs that target nematodes, such as ivermectin and albendazole, reduce microfilarial load but do not eliminate nematode parasites in humans. The filarial nematode *Wolbachia* symbionts are an attractive alternative drug target because, in infected nematode species, all members have *Wolbachia* endosymbionts, and “curing” nematodes of endosymbionts with antibiotic treatment results in lethality and reduced reproductive capacity (Slatko *et al.*, 2010). *Wolbachia* may also contribute to the pathology of onchocerciasis and filariasis, as the human immune system responds more strongly to nematodes that house *Wolbachia*, and there is evidence that *Wolbachia* manipulates the immune response to benefit filarial nematodes (Bouchery *et al.*, 2013; Wan Sulaiman *et al.*, 2019). The antibiotic tetracycline causes extensive apoptosis in the nematode germline, embryos, and larvae, primarily in cells that were previously colonized by *Wolbachia* endosymbionts (Landmann *et al.*, 2011). Several other antibiotics

have been tested as anti-*Wolbachia* therapies, with doxycycline being the first in human clinical trials (Slatko *et al.*, 2010). More recently, focus is shifting from broad-spectrum antibiotics to “anti-*Wolbachia*” drugs that more specifically target the endosymbiont (Taylor *et al.*, 2005; Lentz *et al.*, 2013; Chung *et al.*, 2019; Hong *et al.*, 2019). Increased understanding of filarial nematode–*Wolbachia* symbioses will further inform these targeted efforts.

Advantages of filarial nematodes as a model system for symbiosis research

Current evidence suggests that filarial nematodes and their *Wolbachia* endosymbionts have formed an additive symbiosis, where the filarial nematodes are dependent on their *Wolbachia* endosymbionts for development, reproduction, and survival, instead of actively maintaining the endosymbionts because of benefits (nutrition, defense, etc.) that they provide (Douglas, 2010; Sullivan, 2017). Therefore, although *Wolbachia* endosymbionts of filarial nematodes are commonly referred to as obligate “mutualists”, they might more accurately be thought of as reproductive parasites to which their hosts have become addicted, an idea consistent with the finding that many closely related filarial species with similar lifestyles and diets do not contain *Wolbachia* endosymbionts.

Compared with free-living nematodes and those that can be raised outside of host organisms, filarial nematodes and their *Wolbachia* endosymbionts have a complex life cycle that involves an arthropod vector. *B. malayi* can be raised in the laboratory by passing through *Ochlerotatus togoi* mosquitoes, where they develop until the L3 larval stage (Saeung and Choochote, 2013). L3 larvae are then inoculated into male jirds (gerbils) intraperitoneally by injection where, after a minimum of 3 months, microfilariae and adults can be harvested from infected animals (Weil *et al.*, 1992; Saeung and Choochote, 2013). Over the past decade, genetic approaches for *B. malayi* have advanced greatly. RNA interference (RNAi) was achieved by exposing nematodes to interfering RNAs in mosquitoes (Song *et al.*, 2010), and transiently transgenic animals were created through microinjection, bombardment, or soaking

(Lok and Unnasch, 2018). Recently, stable introduction of transgenes was achieved using the *piggyBAC* transposase system, lipofectamine, and *ex vivo* incubation of the L3 larval stage to insert transgenes stably into the *B. malayi* genome (Liu *et al.*, 2018). The ability through transgenesis to create a luciferase-expressing *B. malayi* facilitated a further advance of *in vivo* imaging of the infection cycle in rodents (Liu *et al.*, 2020a). Similarly, CRISPR-mediated genetic modifications have shown some success, though the process needs improvement through the development of more efficient screening of modified F1s (Liu *et al.*, 2020b). Taken together, the genetic and genomic toolkit for investigating the symbiosis between *B. malayi* and *Wolbachia* is robust and should catalyze exciting advances in understanding the cell biology of intracellular endosymbionts and identification of novel therapeutics to fight neglected filarial parasitic tropical diseases.

Plant-parasitic Nematodes (PPNs)

Plant parasitism is thought to have evolved three times within the Nematoda phylum (Blaxter and Koutsovoulos, 2015), and PPNs are a diverse group of nematode species defined by their ability to infect plant tissues. A handful of significant agricultural pest nematodes have been found to harbor intracellular endosymbionts. It is tempting to speculate that endosymbionts in PPNs, especially those that feed on nutritionally limited tissues such as phloem, might play beneficial roles in nutrient supplementation similar to the endosymbionts of sap-feeding insects (Singh *et al.*, 2019). However, evidence to support this hypothesis is still emerging, and the presence of endosymbionts in PPNs is rare and varies even among closely related nematodes with similar diets and lifestyles. PPNs with co-occurring *Cardinium* and *Wolbachia* endosymbionts provide opportunities to model inter-endosymbiont interactions in host cells, and at the genomic and evolutionary levels.

Cardinium and *Wolbachia* endosymbionts of PPNs

Cardinium (*Cardinium hertigii*) is an endosymbiont of multiple cyst-nematode species (Tylenchida:

Heteroderidae) which are distributed across the globe (for example: *Heterodera glycines*, soybean cyst nematode; *Globodera rostochiensis*, potato cyst nematode; *Pratylenchus penetrans*, root lesion nematode) (Noel and Atibalentja, 2006; Duron *et al.*, 2008; Brown, 2018). Similar to *Wolbachia* endosymbionts, *Cardinium* (Bacteroidetes) is also found in arthropods, where it can impact reproductive modes and change sex ratios by inducing parthenogenesis, cytoplasmic incompatibility, and feminization (Weeks *et al.*, 2001; Zchori-Fein *et al.*, 2001; Gotoh *et al.*, 2007). The genome sequence of *Cardinium hertigii* from a parasitic wasp (cEper) revealed that, similar to many obligate endosymbionts, *Cardinium* has a reduced genome lacking many major anabolic and metabolic pathways, but encoding a large number of transporters, suggesting it relies heavily on the host cytoplasm for energy and nutrients (Penz *et al.*, 2012). *Cardinium*-strain genomes from both arthropods and nematodes contain suites of genes predicted to encode effectors that manipulate host cell physiology (Penz *et al.*, 2012; Santos-Garcia *et al.*, 2014; Brown *et al.*, 2018). Although the mechanisms of arthropod cytoplasmic incompatibility in *Wolbachia* and *Cardinium* are still being defined, genome comparisons of the two endosymbionts suggest that cytoplasmic incompatibility arose independently in *Cardinium* (Penz *et al.*, 2012).

Much less is known about *Cardinium* in PPN hosts than in arthropods. Early studies detailed the ultrastructure and distribution of *Cardinium*, referred to as a “rickettsia-like organism” in *H. glycines*, *Heterodera goettingiana*, and *G. rostochiensis* (Shepherd *et al.*, 1973; Endo, 1979; Walsh *et al.*, 1983; Noel and Atibalentja, 2006). A common ultrastructural feature of many *Cardinium* cells observed by TEM is microfilament-like structures that can span the length of the cells (Endo, 1979; Walsh *et al.*, 1983; Noel and Atibalentja, 2006). *Cardinium* is distributed throughout the nematode, including in the body wall muscle, pseudocoelom, intestine, esophageal glands, the hypodermis of males and juveniles, the ovaries and oocytes (Shepherd *et al.*, 1973; Noel and Atibalentja, 2006), and testis and spermatozoa (Endo, 1979; Walsh *et al.*, 1983; Noel and Atibalentja, 2006). The presence of *Cardinium* in the germline is consistent with its vertical transmission between generations. In nematode esophageal glands, *Cardinium* cells

were associated with the nucleus and endoplasmic reticulum (Endo, 1979), raising the idea that *Cardinium* endosymbionts might aid in parasitism of plant tissues by the secretion of bacterial products (virulence factors, immune evasion factors, cellulase, etc.) through the nematode esophageal glands and stylet. Despite the prevalence in the populations observed to harbor them, and the wide tissue distribution of *Cardinium* within the body of PPN individuals, a role in host physiology has not been identified, and it is not clear where on the range of symbioses *Cardinium* endosymbionts fall: mutualist, commensal, or pathogenic.

In *Pratylenchus penetrans* (Tylenchida), a root-lesion nematode, *Cardinium* and *Wolbachia* endosymbionts co-occur, with *Cardinium* showing high prevalence and *Wolbachia* showing low and variable prevalence (Denver *et al.*, 2016; Wasala *et al.*, 2019). Similar to earlier reports of *Cardinium* distribution in other PPNs by TEM, FISH revealed endosymbionts distributed throughout the *P. penetrans* nematode with a concentration in the esophagus, intestine, and ovaries (Brown *et al.*, 2018). *Cardinium* from *P. penetrans* (cPpe) is an early branching relative of the arthropod *Cardinium* endosymbionts, with both having similarly reduced metabolic capacity based on genomic analyses (Brown *et al.*, 2018). Both a common ancestor (*Amoebophilus*) and cPpe encode a single syntenic secretion system. The anti-feeding prophage-like protein secretion system is distantly related to type VI secretion systems (T6SS) and has been suggested to be responsible for the microfilament-like structures characteristic of *Cardinium* and other anti-feeding prophage-containing organisms (Sarris *et al.*, 2014; Penz *et al.*, 2012; Brown *et al.*, 2018). Comparing the genomes of two co-infecting symbionts, wPpe and cPpe, revealed evidence of horizontal gene transfer, but no evidence of reciprocal metabolic compensation, where each symbiont retains only a partial complement of the metabolic genes it requires, a common feature of some co-occurring endosymbioses (Brown *et al.*, 2018). It is possible that this is an evolving symbiosis, where one symbiont is in the process of being replaced by another. Another possibility is that one symbiont is a hitchhiker pathogen, while the other is more mutualistic, though this is not obvious from the genomic analyses (Brown *et al.*, 2018). Future studies of

the frequency of co-occurrence in sampled populations and the impacts of each symbiont on host fitness are likely to shed light on these issues.

Genome sequencing of the *P. penetrans* *Wolbachia* strain (wPpe) revealed both similarities to and differences from other *Wolbachia* strains. Comparative analysis of wPpe and other *Wolbachia* strains uncovered strong conservation of iron and heme metabolism pathways, but numerous differences: over 200 wPpe predicted proteins are absent in other *Wolbachia* genomes; there are key differences in anabolic pathways (lack of complete riboflavin and biotin synthesis pathways in wPpe); and in wPpe there was a lack of known toxin-antidote systems involved in cytoplasmic incompatibility in other organisms (Brown *et al.*, 2016). Despite the apparent absence of conserved toxin-antidote systems, recent observations suggest that wPpe-mediated feminization may occur in *P. penetrans*. wPpe infection was found variably across *P. penetrans* populations (Wasala *et al.*, 2019). While uninfected populations commonly had a sex ratio of 1:1 (male:female), there was a correlation between wPpe *Wolbachia* infection and female-skewed sex ratios suggesting that *P. penetrans* *Wolbachia* may act as reproductive parasite, as it does in arthropods (Wasala *et al.*, 2019). This presents an interesting opportunity to understand potentially new mechanisms of feminization in a nematode model and an opportunity for comparison with arthropod *Wolbachia*.

Wolbachia has also been found in the PPNs *Radopholus similis* and *Radopholus arabocoffeae* (Tylenchida), which are burrowing nematodes (Haegeman *et al.*, 2009). Phylogenetic analysis positioned the *Wolbachia* from *R. similis* (wRad) at the base of the *Wolbachia* clade with high confidence. Subsequent analyses, which included the wPpe strain, confirmed that the PPN *Wolbachia*, wRad and wPpe, form a monophyletic group (supergroup L) that is a sister to all other known *Wolbachia* supergroups (Brown *et al.*, 2016; Scholz *et al.*, 2020). These results suggest that PPNs may have been the first *Wolbachia* hosts (Haegeman *et al.*, 2009; Brown *et al.*, 2016). Although the role of *Wolbachia* in PPN biology is currently unknown, the symbiosis may be facultative on the nematode side, since the majority of PPNs lack *Wolbachia* endosymbionts, and they are found variably in *P. penetrans*

populations. It has been suggested that ancestral PPN *Wolbachia* endosymbionts played a mutualist role in facilitating plant-specialist lifestyles through nutrient provisioning (Brown *et al.*, 2018), with the obligate dependency between nematodes and *Wolbachia* endosymbionts evolving after the PPN and filaria lineages diverged. However, similar to *Wolbachia* from *B. malayi*, evidence of mutualism is thin. In the future, it will be interesting to further uncover the roles of both *Cardinium* and *Wolbachia* endosymbionts in PPN biology and to explore their potential as targets for biocontrol of PPNs in agricultural settings.

***Xiphinematobacter* endosymbionts**

The PPN family *Xiphinema* spp., known as dagger nematodes, cause crop damage as vectors of viruses and are hosts of the *Candidatus Xiphinematobacter* (Verrucomicrobia family) endosymbionts (Brown, 2018). TEM and fluorescence microscopy of DAPI-stained *Xiphinema brevicollum* nematodes reveal *Xiphinematobacter* symbionts at high concentration in nematode ovarian walls, oocytes, and the anterior intestine (Vandekerckhove *et al.*, 2000; Coomans *et al.*, 2000). In mature oocytes, *Xiphinematobacter* are concentrated at one pole of the embryo, possibly in the endodermal (intestinal) precursor cell, the only tissue where juveniles are found to be colonized (Vandekerckhove *et al.*, 2000; Coomans *et al.*, 2000). This is reminiscent of *Wolbachia* enriching hypodermal cell lineage precursor in the early embryo of *B. malayi* (Landmann *et al.*, 2010). In males, which are very rare in most *Xiphinema* populations, *Xiphinematobacter* symbionts are absent from the testis (Vandekerckhove *et al.*, 2002). Although rare males could be a sign of feminization activities by *Xiphinematobacter* symbionts, it could also simply be a feature of the nematodes' normal reproductive and sex-specification mode. For example, many hermaphroditic species, such as *C. elegans*, have rare males.

The 100% prevalence, intracellular and female germline localization of these symbionts suggests that they are vertically transmitted obligate endosymbionts. *Xiphinematobacter* has a reduced genome size compared with free-living relatives, consistent with an obligate endosymbiont lifestyle (Brown *et al.*, 2015). *Xiphinematobacter*

displays an enrichment of diverse biosynthetic pathways, including those for essential amino acids and heme (Brown *et al.*, 2015). These and other essential vitamins and cofactors produced by *Xiphinematobacter* suggest that it could act as a mutualist, helping to supplement the diet of *Xiphinema* spp. nematodes that use a stylet to penetrate root tips and likely feed on nutrient-poor plant phloem (Brown *et al.*, 2015). Extensive co-evolution between *Xiphinema* spp. and their *Xiphinematobacter* further supports the idea that these endosymbionts are vertically inherited obligate mutualists (Howe *et al.*, 2019). A separate but related clade of *Xiphinema* spp. nematodes harbor a different endosymbiont in the Burkholderiaceae family which shows a similar pattern of distribution within *Xiphinema* nematodes and is related to known fungal and plant endosymbionts (Palomares-Rius *et al.*, 2016; Mobasser *et al.*, 2019). It will be interesting to see how its function compares with other PPN endosymbionts, including *Xiphinematobacter*.

Advantages of PPNs as a model system for symbiosis research

PPNs are emerging as nematode model systems of diverse intracellular endosymbioses. The different types of bacterial endosymbionts, and the co-occurrence of endosymbionts across nematodes with a broadly similar lifestyle, present opportunities to compare these interactions and to ask how they might arise and what their purposes might be. Although it may currently be difficult to manipulate the symbionts because they are intracellular, the nematodes are another story. Most can be raised in laboratory greenhouses on their target plant species. There are multiple reports of successful RNAi both *in vitro* (Chen *et al.*, 2005; Cui *et al.*, 2018; Urwin *et al.*, 2002; Li *et al.*, 2015; Roderick *et al.*, 2018) and *in planta* (Blyuss *et al.*, 2019; Vieira *et al.*, 2015; Li *et al.*, 2017) across multiple nematode taxa. Caution is warranted when interpreting the results of *in planta* experiments, where dsRNAs are likely expressed at extremely high levels in plant tissues. Nonetheless, these approaches enable reverse genetics through targeted gene knockdown. Some of these PPNs are also known vectors of plant viruses, which is how they do

the most damage to crops (Ruark-Seward *et al.*, 2020). In the future, it will be interesting to investigate whether there are any nematode–symbiont–virus interactions and whether targeting the endosymbionts of PPNs could be an effective pest-control strategy.

Chemosynthetic Intracellular Endosymbionts and Ectosymbionts of Marine Nematodes

Multiple species of marine nematodes form symbiotic partnerships with bacteria that are capable of chemosynthesis (the process of fixing CO₂ in the absence of light by harnessing the energy of reduced inorganic molecules through oxidation). Chemosynthetic bacteria associate with diverse marine invertebrates as ectosymbionts, endosymbionts, and intracellular endosymbionts as nutritional mutualists. These nematodes provide the opportunity to explore these nutritional mutualisms in detail. Marine nematode–chemoautotroph symbioses exist in environments containing reduced compounds, such as near hydrothermal vents and cold seeps and in shallow-water sediments (Dubilier *et al.*, 2008). There are two main varieties of nematodes with chemosynthetic symbionts: (i) nematodes with greatly reduced digestive systems which house intracellular endosymbiotic bacteria; and (ii) nematodes that are surface coated with ectosymbiotic bacteria, which facilitates experimental observation. In spite of their external localization, the high degree of congruence between ectosymbiont and host phylogenies suggests a vertical or mixed (vertical and horizontal) model of symbiont transmission between generations (Zimmermann *et al.*, 2016).

Marine nematodes with intracellular endosymbionts

Astomonema, *Parastomonema*, and *Rhabdothyreus* nematodes carry bacterial endosymbionts inside the cells of a highly reduced intestine. *Astomonema*, the best characterized, lacks a mouth, pharynx or digestive tract (Ott *et al.*, 1982). A dense population of endosymbionts fills the cells of the reduced intestine. Ultrastructural analysis of the

endosymbionts, along with their phylogenetic position, suggests that the symbionts of *Astomonema* are chemoautotrophic sulfur-oxidizers (thiotrophic) (Musat *et al.*, 2007). These endosymbionts are almost certainly obligate mutualists, providing energy to their non-ingesting nematode hosts (Fig. 5.1). The *Astomonema* endosymbiont genome also encodes a predicted APS reductase, a key enzyme in sulfate-reducing and sulfur-oxidizing microorganisms (Musat *et al.*, 2007). The *Astomonema* endosymbionts are closely related to the Gamma 1 sulfur-oxidizing endosymbionts from annelid worms and the ectosymbionts of stilbonematine nematodes, with free-living sulfur-oxidizing bacteria being the next closest relatives. Though they occupy similar ecological niches and have related sulfur-oxidizing bacterial symbionts, *Astomonema* are not close relatives of stilbonematine nematodes (Musat *et al.*, 2007) (see below).

Stilbonematine nematodes with ectosymbionts

Stilbonematinae is another group of free-living marine nematodes that form symbiotic associations with chemosynthetic bacteria. The hallmark of this nematode family is that their associations are ectosymbiotic. Almost all of their external body surface, usually with the exception of the head region, is covered by a dense coat of symbiotic bacteria, which are anchored in the nematode cuticle. Different nematodes are coated by morphologically diverse bacterial taxa, from cocci (e.g., *Catanema*), to rod (e.g., *Laxus*, *Robbea*), and crescent-shaped (e.g., *Eubostrichus parasitiferus*) species. Individual associations appear to be monospecific, with each nematode harboring a single ectosymbiont species (Polz *et al.*, 1992). Ectosymbionts are Gram-negative species that show genus-level specificity with their nematode hosts and, usefully, bacterial cell morphology, cuticular attachment, coat structure (single, multi-layer), and distribution appear to be taxonomically diagnostic features of stilbonematine nematode genera (Scharhauser *et al.*, 2020). Taxonomy, TEM, spectroscopy, measurements of stable carbon isotopes, and experiments with radiolabeled substrates confirmed that the ectosymbionts are sulfur-oxidizers (Ott *et al.*, 1991; Polz *et al.*, 1992; Himmel *et al.*, 2009; Polz *et al.*,

1994; Petersen *et al.*, 2016; Zimmermann *et al.*, 2016; Paredes *et al.*, 2020). Additionally, omics-based analysis of one ectosymbiont (*Candidatus Thiosymbion oneisti*) from *Laxus oneistus* suggests that these bacteria express genes necessary to fix nitrogen from the environment, in addition to carbon (Petersen *et al.*, 2016; Paredes *et al.*, 2020), which may also be an important function of these symbionts.

Unlike the *Astomonema* and related taxa described previously, stilbonematine nematodes have a fully formed intestinal tract and are thought to feed on their symbiont coats (Ott *et al.*, 1991). This has led to the long-standing hypothesis that these nematodes and their symbionts participate in a nutrition-based farming symbiosis, similar to the obligate specialist EPNs discussed previously. Here, stilbonematine nematodes are thought to provide an ideal growth environment for the symbiont, both as a scaffold for growth and access to the reduced sulfur compounds and oxygen electron acceptors that the symbionts use to fix CO₂, by migrating between sulfidic layers and oxic layers within sulfidic sands (Ott *et al.*, 1991; Polz *et al.*, 1992; Sogin *et al.*, 2020). A new study examining the physiology of *Laxus oneistus* nematodes and their *Candidatus Thiosymbion oneisti* symbionts in low- and high-oxygen environments has greatly expanded our understanding of thiosymbiont metabolism and challenges aspects of what we understood about these nematodes and their ectosymbionts. By incubating *Laxus oneistus* nematodes in low- and high-oxygen conditions, this study demonstrated that sulfur oxidation in *Candidatus Thiosymbion oneisti* occurs in both oxic and anoxic conditions, and that nitrate is likely the primary terminal electron acceptor under anaerobic conditions (Paredes *et al.*, 2020). Symbiont cells divided most in anoxic seawater with sulfide, suggesting that this environment may be preferred, or closer to the ancestral environment of the symbiont, compared with more stressful, oxygen-rich conditions (Paredes *et al.*, 2020). This study, especially the finding that sulfur oxidation occurs regardless of environmental oxygen, throws doubt on the hypothesis that nematode migration through sand layers is necessary for symbiont respiration. It will be interesting for future studies to uncover mechanisms of environmental sensing and communication in both the nematodes and bacterial

symbionts that allow them to work collaboratively to survive and thrive in the extreme conditions of their ecological niche.

The molecular and cellular basis of ectosymbiosis

The unique morphological constraints of ectosymbioses, in which bacterial cells are anchored at one end (or sometimes both ends) to the nematode cuticle, provide a unique opportunity to understand how fundamental aspects of bacterial cell biology might adapt to facilitate symbiosis with an animal host. Although the recruitment and transmission mechanisms of stilbonematine symbionts are unknown, it was hypothesized that mucus secreted by the robust system of glandular sensory organs in the nematode might be important for recruiting symbionts and/or anchoring them on the cuticle surface. Incubation in D-mannose caused symbiont detachment in *L. oneistus*, while Ca^{2+} was necessary for cuticular attachment (Nussbaumer *et al.*, 2004; Bulgheresi *et al.*, 2006), suggesting that a Ca^{2+} dependent, mannose-binding protein or lectin might be important for endosymbiont attachment to the nematode surface, and indeed, the identified C-type lectin, Mermaid, induced *L. oneistus* symbiont aggregation and was able to compete with the native nematode cuticle for symbiont binding, causing dissociation from the host (Bulgheresi *et al.*, 2006). Furthermore, marine nematodes that occur in the same environment but that have phylogenetically distant ectosymbionts express different Mermaid isoforms with specific affinity for their cognate symbiont, suggesting that these lectins could mediate host–symbiont specificity (Bulgheresi *et al.*, 2011). Such a lectin–carbohydrate recognition and selection strategy may be common among animal hosts and their beneficial bacterial symbionts and is directly relevant to understanding human mucosa-associated microbial communities (Hooper and Gordon, 2001; Chaston and Goodrich-Blair, 2010).

Rod-shaped bacteria, such as *E. coli*, typically divide by growing in length and forming a division ring perpendicular to this long axis. The rod-shaped symbionts of *L. oneistus* appear to divide parallel to their long axis, as observed in

scanning electron micrographs (Polz *et al.*, 1994). This long-axis division strategy might provide the advantage of allowing both daughter cells to stay attached to the host, as opposed to the *E. coli*-like short-axis division, which would result in an unattached, distal symbiont daughter cell that would be released from the nematode surface. However, it was not at all clear how this remarkable division strategy is achieved. Careful measurements of thousands of fixed and immunostained dividing cells revealed that cells first grow in width and are then bifurcated along their long axis by a symmetrical FtsZ protein ring (Leisch *et al.*, 2012). This mechanism is remarkably similar to what occurs during *E. coli* fission, except perpendicular to the familiar strategy. In *Robbea hypermnestra* symbionts, FtsZ localizes medially, but the two poles do not constrict simultaneously. Instead, a proximal FtsZ arc appears to mediate constriction of the proximal anchored pole, whereas a subsequent distal FtsZ arc mediates the constriction of the distal free pole (Leisch *et al.*, 2016). Therefore, an FtsZ ring can only be observed at the final stage of septation, after the proximal FtsZ arc meets the distal one. *R. hypermnestra* symbiont cell polarity was confirmed at the molecular level using an antibody which labeled fimbriae at the host attachment site on one end of the cells (Leisch *et al.*, 2016). Subsequent observation of medial peptidoglycan incorporation at the poles and MreB localization along the long axis of the cell, preceding FtsZ ring localization, provide further contrasts to the *E. coli* model of cell division (Pende *et al.*, 2018). Whether this new mode of cell division is ancestral or adaptive to a symbiotic lifestyle could only be resolved by analyzing the division of a free-living relative, of which none are currently known.

Advantages and future directions of marine nematode–thiosymbiont interactions as a model system for symbiosis research

Although marine nematode–thiosymbiosis is a very specialized interaction between nematodes living in extreme environments and sulfur-oxidizing bacteria, this animal–microbe pair is an excellent model of general principles of symbiosis. In their surface-exposed location, the ectosymbionts are continuously exposed to a harsh environment

and must be newly acquired or maintained between generations and molting events. *L. oneistus* nematodes use glycan recognition to recruit and retain specific symbionts, a potentially common mechanism of symbiont recognition, with parallels to many other systems (Bulgheresi *et al.*, 2006, 2011). Investigations into thio-ectosymbiont cell division revealed that host factors can polarize bacterial cell division, and that the canonical cell division mechanisms we know about from model bacteria such as *E. coli* have been modified to integrate host cues (Leisch *et al.*, 2012, 2016). These studies reveal that conserved molecular and cell biological processes likely facilitate many aspects of animal-microbe symbioses, though they may be modified as host and symbiont coevolve. Because of the unique mode of bacteria energy production and the need for reduced sulfur compounds, understanding how the energy-producing microbes and mobile nematodes sense nutritional status, communicate, and respond appropriately is an interesting avenue of future exploration. Similar marine nematode symbioses with diverse bacteria are still being discovered (Bellec *et al.*, 2019). These fundamental insights into the emergent mechanisms that facilitate this symbiosis have been gleaned despite the fact that these nematodes and their symbionts currently cannot be cultured in a laboratory setting. This field is a prime example of what can be learned by studying nematode-microbe symbioses using available tools, cutting-edge microscopy, and -omics approaches.

Conclusions

In this chapter, we have reviewed what is currently known about several major nematode models of animal-microbe symbiosis. Each nematode-microbe pair has unique features that make it a favorable model for exploring different aspects of symbiosis. There is still much to learn about the functions of the symbionts we have characterized

in this chapter. Some are clearly mutualistic and provide nutritional benefits to their hosts (EPNs and thiosymbionts). Others could be mutualistic and/or pathogenic, and may at times provide significant nutritional benefits to supplement the diet of their host (*C. elegans*-associated microbiome, *Wolbachia*, *Cardinium*, *Xiphinematobacter*). Many of the intracellular endosymbionts (*Wolbachia*, *Cardinium*, *Burkholderia*, *Symbiopectobacterium*) of nematodes we described appear to be “symbiotic professionals” whose close relatives colonize multiple different organisms (for example, many different arthropods, nematodes, plants, etc.). This may suggest a current or former parasitic lifestyle. Only one of the nematode models described in this review (*C. elegans*) harbors an environmentally derived, symbiotic microbial community. However, many other nematode species acquire commensal microbes from their habitat. Some such communities have begun to be characterized (Dirksen *et al.*, 2016; Meyer *et al.*, 2017; Elhady *et al.*, 2017), and we expect an expansion in the number of nematodes for which community membership has been defined. This will provide ample opportunity for comparisons of mechanisms of recruitment and management of symbionts and inter-symbiont competition and cooperation, as well as the functions of symbiont communities in nutrition and immunity, among other processes. Although simple, nematodes are an extremely nutritionally and ecologically diverse phylum, and as models of symbiosis they are equally manifold. There is no doubt that useful discoveries will continue to be made by studying animal-bacterial symbioses through the lens of these tiny, but mighty, roundworms.

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6 Nematode Pharmacology: Neurotransmitters, Receptors, and Experimental Approaches

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Introduction

Pharmacology is the study of the effects of chemical compounds and natural toxins on organisms. It is closely aligned to clinical medicine, and the compounds and toxins are often utilized as therapeutic agents, i.e., drugs. There are two key and interrelated aspects to pharmacology: pharmacokinetics and pharmacodynamics. Pharmacokinetics seeks to model the processes that determine the concentration of the drug at its site of action after administration, whilst pharmacodynamics is the study of the receptors through which it exerts its biological effects. The discipline gained significant traction around the turn of the 20th century with the discovery of the first neurotransmitter, acetylcholine (ACh), and the emerging concept of receptors (Cuthbert, 2006). The application of this pharmacological approach to nematodes (that is, determining the effects of drugs on their tissues, behavior, and viability) has been of increasing interest since then. The earliest studies were performed on the large intestinal parasite *Ascaris* sp. (Baldwin and Moyle, 1949) with the microscopic free-living nematode *Caenorhabditis elegans* coming on to the scene at a much later date. The first studies reported *C. elegans* mutants that

were resistant to the paralytic effects of tetramisole (Brenner, 1974). Since then, exploring the molecular basis of drug effects using the genetic tractability of *C. elegans* has delivered important new insight into intra- and intercellular signaling cascades that are conserved with other animals, including humans, which has contributed to the broad understanding of pharmacological mechanisms. Importantly, these studies have also contributed to understanding the effects of drugs and chemicals that are used to treat parasitic nematode infections of animals and plants.

Main Pharmacological Models: Advantages and Limitations

Much of our understanding of nematode pharmacology is extrapolated from studies on either *Ascaris* spp. or *C. elegans* for the very pragmatic reason that they present the tractable experimental models. Although *C. elegans* and *Ascaris suum* diverged some 500 million years ago, their nervous systems are remarkably conserved (Jorgensen, 2005). These studies have contributed to our understanding of the pharmacological actions of drugs that are used

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to treat parasitic worm infections, i.e., anthelmintics. They have also provided insight into fundamental pharmacological properties of organismal signaling. However, the latter has shown that, whilst nematodes use many of the same neurotransmitters as mammals, and that the molecular characteristics of the receptors are conserved, their discrete pharmacological properties differ, since the nematode receptors are often refractory to definitive mammalian receptor antagonists and modulators (Geary *et al.*, 1992a).

Ascaris lumbricoides is an important human parasite and infects more than 1.2 billion humans, resulting in nutritional problems leading to morbidity and in some cases death. *A. suum* infects pigs and reduces their growth and productivity. Since *A. lumbricoides* and *A. suum* are genetically closely related, the latter provides an excellent model for the study of the former. The *A. suum* draft genome contains over 600 receptors, around 1800 channels, 600 kinases, and 450 peptidases (Jex *et al.*, 2011, 2013). In their chapter on the neurobiology of *Ascaris* in the book *Ascaris: The Neglected Parasite*, Stretton and Maule (2013) listed a number of disadvantages in studying *Ascaris*: (i) due to the success of anthelmintics in pigs, *A. suum* is increasingly hard to find; (ii) it is difficult to maintain for more than a few days in the laboratory in good condition; (iii) it does not provide a tractable genetic system; (iv) it has a bad smell; and (v) it exudes a powerful allergen that can result in asthma. However, they considered the property that negates all these disadvantages is its size, up to 35 cm long in adult females. This means that strips of body wall muscle can be suspended in an organ bath for *in vitro* application of drugs and concentration-response data can be obtained. Electrophysiological recordings from *A. suum* neurons and neuronal commissures have been made using sharp microelectrodes (Cowden *et al.*, 1989; Holden-Dye and Walker, 1994).

While *C. elegans*, pioneered by Brenner (1974), is much smaller (around 1 mm long) with small neurons (around 5 μm), it does have considerable advantages as a laboratory animal over *A. suum*: it is easy to maintain in the laboratory, with a life cycle of about 3 days, and is genetically tractable. However, it is not a parasite and lacks adaptations acquired by parasitic nematodes, and it may have gene products that

may function differently or be absent in parasitic nematodes. In spite of these points, Burns *et al.* (2015) considered *C. elegans* an excellent model for high-throughput screening studies for anthelmintic and nematocidal compounds, and it has also been used for drug discovery for human medicine (see section “*C. elegans* and Drug Discovery”, below). There is a high degree of conservation between the structure of the somatic body wall musculature and nervous system of *C. elegans* and *A. suum*, which makes it possible to extrapolate from studies using both *A. suum* and *C. elegans* to parasitic nematodes that are often less easy to obtain, maintain, and study. Each of the two models has its advantages and these similarities mean that studies which are easier to do in one species complement those done in the other species.

Initial pharmacological studies were undertaken using the body wall somatic muscle system of *Ascaris*, since many of the anthelmintics act at this site to affect locomotion (Geary *et al.*, 1992a), resulting in paralysis and inability to survive in the host (Martin, 1997). Early intracellular recordings from the somatic muscle cells of *A. lumbricoides* were performed by Jarman (1959) and Debell *et al.* (1963). More recently two electrode current-clamp and two electrode voltage-clamp recordings have been made from *A. suum* somatic muscle cells and reviewed by Robertson *et al.* (2008), while cell patch-clamp recordings have been made from somatic muscle cells of *C. elegans* (Richmond and Jorgensen, 1999). Extracellular, intracellular, whole-cell, and single-channel patch-clamp recordings can be made from pharyngeal muscle of *C. elegans* (Cook *et al.*, 2006), while recordings can be made from *A. suum* pharyngeal muscle using a two microelectrode current-clamp system (Martin, 1996). Microfluidic techniques have been developed to record pharyngeal pumping (electropharyngeograms) (EPGs) from *C. elegans* (C. Hu *et al.*, 2013), from *A. suum* L3 larvae, and from *Ancylostoma ceylanicum* L3 larvae (Weeks *et al.*, 2016), and stylet thrusting in the plant nematode *Globodera pallida* (Hu *et al.*, 2014). Automated, high-throughput analysis of nematode motility has been reviewed by Buckingham *et al.* (2014).

A problem in studying *in vivo* pharmacology in nematodes is gaining entry into the animal. The cuticle restricts the entry of compounds,

particularly if they are charged. This impacts on the kinetics of the drug effect on the nematode. Indeed, insight into whether or not a compound is likely to be equilibrating rapidly with the internal milieu of the nematode may be obtained through a careful analysis of the rate of onset and offset of its effect on *C. elegans* behavior. Such a study provided evidence that ethanol rapidly achieves concentrations inside the nematode that equate to those in the external environment (Mitchell *et al.*, 2007), an important consideration for a nematode whose native habitat is rotting vegetation (Kiontke *et al.*, 2011).

Compounds can also be drawn into the mouth through pumping movements of the pharynx. However, once inside the nematode, the rate at which the concentration of the compound increases depends on the rate it is metabolized and this may vary between different species. This is suggested from the comparative *in vitro* and *in vivo* studies of Y. Hu *et al.* (2013), where the clinical value of a drug cannot always be predicted by *in vitro* screening and should include *in vivo* pharmacokinetics/metabolic studies. From their comparative studies these authors found considerable variation between the sensitivity of the nematodes investigated to the drugs tested. Thus, care must be taken from extrapolating data obtained from one species to another. It also may be difficult for a compound to increase to a concentration at which it can interact with receptors on neurons or muscle cells. One solution in the case of *C. elegans* is to cut approximately two-thirds from the anterior end and allow compounds to enter the body cavity directly (Ruiz-Lancheros *et al.*, 2011). For example, the nAChR antagonist, derquantel, was weakly active in intact *C. elegans* but had clear effects on motility in cut animals. These authors considered that the permeability properties of *C. elegans* cuticle are more restrictive than those of the cuticles of parasitic nematodes, which raises doubts on its use as a primary anthelmintic screen. For studies involving the effect of compounds on pharyngeal pumping, a cut head preparation can be used, allowing direct drug access to the pharyngeal nervous system and the pharyngeal muscle cells (Rogers *et al.*, 2001). Another approach is to expose *C. elegans* to a hypotonic environment with agar alone in the absence of nematode growth medium when lower concentrations of compound

are required to induce an effect (Law *et al.*, 2015). Alternatively, *C. elegans* *bus-8* mutants can be used in which the epidermis and cuticle layers are disorganized, which allows enhanced drug sensitivity, a property that can be exploited in drug screening (Partridge *et al.*, 2008).

In addition to studies on *C. elegans* and *A. suum*, other nematodes have been the subject of investigation to discern the molecular pharmacology of nematode signaling, particularly in parasitic nematodes of clinical or economic importance, for example *Haemonchus contortus*, *Oesophagostomum dentatum*, and *Brugia malayi*. The pharmacological insights gleaned from these investigations are described in the following section.

Overview of Neurotransmitter Receptor Systems and Intracellular Signaling Cascades

Many of the chemical neurotransmitters act throughout all or the majority of the animal phyla, including nematodes (Walker and Holden-Dye, 1991; Walker *et al.*, 1996). The following chemicals are used by nematodes as transmitters: acetylcholine (ACh), 5-hydroxytryptamine (5-HT, serotonin), dopamine (3-hydroxytyramine), octopamine (p-hydroxy-phenylethanolamine), tyramine (4-hydroxyphenethylamine), glutamate, and γ -n- amino butyric acid (GABA). A summary of the pre- and postsynaptic components for each transmitter is shown in Fig. 6.1. Among the other chemical transmitters (the biogenic amines, adrenaline, noradrenaline) there is little or no evidence that they function as transmitters in nematodes. *C. elegans* does not contain any obvious orthologs for glycine- or histamine-gated ion channels or for ionotropic or metabotropic purine receptors (Hobert, 2013). Each transmitter will be reviewed for its occurrence and the pharmacology of its receptor subtypes.

Acetylcholine (ACh)

ACh plays key roles in the physiology of body wall somatic muscle (Lewis *et al.*, 1980; Johnson and Stretton, 1985; Lewis *et al.*, 1987), pharyngeal muscle (McKay *et al.*, 2004), central ganglia

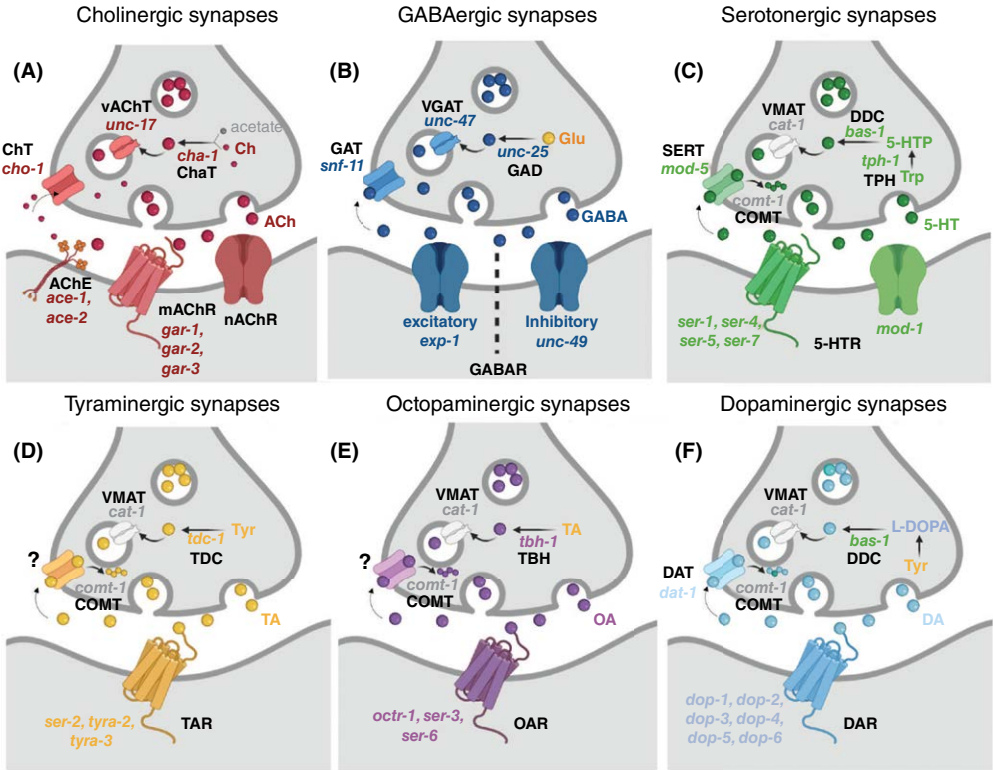


Fig. 6.1. Core molecular components of nematode neurotransmitter synapses. **(A)** Cholinergic synapses. **(B)** GABAergic synapses. **(C)** Serotonergic synapses. **(D)** Tyraminergetic synapses. **(E)** Octopaminergic synapses. **(F)** Dopaminergic synapses.

(Alfonso *et al.*, 1993), and pharyngeal nervous system (Raizen *et al.*, 1995) of nematodes, where it acts at both nicotinic (nAChR) and muscarinic (mAChR) receptors. At nAChR sites ACh acts via pentameric Cys-loop ligand-gated ion channels (CLGICs). The subunits each comprise four transmembrane domains, and α -subunits are defined as those that have two adjacent cysteines (connected by a strained disulfide bond) in the ACh binding site, while those subunits without this motif are termed non- α -subunits (Holden-Dye *et al.*, 2013). The proposed stoichiometry of some nAChRs is shown in Fig. 6.2. The nAChR consists of at least two α -subunits, while the ACh binding site involves both an α -subunit and an adjacent subunit. A remarkable exception to this appears to be the nAChR expressed in the pharyngeal muscle of *C. elegans*, EAT-2, which can form a functional receptor with subunits that lack the vicinal cysteines (Choudhary *et al.*, 2020).

In nematodes, the number of nAChR subunits varies between species, with *C. elegans* having at least 32, including 22 α -subunits. This is considerably more than in many parasitic nematodes and in general parasitic nematodes tend to have smaller genomes (Williamson *et al.*, 2007). However, two subunits, ACR-26 and ACR-27, are present in *Haemonchus contortus* but absent from *C. elegans* (Laing *et al.*, 2013). Interestingly, ACR-26 is present in many parasitic nematodes. *C. elegans* nAChR subunits are divided into five groups: DEG-3 (eight members), ACR-16 (11 members), ACR-8 (three members), UNC-38 (three members), and UNC-29 (four members) (Jones and Sattelle, 2008). Some members of the ACR-16 group closely resemble the vertebrate homomer-forming neuronal $\alpha 7$ receptor (Mongan *et al.*, 2002). Four nAChRs have been identified in *C. elegans*: a neuronal receptor, DEG-3/DES-2 (Treinin *et al.*, 1998); a pharyngeal muscle receptor, EAT-2, that requires a small

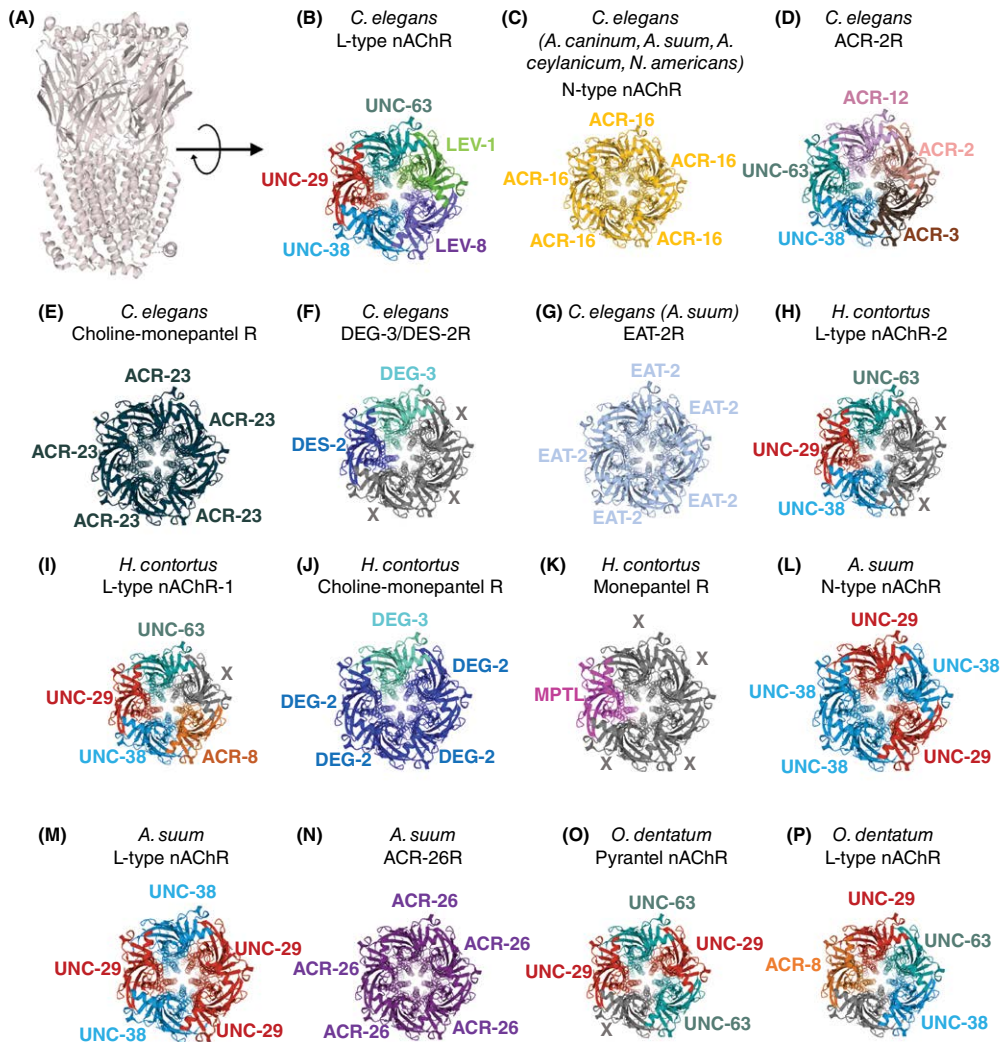


Fig. 6.2. Stoichiometry of nematode nicotinic receptors. **(A)** Front view of a generic structure for nicotinic receptors, with spin arrow indicating top view of all the subtypes. **(B)–(P)** Subtypes. The predicted subunit compositions of each pentameric receptor channel are illustrated.

transmembrane protein, EAT-18, for normal pumping (McKay *et al.*, 2004); and two body wall somatic muscle receptors, one sensitive to levamisole (L) and one sensitive to nicotine (N) (Richmond and Jorgensen, 1999). The L receptor requires both *unc-38* and *unc-29* genes for activity while the N receptor is a homomer of ACR-16. In addition to UNC-38 and UNC-29, the L receptor requires LEV-1 (Fleming *et al.*, 1997), UNC-63 (Culetto *et al.*, 2004), and LEV-8 (also called ACR-13) (Boulin *et al.*, 2008) for full activity. When all five subunit genes were

expressed in *Xenopus laevis* oocytes, the currents were 1000 times greater than those obtained by Fleming *et al.*, (1997). For successful expression of the L-type receptor, three ancillary proteins are also required—RIC-3, UNC-50, and UNC-74 (Boulin *et al.*, 2011)—and under these conditions the receptor responds to ACh and levamisole but not to nicotine. Additional proteins are also involved in L-AChR function in *C. elegans* (for review, see Holden-Dye *et al.*, 2013). Expression of *acr-16* with *ric-3* in *Xenopus* oocytes results in large currents to ACh and nicotine but not to levamisole

(Touroutine *et al.*, 2005). The stoichiometry of the *C. elegans* nAChR is shown in Fig. 6.2B,C and its pharmacology in Table 6.1. Other nAChRs described for *C. elegans* include: DEG-3/DES-2, expressed in neurons (Fig. 6.2F) (Treinin *et al.*, 1998; Yassin *et al.*, 2001); ACR-2, also expressed in neurons (Fig. 6.2D) (Jospin *et al.*, 2009); and ACR-23, a choline-monepantel receptor (Fig. 6.2E) (Kaminsky and Rufener, 2012).

The nAChR of parasitic nematodes has been widely investigated, since many anthelmintics act at this site. Its pharmacology has been studied using dorsal body wall muscle of *A. suum* (Colquhoun *et al.*, 1991). These receptors are insensitive to lophotoxin (Tornøe *et al.*, 1995, 1996) and only weakly blocked by α -bungarotoxin but are sensitive to neuronal bungarotoxin and neosurugatoxin (Walker *et al.*, 1993). Patch-clamp analysis of *A. suum* muscle cells demonstrated the presence of three receptor types: L-type activated by levamisole; N-type activated by nicotine; and B-type activated by buphenium (Robertson *et al.*, 2002; Qian *et al.*, 2006). Levamisole can activate all three receptors but preferentially activates L-type. In the strongylid nematode *Oesophagostomum dentatum*, four levamisole-sensitive channels have been identified, suggesting four different receptors (Robertson *et al.*, 1999). By

comparison with *A. suum*, one of these channels, 35 pS channel, can be identified as the L-type nAChR. A later paper (Buxton *et al.*, 2014) characterized four *O. dentatum* nAChR subunits: the α -subunits Ode-UNC-38, Ode-UNC-63, Ode-ACR-8, and the non- α -subunit Ode-UNC-29i by expression in *X. laevis* oocytes. Injecting different combinations of subunits identified four pharmacologically different nAChRs with different pharmacological properties. For example, the levamisole-sensitive receptor was made up of Ode-UNC-29, Ode-UNC-63, Ode-UNC-38, and Ode-ACR-8 subunits with a single channel conductance of 35 pS (Fig. 6.2P). This receptor combination was the most sensitive to ACh. Ode-UNC-29 and Ode-UNC-63 are essential for a functional receptor which was particularly sensitive to pyrantel (Fig. 6.2O), where X is either UNC-29 or UNC-63. A single channel, 26–28 pS, has been found in *B. malayi* muscle which is activated by levamisole and ACh (Robertson *et al.*, 2011). More recently four different nAChRs have been identified in this nematode (Verma *et al.*, 2017).

The cloning and sequencing of two nAChR subunit cDNAs from *A. suum* that are similar to *C. elegans* UNC-29 and UNC-38 have been described (Williamson *et al.*, 2009). These subunits

Table 6.1. The pharmacological profile of *C. elegans* nAChRs. After Holden-Dye *et al.* (2013).

Drug	L-AChR	N-AChR
Acetylcholine	Agonist	Agonist
Nicotine	Antagonist	Agonist
Levamisole	Agonist ^a	Antagonist
Dihydro- β -erythroidine	Weak antagonist or no effect	Antagonist
d-Tubocurarine	Antagonist	Antagonist
DMPP ^b	Agonist	
Mecamylamine	Antagonist	
Pyrantel	Agonist	
Hexamethonium	Antagonist	Antagonist
Oxantel		Antagonist
Morantel	Agonist	Antagonist
Methyridine		No effect
Butamisole		Antagonist
α -Bungarotoxin	Weak antagonist or no effect	Antagonist, very weak
Methilylcanonitine	Antagonist	Weak antagonist
Meosurugatoxin	Antagonist	
Bipinnatin B	No effect	
Tribendimidine	Agonist	

^aAntagonist at 500 μ M in *Xenopus* oocytes;

^bDMPP 1,1-dimethyl-4-phenylpiperazinium

are expressed in *A. suum* body wall muscle cells. When expressed in *X. laevis* oocytes, a receptor is formed that responds to ACh, levamisole, nicotine, and pyrantel. However, the sensitivity to these compounds depends on the ratio of UNC-29 and UNC-38 subunits composing the receptor. For example, when the ratio was 5:1 in favor of Asu-UNC-38, nicotine was a full agonist and levamisole was a partial agonist. This receptor was sensitive to oxantel but not to pyrantel. When the ratio was reversed, levamisole was a full agonist and nicotine was a partial agonist. In this case the receptor responded to pyrantel but not to oxantel. The authors concluded that there were differences between the nAChRs of *A. suum* and *C. elegans*. Two of their proposed receptor combinations are shown in Fig. 6.2L,M. While many of the nAChR subunits present in *C. elegans* have been identified in *A. suum*, (ACR-17, DEG-3, ACR-21, ACR-8, UNC-38, UNC-63, UNC-29, ACR-15, ACR-16), one, ACR-26, is absent (Bennett *et al.*, 2012). While ACR-26 is found in a number of parasitic nematodes (for example, *B. malayi*, *Dirofilaria immitis*, *Loa loa*, *Wuchereria bancrofti*, *Strongyloides ratti*, *Cooperia oncophora*, *Ostertagia ostertagi*, *Teladorsagia circumcincta*), it is absent in *Trichinella spiralis* and plant nematodes of the genus *Meloidogyne*, and the free-living nematode *Pristionchus pacificus*. The gene *acr-26* is expressed on head muscle cells of *A. suum* but absent from body wall muscle cells. When *X. laevis* oocytes were injected with *Asu-acr-26*, expression was unreliable but when it did express it responded to ACh and nicotine with an EC₅₀ to ACh of 10–100 nM. Interestingly, no functional channels were found when oocytes were injected with *Hco-unc-26* cRNA. Bennett *et al.* (2012) suggested that this receptor might provide a novel target for the development of cholinergic anthelmintics specific for animal parasites. The structure of this receptor is shown in Fig. 6.2N. A homomeric N-type nAChR has been found in *A. suum* which is made up of ACR-16 (Abongwa *et al.*, 2016), similar to that found in *C. elegans* and with a similar pharmacology (Fig. 6.2C). The *A. suum* receptor is present in a number of tissues, including somatic and pharyngeal muscle; it is activated by ACh and nicotine but insensitive to levamisole and pyrantel, and has been proposed as a target for anthelmintics (Zheng *et al.*, 2016). There is also a homomeric

ACh receptor formed from ACR-16 in *Ancylostoma caninum* (Choudhary *et al.*, 2019), *A. ceylanicum*, and *Necator americanus* (Kaji *et al.*, 2020).

Genes orthologous to *unc-29*, *unc-63*, *unc-38*, and *lev-1* of *C. elegans* have been identified in *H. contortus*, *T. circumcincta*, and *Trichostrongylus colubiformis* but not for *lev-8* (Fauvin *et al.*, 2010). However, a truncated variant of *Hco-acr-8*, *Hco-acr-8b*, was found which might play a role as a marker for levamisole resistance (Fauvin *et al.*, 2010). When cRNAs for AChR subunits—*Hco-acr-8*, *Hco-unc-29.1*, *Hco-unc-38*, *Hco-unc-63a* plus *Hco-ric-3.1*, *Hco-unc-50*, and *Hco-unc-74*—were injected into *X. laevis* oocytes, currents were obtained following application of both ACh and levamisole (Boulin *et al.*, 2011). This receptor was named Hco-L-AChR1 and did not require *Hco-lev-1* for activation by levamisole (Fig. 6.2I). When *Hco-acr-8* was removed from the combination, the expression was reduced by 93%; this receptor was named Hco-L-AChR2 and was less sensitive to both ACh and levamisole (Fig. 6.2H). The pharmacology of the two receptors was different, with Hco-L-AChR2 being more sensitive to pyrantel and less sensitive to DMPP compared with Hco-L-AChR1. In addition, the ACh response to the nicotine-sensitive Hco-L-AChR2 was blocked by dihydro- β -erythroidine but not that of the nicotine-insensitive Hco-L-ACh1, while tubocurarine blocked the ACh response of both receptors. This supports the finding in *C. elegans* that the nicotine-insensitive L-AChR is not blocked by dihydro- β -erythroidine but the nicotine-activated N-AChR is blocked. Bephenium selectively activates Hco-L-AChR1, Hco-L-AChR2 being insensitive, indicating that bephenium activation requires ACR-8 (Charvet *et al.*, 2012). LEV-8 appears to be absent in *H. contortus* and some other parasitic nematodes, but it is likely that ACR-8 can functionally substitute for LEV-8 in *H. contortus* (Blanchard *et al.*, 2018). LEV-1 is present in *H. contortus* but does not appear to be required for a functional nAChR. A cholinomonepantel receptor has also been proposed for *H. contortus*, involving DEG-3 and DES-2 subunits and an MPTL-1 receptor (Rufener *et al.*, 2010) (Fig. 6.2J,K).

In an attempt to investigate whether a levamisole-sensitive receptor composed of multiple subunits will express a parasitic nematode subunit in *C. elegans*, *unc-38* and *unc-29* genes

from *H. contortus* and *A. suum* were expressed in *C. elegans* mutants (Sloan *et al.*, 2015). These authors then looked to see whether the parasitic genes could rescue locomotor defects and levamisole resistance in these mutants. The UNC-38 subunit of *H. contortus* completely rescued the locomotor defects and levamisole resistance of the *C. elegans* (Putrenko *et al.*, 2005) mutant but there was no expression of the *A. suum* UNC-38. The *H. contortus* UNC-29.1 subunit partially rescued levamisole resistance in a *C. elegans* unc-29 mutant but failed to rescue the motility defect in a thrashing assay. *C. elegans* mutants expressing the *A. suum* UNC-29 subunit showed partial recovery of levamisole resistance. It is worth noting that *H. contortus* and *C. elegans* are in clade 5 while *A. suum* is in clade 3 (Blaxter *et al.*, 1998), which shows that the evolutionary relationship between nematodes is important when comparing the pharmacological properties of subunits of parasitic nematodes with those of *C. elegans*.

Acetylcholine-sensitive Cys-loop ligand-gated chloride channel (LGCC) subunits have been cloned, expressed, and characterized in *C. elegans* (Putrenko *et al.*, 2005). This type of LGCC is not found in vertebrates (Jones and Sattelle, 2008). Two subunits, ACC-1 and ACC-2, form homomeric channels when expressed in *X. laevis* oocytes which are activated by ACh and arecoline but only very weakly by nicotine. Tubocurarine and strychnine were the most potent antagonists of ACh at ACC-1, followed by atropine, dihydro- β -erythroidine, and hexamethonium. Hexamethonium had both agonist and antagonist activity. α -Bungarotoxin did not block these receptors. These authors also found evidence for two further subunits, ACC-3 and ACC-4. A member of the ACC channel family, LGC-46, is located on presynaptic terminals of cholinergic motoneurons of *C. elegans* and regulates synaptic vesicle-mediated ACh release (Takayanagi-Kiya *et al.*, 2016). In *lgc-46* mutants there is prolonged release of ACh but no change in spontaneous release. The authors suggested that activation of this receptor can modulate over-excitation due to excess release of ACh. ACC-4 is also required for LGC-46 function. There is a total of eight receptor subunit genes in *C. elegans*—*acc-1* to *acc-4* and *lgc-46* to *lgc-49* (Jones and Sattelle, 2008)—while *H. contortus* contains seven of these genes, with the absence of *lgc-48* (Laing

et al., 2013). ACC-1 has been isolated from *H. contortus*, Hco-ACC-1, but failed to form a functional homomeric channel when expressed in *X. laevis* oocytes (Callanan, 2007; Callanan *et al.*, 2018). However, when co-expressed with Hco-ACC-2, it forms a channel which is sensitive to ACh and carbachol. When expressed in *C. elegans*, *Hco-acc-1* only localizes to pharyngeal neurons and enhances reversal avoidance to octanol, showing that it can function *in vivo*. However, in *H. contortus* ACC-1 is present in muscle cells in the anterior region of the pharynx. The pharmacology of ACC-2 from *H. contortus*, Hco-ACC-2, has been investigated by Habibi *et al.* (2018) using expression in *X. laevis* oocytes where ACh has an EC_{50} of 21 μ M. Hco-ACC-2 is also activated by pyrantel, carbachol, levamisole, methacholine, urecholine, and choline.

The mAChRs in nematodes are less complex than the nACRs in terms of number and diversity. Three subtypes have been identified in *C. elegans*: GAR-1 (Hwang *et al.*, 1999), GAR-2 (Lee *et al.*, 2000), which are both neuronal, and GAR-3 (Hwang *et al.*, 1999). Muscarinic acetylcholine receptors are involved in regulating the pharyngeal muscle of *C. elegans* and the mAChR subunit GAR-3 regulates calcium-dependent processes in the muscle (Steger and Avery, 2004). The receptor encoded by *gar-3* is an arecoline receptor and, when activated, prevents pharyngeal muscle relaxation through an L-type calcium channel. When expressed in Chinese hamster ovary (CHO) cells, GAR-3 activates phospholipase C (Hwang *et al.*, 1999), phospholipase D (Min *et al.*, 2000), adenylyl cyclase (Park *et al.*, 2006), and extracellular signal-regulated kinases 1 and 2 (ERK1 and 2) (Kim *et al.*, 2008) when stimulated with carbachol (Shin and Cho, 2014). Its biochemistry is similar to mammalian M1/M3/M5 subtypes in terms of their biochemistry. Shin and Cho (2014) found that the *C. elegans* proteins VIG-1 and FRM-1 modulate carbachol-stimulated ERK1/2 activation in CHO cells expressing GAR-3. Evidence suggests that these proteins achieve this by interacting with the i3 loop of GAR-3.

5-Hydroxytryptamine (5-HT, serotonin)

5-HT has many key roles in the physiology of nematodes and regulates behaviors including

locomotion, metabolic control, feeding, aversive learning, egg-laying, male mating behavior, and regulation of insulin-like signaling pathways. Insight into the role of 5-HT in *C. elegans* was provided with the identification of the gene encoding the rate-limiting enzyme for 5-HT synthesis, tryptophan hydroxylase, *tph-1* (Sze *et al.*, 2000). Mutants of *tph-1* exhibit pleiotropic effects and the expression pattern of *C. elegans* in the nervous system identifies a small subset of neurons as serotonergic, including the pharyngeal neurosecretory motoneurons (NSMs) and the hermaphrodite specific neurons (HSNs) (Horvitz *et al.*, 1982; White *et al.*, 1986; Sze *et al.*, 2000; Carnell *et al.*, 2005). Acute increases in 5-HT and chronic deficiency cause changes in gene expression in *C. elegans* linked with stress pathways and development (Cruz-Corchado *et al.*, 2020).

The first *C. elegans* 5-HT receptor to be characterized was SER-4 (Y22D7AR.13), which was classified as a 5-HT-1-like receptor (Olde and McCombie, 1997) and has been named SER-4. The pharmacological profile of SER-4 was examined by competitive displacement studies using radiolabeled LSD. The most potent 5-HT antagonists to inhibit LSD binding were methiothepin, (+)-butaclamol, prazosin, and clozapine, with K_i values of 0.0016, 0.025, 0.034, and 0.036 μM , respectively, while for agonists the order was lisuride, 1-(1-naphthyl)piperazine, 5-OMeDMT, 5-HT, DOI, and 5-methoxytryptamine, with K_i values of 0.0023, 0.76, 8.2, 16, 55, and 58 μM . Using a *ser-4::gfp* reporter gene, consistent expression of *ser-4* occurred in RIB and RIS head neurons (Tsalik *et al.*, 2003).

This characterization of SER-4 was followed by SER-1 (F59C12.2), a 5-HT-2-like receptor (Hamdan *et al.*, 1999), and then by SER-7 (C09B7.1), 5-HT-7-like (Hobson *et al.*, 2003). These are all classified as G-protein-coupled receptors (GPCRs) and it has been proposed that SER-1, SER-4, and SER-7 act through G_{α_q} , G_{α_o} , and G_{α_s} , respectively (Harris *et al.*, 2009). SER-1 is coupled to calcium signaling while SER-4 and SER-7 are coupled to adenylate cyclase, the former inhibiting while the latter stimulates adenylate cyclase.

The SER-1 receptor pharmacology showed a mixture of 5-HT-1 and 5-HT-2 receptor properties. The EC_{50} values for 5-HT for the long and short isoform variants were 0.72 and 0.8 μM ,

respectively. SER-1 also responded to lisuride but was less sensitive, and the maximum response was only 25% that of 5-HT. Methiothepin and cyproheptadine antagonized calcium activation by 5-HT with K_i values for the long isoform of 0.009 and 0.032 μM , respectively. Clozapine, metergoline, ketanserin, and DOI were all less potent. SER-1 did not respond to dopamine, octopamine, or histamine. In a subsequent study (Xie *et al.*, 2005) using labeled mesulergine binding, it was found that SER-1 had a low affinity for 5-HT, with K_i values of 9.7 and 7.5 μM for 5-HT and 5-methoxytryptamine, respectively. However, the affinity of 5-HT can be increased by mutagenesis of SER-1 conserved residues helix 4 and 7; for example, the K_i value for 5-HT decreased from 9.7 to 0.39 μM but the E_{max} decreased by up to 65% (Xie *et al.*, 2005). Using a *ser-1::gfp* reporter gene, SER-1 is localized in most pharyngeal muscle cells (Tsalik *et al.*, 2003).

Subsequent interrogation of the *C. elegans* genome to look for additional 5-HT receptors identified a further five genes that encoded putative 5-HT receptors (Carre-Pierrat *et al.*, 2006). These genes included K02F2.6, identified as SER-3 which may be a 5-HT-4 type but later identified as an octopamine receptor (see section "Octopamine" below).

5-HT regulates *A. suum* metabolism, motility, and feeding (Donahue *et al.*, 1981; Brownlee *et al.*, 1995; Reinitz and Stretton, 1996). A 5-HT receptor that has been cloned and sequenced from *A. suum*, and named AS1, resembles a 5-HT-2-like receptor (Huang *et al.*, 1999). When transiently expressed in COS-7 cells, isolated membranes showed high affinity binding for radiolabeled LSD which was inhibited by 5-HT but not by dopamine, octopamine, or histamine. In addition, two further isoforms were identified and named AS2 and AS3. All these isoforms were expressed in the pharynx while AS1 and AS2 were also present in body wall muscle. These isoforms were subsequently named 5-HT_{2AS1}, 5-HT_{2AS2}, and 5-HT_{2AS1Δ4} and their pharmacology was investigated following expression in HEK293 cells (Huang *et al.*, 2002). Competitive binding studies yielded the following order of potency for 5-HT_{2AS1}: (+)-butaclamol, 12.10 pM; > mesulergine > methiothepin > R(+)-lisuride > cyproheptadine > 5-HT, all in 3.05–37.75 nM; > clozapine > mianserin > metergoline > 5-methoxy-*n,n*-dimethyltryptamine,

0.1–0.42 μM ; > ritanserin > α -methyl-5-HT > 5-carboxamidotryptamine (5-CT) > ($\pm$)-2,5-dimethoxy-4-iodoamphetamine (DOI) > quipazine > ($\pm$)-8-hydroxy-2-(di-*n*-propylamine)tetralin (8-OH-DPAT) > ketanserin, range 1.17–29.5 μM . These authors concluded that this profile was similar to that of the *C. elegans* homolog SER-1, from the study of Hamdan *et al.* (1999), with respect to high affinity for the antagonists cyproheptadine, metergoline, mianserin, and ritanserin and low affinity for the agonists 5-CT, quipazine, and 8-OH-DPAT. However, there were also differences between 5-HT_{As1} and SER-1. There was an 81% identity between 5-HT_{2As} and SER-1 which the authors suggested may reflect adaptations associated with the parasitic lifestyle. Huang *et al.* (2002) found that 5-HT stimulated phosphoinositol turnover in HEK293 cells expressing either 5-HT_{2As1} or 5-HT_{2As2} and they presumed that 5-HT_{2As} receptors couple physiologically to G_{oq}. They confirmed that the 5-HT_{2As} receptors were localized in body wall muscle, nerve cord/hypodermis, and in both neurons and muscle cells of the pharynx.

A 5-HT receptor has been cloned and characterized from *H. contortus*, 5-HT_{1Hc1}, which has high sequence homology to mammalian 5-HT-1 receptors and is present in eggs, L1/L2, L3, and adults (Smith *et al.*, 2003). Membranes prepared from insect Sf9 cells expressing 5-HT_{1Hc} receptor cDNA had nM binding affinity to 5-HT. However, its pharmacology differed from that of either *C. elegans* SER-4 or the mammalian 5-HT-1a subfamily of receptors. The K_i values for labeled 5-HT binding to Sf9 membranes expressing 5-HT_{1Hc} receptors were in the low nM range for p-amino-phenethyl-m-trifluoromethylphenyl piperazine (PAPP), 5-HT, BMY7378, WAY100635, dihydroergotamine, methysergide, NAN-190, (R+)-lisuride, 5-CT, and ritanserin. This receptor had a relatively low affinity for LSD, clozapine, spiperone, and DOI.

A mammalian 5-HT-7-like receptor has been identified and functionally characterized from *C. elegans*; it has been named SER-7b and proposed to act through G_os (Hobson *et al.*, 2003). When SER-7b is transiently expressed in COS-7 cells, it is constitutively active and stimulates basal levels of cAMP in the absence of ligand. These levels are raised further in the presence of 5-HT and tryptamine. In contrast, methiothepin, ritanserin, clozapine, and ketanserin all

reduced basal cAMP production, while mesulergine stimulated basal cAMP. The pharmacological profile of SER-7b was investigated by the extent to which ligands would displace radiolabeled LSD from COS-7 cell membranes. Methiothepin, metergoline, ritanserin, clozapine, 5-HT, and tryptamine all displaced labeled LSD binding from these membranes. Only 5-HT and tryptamine bound with high affinity, while other naturally occurring amines failed to do so. Interestingly, 5-carboxyaminotryptamine, a potent agonist at mammalian 5-HT-7 receptors, failed to bind with high affinity. In a second paper these authors found that *ser-7::gfp* was expressed in the following pharyngeal neurons: MC, M2, M3, M4, and M5, and in vulval muscle, and concluded that SER-7b plays a key role in the regulation of pharyngeal pumping, feeding, and egg-laying by activating adenyl cyclase to raise levels of cAMP (Hobson *et al.*, 2006).

An equivalent of the mammalian ligand-gated cation channel 5-HT-3 has not been found in nematodes. However, MOD-1 has been identified as a 5-HT-gated chloride channel in *C. elegans*. MOD-1 protein has four predicted transmembrane regions and a large cytoplasmic domain between M3 and M4 (Ranganathan *et al.*, 2000). When deprived of food, *mod-1* mutants are defective in the enhanced slowing response but not defective in the basal slowing response. Expression of *mod-1* in *X. laevis* oocytes voltage-clamped at –70 mV resulted in a large rapidly developing inward current in response to 1 μM 5-HT. These channels were insensitive to 1 mM glycine, ACh, GABA, glutamate or histamine, or to 10 μM ivermectin. Granisetron (130 μM) failed to block the response to 5-HT, while mianserin (50 μM) did antagonize the response. The K_i values for mianserin and methiothepin were about 19 and 32 μM , respectively. The 5-HT-dependent current from MOD-1 expressing oocytes reversed at around –20 mV, which is the chloride reversal potential, E_{Cl}, for oocytes (Weber, 1999). HEK293 cells transiently transfected with *mod-1* DNA confirmed that the MOD-1 channel is selective for chloride ions, thus it is a 5-HT-gated chloride channel and unique to the invertebrates. Expression of *mod-1::gfp* reporter was found in cell bodies and axons of neurons in the head, ventral cord, and tail of *C. elegans* but was absent from muscle cells (Ranganathan *et al.*, 2000). However, a later paper found that *mod-1* reporter is

expressed in all body wall muscle cells that directly execute body bends during locomotion in *C. elegans* (Gürel *et al.*, 2012). A 5-HT-gated chloride channel has been isolated from *H. contortus*, *Hco-mod-1*, and a predicted ortholog has been identified from *L. loa*, *B. malayi*, and *W. bancrofti* (Beech *et al.*, 2013). There is also a low-affinity ligand-gated chloride channel to 5-HT in *C. elegans*, LGC-40, with an EC_{50} of 907 μ M, when expressed in *X. laevis* oocytes (Ringstad *et al.*, 2009). LGC-40 currents induced by 5-HT were blocked by d-tubocurarine and hemicholinium, IC_{50} values of 8.3 and 2.0 μ M, respectively, but this receptor was relatively insensitive to atropine.

In their study on chemosensation in *C. elegans*, Harris *et al.* (2009) identified a new 5-HT GPCR, SER-5 (F16D3.7), first proposed as a 5-HT receptor by Carre-Pierrat *et al.* (2006), which is expressed on ASH sensory neurons. In addition, SER-5 is expressed in vulval and body wall muscles, in head and tail neurons, and in sensory neuron AWB. This receptor is required for the 5-HT-dependent increase in octanol sensitivity in *rrf* but not wild-type *C. elegans*. Evidence suggests that SER-5 is most closely related to mammalian 5-HT-6 receptors. SER-5, together with MOD-1 and SER-1, are required for 5-HT-dependent increase in aversive responses to dilute octanol.

Orthologs of *C. elegans* 5-HT receptors have been identified in the plant-parasitic nematode *Globodera pallida* (Crisford *et al.*, 2020). While potential orthologs were identified for SER-4, SER-7, and MOD-1, orthologs were not identified for SER-1 or SER-5. The closest homolog of *C. elegans*, SER-7 from *G. pallida*, was cloned and tested for its ability to rescue the lack of 5-HT-stimulated pharyngeal pumping in a *ser-7* mutant of *C. elegans*. This SER-7 from *G. pallida* restored 5-HT-activated pumping in the mutant. This 5-HT effect was blocked by methiothepin, which antagonizes the 5-HT effect on stylet thrusting in *G. pallida*. Crisford *et al.* (2020) also cloned a putative ortholog of *mod-1* from *G. pallida* and found that it could rescue the 5-HT-induced phenotype of *C. elegans* mutants.

5-HT receptors have been expressed ectopically in *C. elegans* in muscle and neurons at sites likely to yield clear phenotypes following stimulation by 5-HT and other monoamines (Law *et al.*, 2015). These authors used receptors

involved in 5-HT-dependent inhibition of locomotion in *C. elegans*, an effect that requires both SER-4 and MOD-1. MOD-1 receptors from *H. contortus* were expressed in cholinergic motoneurons and body wall muscle of these *C. elegans* mutants that lacked any 5-HT receptors. 5-HT rapidly paralyzed these animals with EC_{50} values of 0.2 mM for both sites. It was concluded that chimeric *C. elegans* provided a model to test potential drug targets from parasitic nematodes and hence for parasitic screening.

Table 6.2 summarizes the 5-HT receptor subtypes, their K_i values, and tissue distribution in *C. elegans*, *A. suum*, *H. contortus*, and *G. pallida*.

Dopamine (DA)

There are eight dopaminergic mechanosensory neurons in the hermaphrodite *C. elegans*: a pair of ADEs, four CEPs, and a pair of PDEs (Sulston *et al.*, 1975). The dopamine they release signals through four GPCRs—DOP-1 (F15A8.5), DOP-2 (K09G1.4), DOP-3 (T14E8.3), DOP-4 (C52B11.3) (Suo *et al.*, 2004; Chase and Koelle, 2007)—and one DA-gated chloride channel, LGC-53 (Ringstad *et al.*, 2009). DA is involved in a range of functions in *C. elegans*, including locomotion, egg-laying behavior, responses to food, defecation, and habituation to touch. DOP-1 and DOP-4 have similarities to the mammalian DOP-1 receptor, while DOP-2 and DOP-3 are similar to the mammalian DOP-2 receptor. The molecular basis of signaling pathways activated through DOP-2 has been shown to involve the G_{ai} protein, GPA-14 (Pandey and Harbinder, 2012).

The first *C. elegans* DA receptor to be cloned was DOP-1, which has three splice variants: CeDOP-1L, CeDOP-1M, and CeDOP-1S (Suo *et al.*, 2002). DOP-1 showed the highest homology with mammalian D1-like receptors and activated adenylyl cyclase. COS-7 cells were transiently transfected with pCeDOP-1M; their membranes were tested for their ability to bind labeled LSD, and the K_D and B_{max} were determined. DA inhibited this LSD binding with K_i of 186 nM while other biogenic amines required μ M levels to inhibit binding. No specific binding was obtained when COS-7 membranes were exposed to labeled SCH233390, a D1-like antagonist, or the D2-like antagonist, spiperone. DOP-1 has been identified in a number of neurons (Tsalik *et al.*, 2003). Suo

Table 6.2. Summary of 5-HT neurotransmitter system. Tissue distribution and/or developmental stage of expression is for the receptor class.

Species	Lifestyle	Receptor subtypes	Receptor class	K _i	Tissue distribution / developmental stage
<i>C. elegans</i>	Free-living	SER-1 (5-HT _{2ce})	GPCR	9.7 μ M	Neuronal, body wall muscle, pharyngeal muscle
		SER-4 (5-HTCe)	GPCR	16 μ M	
		SER-5	GPCR	n.d.	
		SER-7	GPCR	n.d.	
		MOD-1	Ion channel	n.d.	
<i>A. suum</i>	Parasitic (porcine)	AS1 (5-HT _{2AS1})	GPCR	37.8 nM	Pharynx and body wall muscle
		AS2 (5-HT _{2AS2})	GPCR	32.2 nM	
		AS3 (5-HT _{2AS1Δ4})	GPCR	4.87 nM	
<i>H. contortus</i>	Parasitic (ruminant)	5-HT _{1Hc1}	GPCR	0.8 nM	Developmental stages L1, L2, L3, and adult
		Hco-mod-1	Ion channel	n.d.	
<i>G. pallida</i>	Parasitic (plant)	<i>gpa-ser-7</i> (GPLIN_000340600)	GPCR	n.d.	n.d.

n.d. = not defined

et al. (2003) also cloned a cDNA encoding a putative *C. elegans* D2-like receptor with two splice variants, CeDOP-2S and CeDOP-2L, which showed a higher sequence homology to mammalian D2-like receptors than to D1. DOP-2 showed a high affinity binding for labeled LSD, with dissociation constants K_i of 2.98 and 2.24 μM for the S and L forms, respectively. Other biogenic amines were 10–100 times less potent. Butaclamol showed the highest affinity to CeDOP-2, with K_i value of 35 nM, while other DA antagonists had lower affinity, with clozapine > SCH23390 > haloperidol > spiperone > chlorpromazine > sulpiride. Both CeDOP-2S and L inhibited adenyl cyclase when transiently expressed in CHO cells, with EC_{50} values of 73–75 nM. Butaclamol, 10 μM , inhibited the effect of 0.1 μM DA in these cells, while the other antagonists mentioned above failed to inhibit. CeDOP-2 was expressed in all the DA-containing neurons plus some other neurons. *dop-2* is strongly expressed in RIA interneurons (Tsalik *et al.*, 2003).

DOP-3 and DOP-4 have been cloned and characterized in *C. elegans* by Sugiura *et al.* (2005). DOP-3 encodes a mammalian D2-like receptor and also has a truncated splice variant, DOP-3nf. When expressed in CHO cells, DOP-3 inhibits adenyl cyclase production. DOP-3 is required for the ability of food and exogenous DA to rescue the octanol avoidance defect of *rgs* mutant animals (Ezak and Ferkey, 2010). DOP-4 encodes a D1-like receptor and when CHO cells expressing *dop-4* were exposed to DA, DA stimulated cAMP production, an effect absent when these cells were challenged with other biogenic amines. DOP-4 most closely resembles a D1-like receptor found in other invertebrates which is distinct from other mammalian D1-like receptors. DOP-3 is expressed in head, ventral cord, and tail neurons and in some body wall muscle cells (Chase *et al.*, 2004), while DOP-4 is expressed in pharyngeal neurons I1 and I2, neurons ASG, AVL, CAN, and PQR (Sugiura *et al.*, 2005).

The pharmacology of the DA-gated chloride channel, LGC-53, in *C. elegans* has been investigated using its expression in *X. laevis* oocytes (Ringstad *et al.*, 2009). DA had an EC_{50} of 4.4 μM , while the antagonists risperidone, haloperidol, and spiperone had IC_{50} values of 40, 32, and 28 μM , respectively. A ligand-gated chloride channel has been isolated and characterized from *H. contortus*, HcGGR3, which is activated by DA

and shares 84% similarity with its presumed *C. elegans* ortholog, CeGGR3, and 46% similarity with *C. elegans* MOD-1 (Rao *et al.*, 2009). When expressed in *X. laevis* oocytes this channel formed a homomer which was activated by 10 μM DA. At 1 mM, octopamine, 5-HT, and tyramine induced small to moderate responses. The highest expression of HcGGR3 is found in the eggs > adult females > L3, with least expression in adult males. Immunolocalization of HcGGR3 occurred in tissue associated with anterior deirid socket/sheath cells, suggesting a role in mechanosensation.

Table 6.3 summarizes the dopamine receptor subtypes, their K_i values for dopamine, and tissue distribution in *C. elegans*, *H. contortus*, and *B. malayi*.

Tyramine (TYR)

TYR is synthesized from tyrosine by tyrosine decarboxylase (Fig. 6.1D). In *C. elegans* tyrosine decarboxylase is encoded by *tdc-1*, which is expressed in a pair of motoneurons, RIM. RIM does not express *tbh-1*, the gene that encodes tyramine- β -hydroxylase (Alkema *et al.*, 2005), the enzyme required to synthesize octopamine from TYR, thus identifying RIM as a tyraminer-gic neuron. A TYR GPCR has been isolated and characterized, designated SER-2 (Rex and Komuniecki, 2002; Rex *et al.* 2004). Two cDNAs were isolated, SER-2 and SER-2a; SER-2 was transiently transfected in COS-7 cells, the membranes were analyzed for radiolabeled LSD binding, and the SER-2 pharmacological profile was determined. SER-2 showed the highest affinity for TYR with a K_i of 73 nM compared with K_i values of 2.19, 3.12, and 13.7 μM for OA, DA, and 5-HT, respectively. Low nM K_i values were obtained for chlorpromazine > mianserin = phentolamine > cyproheptadine. Other compounds tested were less potent, with rank potency of yohimbine > clonidine > naphazoline > synephrine > metoclopramide > tolazoline. TYR, with an IC_{50} of around 360 nM, depressed forskolin-stimulated production of cAMP in HEK293 cells, probably acting through $G_{\alpha/o}$.

A second GPCR, TYRA-2 (F01E11.5), has been identified in *C. elegans* and when expressed in COS-7 cell membranes it binds radiolabeled TYR with a high affinity, K_d of 20 nM (Rex *et al.*, 2005). Other biogenic amines, OA and DA, inhibit

Table 6.3. Summary of the dopamine receptors. Tissue distribution and/or developmental stage of expression is for the receptor class.

Neurotransmitter: Dopamine					
Species	Lifestyle	Receptor subtypes	Receptor class	K _i	Tissue distribution / developmental stage
<i>C. elegans</i>	Free-living	<i>dop-1</i> (F15A8.5)	GPCR	186 nM	Neuronal (mechanosensory, pharyngeal, ventral cord, tail), body wall muscle
		<i>dop-2</i> (K09G1.4)	GPCR	DOP-2S 2.98 μ M DOP-2L 2.24 μ M	
		<i>dop-3</i> (T14E8.3)	GPCR	n.d.	
		<i>dop-4</i> (C52B11.3)	GPCR	n.d.	
<i>H. contortus</i>	Parasitic (ruminant)	<i>lgc-53</i>	Ion channel	n.d.	Eggs, adult, females, L3 stage, sheath cells
		<i>HcGGR3</i>	Ion channel	n.d.	
<i>B. malayi</i>	Parasitic (human)	<i>Bm-dop-6</i> (Bm1_31090(14954.m01687))	GPCR ^a	n.d.	n.d.
		<i>Bm-dop-7</i> (Bm1_52070(14992.m11197))	GPCR ^a	n.d.	

^aPutative; n.d. = not defined.

this binding with low affinity, 1.55 and 1.78 μM . The rank order of the most potent compounds tested for their ability to compete with labeled TYR were cyproheptadine > chlorpromazine = mianserin > naphazoline > phentolamine > synephrine, while the least potent were metoclopramide, clonidine, and 5-HT. TYR also increased GTP γ S binding to COS-7 membranes expressing TYRA-2 with an EC_{50} of 50 nM and this GTP γ S binding is PTX-sensitive, indicating that TYRA-2 may couple to $G_{\alpha i/o}$. In *C. elegans*, TYRA-2 expression appears to be entirely neuronal, being located on MC and NSM pharyngeal neurons, suggesting a role in the regulation of pharyngeal pumping, on amphid neurons and neurons in the nerve ring, body, and tail.

A third GPCR for TYR, TYRA-3, has been identified in *C. elegans* (Wragg *et al.*, 2007). TYRA-3 was transiently expressed in COS-7 cells and isolated membranes were examined for labeled LSD binding. These membranes showed saturable LSD binding with K_d and B_{max} of 31.1 nM and 5.4 pmol/mg, respectively. LSD binding was inhibited by TYR and OA with apparent IC_{50} s of 0.7 and 65 μM , respectively, while DA and 5-HT had values > 100 μM . *tyra-3::gfp* is not expressed in sensory neurons but is expressed in some head and tail neurons, including ADE/CEP DA-containing neurons and in vulval muscle.

TYR also acts through a chloride-gated channel, LGC-55 (Pirri *et al.*, 2009). These authors expressed *lgc-55* in *X. laevis* oocytes and recorded currents in response to biogenic amines. The EC_{50} s for TYR, DA, and OA were 12.1, 158.8, and 215.2 μM , respectively. When whole-cell patch-clamp recordings were made from *C. elegans* body wall muscle cells expressing *myo-3::lgc-55* and 200 μM TYR applied, TYR elicited a current which was absent in wild-type muscle cells, demonstrating that this receptor could be activated by TYR *in vivo*. LGC-55 was expressed in cells that are postsynaptic to RIM motoneurons, including AVB and in a subset of neck muscle cells. However, TYR receptors are also expressed in cells that do not receive synaptic inputs from RIM neurons, suggesting that tyramine can act as a neurohormone (Pirri *et al.*, 2009). A TYR receptor has been cloned and characterized from *H. contortus*, Hco-LGC-55, and is an ortholog of Cel-LGC-55 that can form a homomeric chloride channel, gated by TYR, OA, and DA (Rao *et al.*, 2010). This channel is

expressed in all life stages of *H. contortus*. Heterologous expression of *Hco-lgc-55* in *X. laevis* oocytes results in a homomeric amine-gated chloride channel with EC_{50} values for TYR, DA, and OA of 5.8, 25.2, and 133 μM , respectively. The threshold response for TYR was 0.5 μM . Hco-LGC-55 is chloride selective, since its E_{Cl} was dependent on external chloride.

Table 6.4 summarizes the tyramine receptor subtypes, their K_i values for tyramine, and tissue distribution in *C. elegans*, *H. contortus*, and *B. malayi* (Smith *et al.* 2007).

Octopamine (OA)

OA is synthesized in two stages: firstly, tyrosine is decarboxylated to TYR by tyrosine decarboxylase, which is then β -hydroxylated by tyramine- β -hydroxylase to octopamine (Fig. 6.1E). OA inhibits egg-laying and pharyngeal pumping in *C. elegans* and occurs in a pair of head interneurons, RIC, which contain both enzymes (Rogers *et al.*, 2001; Alkema *et al.*, 2005). This inhibitory effect of OA on *C. elegans* pharyngeal pumping is blocked by epinastine, a mammalian histamine receptor H-1 antagonist (Packham *et al.*, 2010).

Three OA receptors have been identified in *C. elegans*: SER-3, SER-6, and OCTR-1. SER-3 (K02F2.6) is most closely related to amine receptors that are $G_{\alpha o}$ -coupled, raise intracellular calcium, and increase cAMP when activated (Suo *et al.*, 2006). *C. elegans* containing the reporter fusion transgene *ser-3::gfp* expressed GFP in many head and tail neurons, head muscle, intestine, phasmid socket cells, spermatheca, and eggs. SIA neurons were among the head neurons that were GFP positive and studies by Suo *et al.* (2006) showed that SER-3 in SIA neurons mediated starvation- and OA-induced activation of CREB (cAMP response element-binding protein) in SIA. OA, also acting on SER-3 and SER-6 receptors in SIA neurons, promotes roaming behaviors that are characteristic of fasting *C. elegans* (Churgin *et al.*, 2017). SER-6 is another G_q OA receptor that is highly homologous to SER-3 (Yoshida *et al.*, 2014) and is required for OA-mediated changes in octanol sensitivity (Mills *et al.*, 2012). When SER-6 was heterologously expressed in *X. laevis* oocytes they responded strongly to OA, 1 μM , but only weakly to 2 μM TYR and not at all to DA or 5-HT (Mills *et al.*,

Table 6.4. Summary of the tyramine and octopamine receptors. Tissue distribution and/or developmental stage of expression is for the receptor class.

Neurotransmitter: Tyramine					
Species	Lifestyle	Receptor subtypes	Receptor class	K _i	Tissue distribution / developmental stage
<i>C. elegans</i>	Free-living	<i>ser-2</i> <i>tyra-2</i> <i>tyra-3</i> <i>lgc-55</i>	GPCR GPCR GPCR Ion channel	73 nM 26 nM n.d. n.d.	Neuronal, vulval muscle
<i>H. contortus</i>	Parasitic (ruminant)	<i>Hco-LGC-55</i>	Ion channel	n.d.	All life stages
<i>B. malayi</i>	Parasitic (human)	<i>BM4</i>	GPCR ^a	12.3 nM	n.d.
Neurotransmitter: Octopamine					
<i>C. elegans</i>	Free-living	SER-3 SER-6 OCTR-1	GPCR GPCR GPCR	K _i n.d. K _i n.d. K _i n.d.	Head and tail neurons, head muscle, intestine, phasmid socket cells, spermatheca and eggs.

n.d. = not defined

2012). This inward current was presumed to be mediated by endogenous calcium-gated chloride channels with an EC_{50} of 2 μ M. A third OA receptor, OCTR-1 (F14D12.6), has been described which activates G_{ao} , functions directly in ASH neurons to decrease sensitivity to octanol (Wragg *et al.*, 2007), and plays a role in innate immunity in *C. elegans* (Sun *et al.*, 2011). The OCTR-1-expressing neurons, ASH and ASI, negatively regulate innate immunity, since *octr-1* mutants showed enhanced resistance to killing by *Pseudomonas aeruginosa*, suggesting that loss of OCTR-1 signaling increases the immune function in nematodes. OCTR-1 regulates innate immunity by suppressing translation and the unfolded protein response pathways at the protein level (Liu *et al.*, 2016). The exogenous application of OA, 10–25 mM, suppressed OCTR-1-dependent immune responses, while the estimated level in an animal was 3 μ M (Sellegounder *et al.*, 2018). There is an immunoinhibitory pathway in *C. elegans* whereby release of OA from RIC neurons acts on OCTR-1 present in ASH neurons to suppress innate immune responses in pharyngeal and intestinal tissues. Exposure to low doses of nanopolystyrene, 100 nM, 10–100 μ M, activates neuronal JNK MAPK signaling in *C. elegans* and affects OA signaling via SER-6 and OCTR-1 receptors in the intestine (Qu *et al.*, 2020).

Table 6.4 summarizes the octopamine receptor subtypes and tissue distribution in *C. elegans*.

Glutamate

Glutamate functions widely as a transmitter in *C. elegans* since the vesicular glutamate transporter, EAT-4, is co-expressed in at least 15 different classes of neurons in *C. elegans* (Lee *et al.*, 1999). There are both ionotropic and metabotropic glutamate receptors, iGluR and mGluR, respectively, in *C. elegans* (Brockie and Maricq, 2006; Dillon *et al.*, 2015). There are ten putative cation-selective iGluR subunits: GLR-1 to GLR-8, which are AMPA/kainate-like, and two NMDA-like subunits, NMR-1 and NMR-2, whose genes are expressed in *C. elegans* (Brockie *et al.*, 2001a); and three GPCR mGlu: MGL-1 to MGL-3, which play a key role in circuits underlying feeding behavior (Dillon *et al.*, 2015). In addition, there are at least six glutamate-gated chloride channel genes. First identified by Cully *et al.* (1994), these

translate into seven subunits: *glc-1*, GluCl α 1; *glc-2*, GluCl β ; *glc-3*, GluCl α 4; *glc-4*, none; *avr-14*, GluCl α 3A (GBR-2A) and GluCl α 3B (GBR-2B); and *avr-15*, GluCl α 2A and GluCl α 2B (Yates *et al.*, 2003). GluCl channels have also been identified in *A. suum*, *H. contortus*, and *D. immitis* (Jagannathan *et al.*, 1999; Cheeseman *et al.*, 2001; Forrester *et al.*, 2003; Yates and Wolstenholme, 2004).

The detailed distribution of iGluRs has been studied in *C. elegans* by Brockie *et al.* (2001a), who found that many neurons expressed multiple non-NMDARs in various combinations, suggesting heteromeric receptors, while other neurons expressed only one, suggesting homomeric receptors. Two genes, *glr-7* and *glr-8*, were primarily expressed in pharyngeal neurons, including NSM, M3, and M4; all play a key role in motor control of pharyngeal pumping. Both GLR-1 and GLR-2, together with NMR-1, mediate osmotic avoidance behavior in *C. elegans* (Mellem *et al.*, 2002). Recordings from interneuron AVA showed currents activated by glutamate, kainate, and NMDA but not by AMPA, and that the glutamate current could be partially antagonized by CNQX (Brockie *et al.*, 2001b; Mellem *et al.*, 2002). Overall, Brockie and Maricq (2006) concluded that glutamate plays a key role in locomotion, pharyngeal pumping, and sensory processing in *C. elegans*. *C. elegans* AIB neurons have GLR-1 and GLR-5 channels with currents gated by glutamate but these channels have different activation thresholds and kinetics (Zou *et al.*, 2018). When activated, the GLR-5 high-threshold, slow-inactivation currents induced sustained calcium release from IP₃-sensitive calcium stores.

The first *C. elegans* glutamate-gated chloride channel subunits to be isolated and expressed in *X. laevis* oocytes were GluCl α 1 and GluCl β , which were sensitive to glutamate, ibotenate, ivermectinPO₄, and a series of avermectin analogs but insensitive to NMDA, kainate, quisqualate, AMPA, and ACh, all at 1 mM (Cully *et al.*, 1994). Picrotoxin reduced both glutamate and ivermectinPO₄ currents, while strychnine, bicuculline, and CNQX had no blocking effect. Each subunit can form pentameric chloride channels. The combination of both α and β subunits responded to both glutamate and ivermectinPO₄, 1 mM and 1 μ M, respectively. The glutamate current was rapid and reversible

while that of ivermectinPO₄ was slow and irreversible. The homomeric GluCl α 1 subunit alone failed to respond to glutamate but did respond to ivermectinPO₄. Conversely, the homomeric GluCl β subunit alone responded to glutamate but not to ivermectinPO₄. Low nM ivermectinPO₄ also potentiated the response to 300 μ M glutamate. Interestingly, the EC₅₀ for glutamate was 380 μ M for the α subunit but for the co-expressed α/β subunits it was 1.36 mM, while for ivermectinPO₄ the EC₅₀s for the α and α/β subunits were 140 and 190 μ M, respectively.

Dent *et al.* (1997) identified *avr-15* as a gene encoding a chloride channel subunit that mediates inhibitory glutamate transmission from M3 on to pharyngeal muscles pm4 and 5 of *C. elegans*. *avr-15* encodes alternatively spliced GluCl α 2A and 2B subunits, which both form homomeric receptors that are sensitive to glutamate, with EC₅₀s of 2.0 and 0.208 mM, respectively, which are also sensitive to ivermectin. *avr-15* mutants are insensitive to both glutamate and ivermectin. When GluCl α 2 was expressed in *X. laevis* oocytes, glutamate induced a rapidly desensitizing current with an EC₅₀ of 2 mM, while ivermectin induced a slowly desensitizing current which failed to reverse on removal. Ibotenic acid, 0.5 mM, induced a weak current. In a separate series of experiments when GluCl α 2 was expressed, the EC₅₀s for glutamate and ivermectinPO₄ were 208 μ M and 107.8 nM, respectively (Vassilatis *et al.*, 1997).

Intracellular recordings were made from *C. elegans* pharyngeal muscle cells and the effects of glutamate and ivermectin were investigated (Pemberton *et al.*, 2001). The glutamate response was a rapidly desensitizing reversible Cl-dependent depolarization with an EC₅₀ of 166 μ M, weakly antagonized by picrotoxin, while the EC₅₀ for ivermectin was 2.7 nM, five orders of magnitude more potent. Ivermectin induced a slow irreversible depolarization. The EC₅₀ for ibotenate was 17.5 μ M, around ten times more potent than glutamate, while quisqualate and kainate were considerably less potent than glutamate. Using the glutamate uptake blocker, pyrrolidine-2,4-dicarboxylic acid, evidence was found for tonic release of glutamate. In *avr-15* mutants, the EC₅₀ for glutamate was 1.15 mM, a ten-times reduction in potency compared with wild-type animals, while ivermectin failed to induce a response, showing that AVR-15 is partially

required for the pharyngeal response to glutamate and essential for the response to ivermectin.

C. elegans avr-14 encodes two alternatively spliced glutamate-gated chloride channel subunits: GluCl α 3A and GluCl α 3B (Dent *et al.*, 2000). GluCl α 3B forms homomeric channels which were activated by glutamate, 10 mM, with a fast, rapidly desensitizing response, while ivermectin, 10 μ M, activated a slow, irreversible response in *X. laevis* oocytes. GluCl α 3A failed to respond to either glutamate or ivermectin when expressed in *X. laevis* oocytes. *C. elegans* GluCl α 4 subunits also formed homomeric receptors when expressed in *X. laevis* oocytes that responded to glutamate and ivermectin with EC₅₀s of 1.9 mM and 400 nM, respectively (Horoszkow *et al.*, 2001), but the glutamate response was insensitive to picrotoxin. However, both BIDN and fipronil blocked glutamate with IC₅₀s of 0.2 and 11.5 μ M, respectively. Four GluCl receptor subunits have been cloned from *H. contortus*—HcGluCl α (*avr-15*), HcGluCl α 3A and 3B (*avr-14*), and HcGluCl β (*glc-2*)—and one from *A. suum*: AsGluCl3 (Forrester *et al.*, 1999; Jagannathan *et al.*, 1999; Cheeseman *et al.*, 2001; Forrester *et al.*, 2002). HcGluCl α and HcGluCl α 3B have high affinity for ivermectin binding while HcGluCl α 3A and HcGluCl β fail to bind ivermectin in COS-7 cell-binding studies. Picrotoxin, fipronil, glutamate, and GABA failed to compete for ivermectin binding to either of the high-affinity subunits (Cheeseman *et al.*, 2001). *H. contortus* homomeric GluCl α 3B receptor subunits have been investigated by recording single-channel conductance and current voltage relations in HEK AD293 cells transfected with cDNAs encoding GluClR subunit, AVR-14B (GluCl α 3B) (Atif *et al.*, 2017). Both glutamate and ivermectin (low nM) activate this receptor. The conductance of this channel was around 23 pS. The authors also studied the properties of a G36'A mutation of the channel which increased receptor desensitization for both glutamate and ivermectin. In a subsequent paper, it was shown that HcGluCl β can form functional receptors and assemble with α subunits to form $\alpha\beta$ GluClRs in *X. laevis* oocytes (Atif *et al.*, 2019). GluClRs are widely distributed in *H. contortus*, including pharyngeal neurons, and have important roles in regulating locomotion, pharyngeal function, and sensory processing (Portillo *et al.*, 2003).

The molecular characteristics of three metabotropic glutamate receptors in *C. elegans*—MGL-1, MGL-2, and MGL-3—have been defined (Dillon *et al.*, 2015) and these GPCRs appear to be exclusively expressed in the nervous system (Dillon *et al.*, 2015). Their expression in the pharyngeal nervous system indicates that they play a role in the regulation of feeding. Evidence suggests that MGL-1 and MGL-3, but not MGL-2, modulate activity in the pharyngeal neuronal circuit, with MGL-1 making a key contribution to changes in feeding behavior following acute food removal and to the context-dependent modulation of feeding behavior. The same group identified MPZ-1 and PTP-1 as candidate scaffolding proteins for MGL-1 receptors and found that their genes co-express with *mgl-1* in a subset of pharyngeal neurons (Dillon *et al.*, 2018). Presynaptic MGL-2 has been implicated in feedback excitation on to AVA (a major neuron governing reversals) following glutamate spill-over, due to impaired clearance, which induces repetitive reversal behavior in *C. elegans* (Katz *et al.*, 2019). These authors suggest that MGL-2 acts through $G_{\alpha q}$.

GABA

There are seven genes for GABA-gated ion channels in *C. elegans*: *gab-1*, *unc-49*, *lgc-35*, *lgc-36*, *lgc-37*, *lgc-38*, and *exp-1* (Hobert, 2013). GABA occurs in both neuronal and non-neuronal tissue (Gendrel *et al.*, 2016). Cys-loop GABA-chloride-gated ion channels in nematodes have been reviewed in depth (Accardi *et al.*, 2012). UNC-49 occurs only on muscle cells, while EXP-1 and LGC-35 occur on both motoneurons and muscle (Beg and Jorgensen, 2003). The UNC-49 GABA-A-like receptor occurs on body wall and head muscle cells of *C. elegans* where *unc-49* can generate three distinct subunits: UNC-49-A, UNC-49-B, and UNC-49-C (Bamber *et al.*, 1999). However, only UNC-49-B and UNC-49-C are co-expressed at high levels and co-localized to muscle synaptic regions, while UNC-49-A is hardly detectable. Using *X. laevis* oocytes and HEK293 cells, it would appear that UNC-49-B and UNC-49-C co-assemble at the neuromuscular junction to encode a heteromeric GABA-R, though UNC-49-B is essential for GABA function. When UNC-49-B.1 was expressed alone, it

formed a homomer, producing a strong desensitizing current to GABA with an EC_{50} of 43.7 μ M. Using whole-cell voltage-clamp recordings from individual muscle cells, it has been shown that UNC-49 is essential for the chloride-mediated GABA response of *C. elegans* muscle cells (Richmond and Jorgensen, 1999). *unc-49* mutants failed to respond to either GABA or muscimol. In a further study, using expression of subunits in *X. laevis* oocytes, the pharmacological properties of the UNC-49B homomer with the UNC-49B/C heteromer were compared (Bamber *et al.*, 2003). Bicuculline failed to inhibit the GABA-activated currents and diazepam failed to enhance GABA currents of either receptor. The homomer was much more sensitive to picrotoxin than the heteromer, with IC_{50} s of 0.9 and 166 μ M, respectively. Pentobarbitone enhanced the homomer more than the heteromer, whereas propofol slightly enhanced the homomer while slightly inhibiting the heteromer. Pregnanolone strongly inhibited both receptors. Patch-clamp electrophysiology recordings from *C. elegans* muscle cells provided evidence that the *in vivo* UNC-49 receptor was a heteromer composed of UNC-49-B and UNC-49-C (Bamber *et al.*, 2005). Evidence for this comes from using pregnenolone SO_4 which strongly inhibited the GABA current of wild-type muscle cells, IC_{50} 21.7 μ M, and in UNC-49-B/C transgenics, IC_{50} 22.9 μ M, while for UNC-49-B transgenics, the IC_{50} value was greatly increased to > 300 μ M. Similarly, for picrotoxin, wild-type muscle and UNC-49-B/C transgenics were insensitive to picrotoxin while UNC-49-B transgenics muscle was picrotoxin sensitive. Overall, Bamber *et al.* (2003, 2005) concluded that UNC-49 receptors shared significant structural and pharmacological similarities with mammalian GABA-A receptors and that the properties of nematode GABA receptors are more similar to the UNC-49-B/C heteromer than the UNC-49-B homomer.

In general, the pharmacology of *C. elegans* UNC-49 resembles the pharmacology of the GABA receptor studied using *A. suum* body wall muscle (Holden-Dye *et al.*, 1989). GABA hyperpolarized *A. suum* muscle cells with a threshold of 5–10 μ M and EC_{50} of 26.3 μ M. Compared with GABA, muscimol had a relative potency of 0.4 while dihydromuscimol was 7.53 times more potent. GABA responses were not potentiated by pentobarbitone or flurazepam or blocked

by dieldrin, bicuculline, and picrotoxin. Ivermectin blocked GABA responses with an IC_{50} of $8.86 \mu M$ (Holden-Dye and Walker, 1990).

Two genes, *Hco-unc-49B* and *-49C*, have been identified from *H. contortus* and expressed in *X. laevis* oocytes which show similar properties to *unc-49* from *C. elegans*, though differences were also observed (Siddiqui *et al.*, 2010). While oocytes expressing *Hco-unc-49B* and *-49B/C* responded to GABA, with EC_{50} s of 64 and $39.9 \mu M$, respectively, *Hco-unc-49C* failed to respond to $100 \mu M$ GABA. *Hco-unc-49B* was more sensitive to picrotoxin than was *-49B/C* but *-49B/C* had a higher sensitivity to muscimol than *-49B*. These authors found *Hco-unc-49B/C* more sensitive than *-49B* to GABA; the opposite was the case with *C. elegans*. Molecular characterization of the binding loop E of UNC-49 in *H. contortus* has revealed differences in the agonist binding pocket between it and the mammalian GABA receptor that could be useful in the design of novel anthelmintics (Kwaka *et al.*, 2018).

Apart from *H. contortus*, *unc-49* has been identified in other parasitic nematodes, including *B. malayi*, *T. spiralis* (Williamson *et al.*, 2007), *A. suum*, and *L. loa* (Accardi *et al.*, 2012). In addition, all seven GABA genes have been identified in *A. suum* and *H. contortus*, and are probably all present in *L. loa* and *T. spiralis*, but *gab-1* is absent from *B. malayi* (Accardi *et al.*, 2012). Alleles of the *hg1* gene which encodes a putative GABA receptor α/β subunit isolated from *H. contortus* respond differently to ivermectin. HG1A alleles are sensitive to ivermectin while HG1E alleles are resistant (Feng *et al.*, 2002). When these alleles were co-expressed with *gab-1* from *C. elegans* in *X. laevis* oocytes, they did not respond to ivermectin, but when co-applied with $10 \mu M$ GABA, the GABA current was potentiated compared with GABA alone in GAB-1/HG1A receptor but reduced in the GAB-1/HG1E receptor. These authors concluded that GABA receptors may be involved in ivermectin resistance.

While GABA is mainly an inhibitory transmitter in *C. elegans* and other nematodes, one receptor, EXP-1, mediates excitation from two GABA motoneurons (AVL and DVB) on to enteric muscle through a GABA-gated cation channel (McIntire *et al.*, 1993; Beg and Jorgensen, 2003). Beg and Jorgensen (2003) cloned *exp-1* and found that its receptor was localized on enteric muscle neuromuscular junctions. When

this receptor was expressed in *X. laevis* oocytes, GABA had an EC_{50} of $26.4 \mu M$ and the GABA current depended on sodium with negligible divalent cation permeability. EXP-1 was activated by muscimol, EC_{50} $420.5 \mu M$, but not blocked by bicuculline, picrotoxin, or mecamylamine. LGC-35 is probably also a GABA-gated cation channel subunit (Jones and Sattelle, 2008).

The body wall muscle GABA-gated chloride channel receptor is the target for the anthelmintic piperazine, which acts as a weak partial agonist (Martin, 1982) eliciting flaccid paralysis. Further studies have shown that piperazine activates currents in L1 muscle cells of *C. elegans* and, while ivermectin fails to activate currents, it does inhibit GABA-induced currents (Hernando and Bouzat, 2014). Single-channel currents were also activated by muscimol and piperazine.

The *C. elegans* genome encodes two metabotropic GABA_B-like GPCRs: *gbb-1* and *gbb-2* (Dittman and Kaplan, 2008); *gbb-1* mutants but not *gbb-2* mutants are long lived, indicating that *gbb-1* has a role in regulating lifespan (Chun *et al.*, 2015). In contrast, *unc-49* mutants had normal lifespans. The *gbb-1* cDNA is expressed in neurons, muscle, and intestine but only *gbb-1* expression in neurons rescued the *gbb-1* phenotype. GBB-1 regulates lifespan through G protein-PLC β signaling which transmits longevity signals to the transcription factor DAF-16/FOXO, a regulator of lifespan. Subsequent research found that mutations in four GABA receptors, *gbb-1*, *unc-49*, *lgc-36*, and *exp-1*, extended healthspan in *C. elegans* by promoting resistance to oxidative/heat/UV stress and pathogen resistance and two of these mutants, *gbb-1* and *exp-1*, also showed extended lifespan (Yuan *et al.*, 2019). The authors concluded that GABA receptors in *C. elegans* play a role in aging.

Table 6.5 summarizes the GABA receptor subtypes and tissue distribution in *C. elegans*, *H. contortus*, *B. malayi*, *T. spiralis*, *A. suum*, and *L. loa*.

Nitric oxide (NO)

NO is synthesized from arginine and oxygen by NO synthase (NOS). Intriguingly, while there is no evidence in the *C. elegans* genome for a gene for NO synthase, there is evidence that parasitic nematodes can synthesize NO (Masetti *et al.*, 2004). However, NO derived from bacteria in the gut of

Table 6.5. Summary of GABA receptors. Tissue distribution and/or developmental stage of expression is for the receptor class.

Species	Lifestyle	Receptor subtypes	Receptor class	Tissue distribution / developmental stage
<i>C. elegans</i>	Free-living	<i>unc-49</i> <i>lgc-35</i> <i>lgc-36</i> <i>lgc-37</i> <i>lgc-38</i> <i>exp-1</i> <i>gab-1</i> <i>gbb-1</i> <i>gbb-2</i>	Ion channel (Cl ⁻) Ion channel (Na ⁺ /K ⁺) Ion channel (Cl ⁻) Ion channel (Cl ⁻) Ion channel (Cl ⁻) Ion channel (Na ⁺ /K ⁺) Ion channel (Cl ⁻) GPCR GPCR	Motoneurons, body wall muscle, head muscle, enteric muscle
<i>H. contortus</i>	Parasitic (ruminant)	<i>Hco-unc-49</i> <i>Hco-lgc-35</i> <i>Hco-lgc-36</i> <i>Hco-lgc-37 (Hco-hg-1)</i> <i>Hco-lgc-38</i> <i>Hco-exp-1</i> <i>Hco-gab-1</i>	Ion channel Ion channel Ion channel Ion channel Ion channel Ion channel Ion channel	Neuronal, ventral nerve cord, nerve ring around the pharynx
<i>B. malayi</i>	Parasitic (human)	<i>Bma-unc-49</i> <i>lgc-37 (AAQA01024874.1)</i> <i>lgc-38 (XP_001891574)</i> <i>Bma-exp-1</i> <i>Bm1_48105</i>	Ion channel Ion channel Ion channel Ion channel GPCR	n.d.
<i>T. spiralis</i>	Parasitic (human)	<i>Tsp-unc-49</i> <i>Tsp-lgc-38</i> <i>Tsp-exp-1</i> <i>Tsp_03091</i>	Ion channel Ion channel Ion channel GPCR	n.d.
<i>A. suum</i>	Parasitic (porcine)	<i>Asu-unc-49</i> <i>lgc-35 (AEUI01011595)</i> <i>Asu-lgc-36</i> <i>Asu-lgc-37</i> <i>lgc-38 (AEUI01077442)</i> <i>exp-1 (AEUI01071915)</i> <i>Asu-gab-1</i>	Ion channel Ion channel Ion channel Ion channel Ion channel Ion channel Ion channel	Neuronal, nerve ring around the pharynx, body wall muscle

Continued

Table 6.5. Continued.

Species	Lifestyle	Receptor subtypes	Receptor class	Tissue distribution / developmental stage
<i>L. loa</i>	Parasitic (human)	<i>Llo-unc-49</i> <i>Llo-lgc-37</i> <i>Llo-lgc-38</i> <i>Llo-exp-1</i> <i>Llo-gab-1</i> <i>LOAG_07586</i>	Ion channel Ion channel Ion channel Ion channel Ion channel GPCR	n.d.

Ion conductance is indicated in parentheses. n.d. = not defined

C. elegans can increase thermotolerance and extend lifespan in *C. elegans* through a defined group of 65 genes under the dual control of DAF-16 and HSF-1 transcription factors (Gusarov *et al.*, 2013). L1 larvae of *Trichinella britovi* possess an enzyme capable of producing NO (Masetti *et al.*, 2004). Using NADPH-diaphorase histochemistry, NOS III antibody, and the anti-nitrotyrosine antibody, this activity was present in the cuticle and stichocytes. Evidence suggested that this NOS isoform differed from mammalian NOS. Positive NADPH-diaphorase staining occurs in neurons in the ventral ganglion, the retrovesicular ganglion, ventral and dorsal cords, and sublateral line of *A. suum* (Bascal *et al.*, 1995). Intense staining occurred in the motoneuron commissures, suggesting a role for NO at the neuromuscular junction. Staining was also present in the pharyngeal nervous system and peripheral nervous system innervating the sensory organs. These results indicate a widespread role for NO in the *A. suum* nervous system. There is also evidence that NADPH-diaphorase staining and SALMFamide-like immunoreactivity are co-localized, suggesting that NO may modulate the release of neuropeptides in *A. suum* and probably other parasitic nematodes (Bascal *et al.*, 1996). This suggestion is supported by the finding that NO is involved in the inhibitory action of two FMRFamide-related peptides on *A. suum* muscle (Bowman *et al.*, 1995). Both PF1 (SDPNFLRFamide) and PF2 (SADPNFLRFamide) hyperpolarize the muscle membrane potential and induce flaccid paralysis. The reversal of levamisole-induced contraction of the muscle by PF1 is blocked by *N*-nitro-*L*-arginine, which interferes with NO synthesis, while sodium nitroprusside, which releases NO in solution, mimics the effects of PF1 and PF2. Bowman *et al.* (1995) found evidence for the presence of NOS in *A. suum* hypodermis and body wall muscle. NOS has also been identified in body wall muscle of *B. malayi* and in *Acanthocheilonema viteae* and *D. immitis* (Pfarr and Fuhrman, 2000; Pfarr *et al.*, 2001). Overall, these observations suggest that NO may act as transmitter or modulator in nematodes.

Neuropeptides

Neuropeptides play important roles in the regulation of physiological processes in nematodes and can act locally at synapses, co-localized with

classical transmitters, or generally as neurohormones. It is likely that all neurons can synthesize and release one or more neuropeptides, but they also occur in non-nervous tissue. *C. elegans* has two major families of neuropeptides: insulin-like peptides (ILPs) and FMRFamide-like peptides (FaRPs or FLPs) (Li and Kim, 2008; Hobert, 2013). In addition, peptides that bear no resemblance to FLPs or ILPs are called non-insulin, non-FLP peptides (NLPs). Li and Kim identified 31 genes encoding over 70 possible distinct FLPs in *C. elegans* plus nine non-FLP peptides (Nelson *et al.*, 1998; Li *et al.*, 1999; Li and Kim, 2008). Over 40 FLPs have been isolated from *C. elegans* (Peymen *et al.*, 2014). The only *flp* gene absent from *C. elegans* is *flp-31*. There are 40 genes, *ins-1/39*, *daf-28*, encoding over 80 ILPs and 51 genes encoding over 120 NLPs in *C. elegans*. Neuropeptides are derived from pre-propeptides that are proteolytically cleaved and post-translationally modified to produce active peptides. The propeptides are cleaved by convertases. FLPs have been shown to affect locomotion, pharyngeal pumping, egg-laying, metabolism, aggregation, stress responses, sleep-like behavior, learning and memory, heat avoidance, and male mating (Li and Kim, 2014). Neuropeptides signal through GPCRs, which make up more than 2% of the *C. elegans* genome (Keating *et al.*, 2003), though there are exceptions; for example, FMRFamide from *Cornu aspersum* (formerly *Helix aspersa*) signals through an amiloride-sensitive sodium channel (Lingueglia *et al.*, 1995), which might also occur in nematodes. There are 12 FLP GPCRs in *C. elegans* but these can be activated by more than one FLP with a range of sensitivity and EC₅₀s from nM to mM. The *C. elegans* insulin/IGF-1 signaling pathways connect nutrient levels to metabolism, growth development, longevity, and behavior through a single ILP receptor, DAF-2/IGFR-like tyrosine kinase (Murphy and Hu, 2013), but other insulin receptor-related proteins, *irl*d genes, lack tyrosine kinase domains (Dlakić, 2002). Forty ILP genes have been overexpressed in *C. elegans* and assigned for phenotypes for roles, including dauer formation, L1 arrest life cycle, and fat accumulation (Zheng *et al.*, 2018). There are over 150 genes in *C. elegans* whose products share significant homology to known vertebrate or insect neuropeptide receptor families, both secretin-type but mainly rhodopsin-type, including neuropeptide F/Y,

ghrelin-obestatin/neuromedin U, neurokinin/neuropeptide FF/orexin, somatostatin, galanin, gonadotropin-releasing hormone, gastrin-cholecystokinin, vasopressin, *Drosophila* FMR-Famide and dromyosuppressin, but bombesin was absent (Cardoso *et al.*, 2012; Frooninckx *et al.*, 2012; Hobert, 2013).

To determine the neuropeptide ligand for an orphan GPCR, the receptor is expressed in a heterologous expression system, for example Chinese hamster ovary (CHO) or human embryonic kidney (HEK) cells, and the receptor-expressing cells are challenged with a library of potential peptide ligands (Frooninckx *et al.*, 2012). As already mentioned, a number of neuropeptides will probably activate the receptor, but those that are most potent are likely candidates as the endogenous ligand. The first nematode neuropeptide GPCR to be de-orphanized was the *C. elegans* gene C10C6.2 (Kubiak *et al.*, 2003), which was activated by neuropeptides encoded by flp-15: GGPGGLRF-NH2 (FLP15-1), RGPGLRF-NH2 (FLP15-2A), and its des-Arg1 counterpart, GPSGLRF-NH2 (FLP15-2B). A gene knockout study using RNAi has also shown that the *C. elegans* NPY receptor C10C6.2 is involved in locomotion (Keating *et al.*, 2003).

NPR-1, a homolog of vertebrate NPY, plays a role in feeding behavior, oxygen sensing, thermal avoidance, ethanol tolerance, and innate immunity in *C. elegans* (for review, see Frooninckx *et al.*, 2012), and is activated by FLP-18 and FLP-21. It has two alleles: both FLP-18 and FLP-21 activate the Val-215 allele, while only FLP-21 activates the Phe-215 allele. *C. elegans* with the Val allele are solitary feeders, while those with Phe allele are social (de Bono and Bargmann, 1998).

FLP genes have been identified in many parasitic nematodes, including *A. suum*, *A. ceylanicum*, *B. malayi*, *G. pallida*, *H. contortus*, *Meloidogyne incognita*, and *T. spiralis* (Peymen *et al.*, 2014). For example, *M. incognita* has 19 *flp* and 21 *nlp* genes (Abad *et al.*, 2008). The first FLP to be biochemically isolated was KNEFIRFamide (AF1) encoded by *flp-8* in *A. suum* (Cowden *et al.*, 1989). The diversity of FLPs across nematodes has been analysed using systematic BLAST searches of nematode ESTs on the GenBank database using known FLPs from *C. elegans* and *A. suum* (McVeigh *et al.*, 2005). A total of 1062 FLP-encoding ESTs from 46 species of nematodes were identified, with *flp-1*, *flp-11*, *flp-14*, and *flp-16*

being the most highly expressed genes. From this study it would appear that the level of structural conservation between species is high, though there is some interspecies variation. It is likely that all nematodes have an *flp* gene repertoire similar to *C. elegans* and that *flp* genes have distinct but overlapping expression patterns. In an attempt to de-orphanize FLPs, McCoy *et al.* (2014) identified putative *flp* and *flp*-GPCR gene homologs in 17 parasitic nematodes, including *A. suum*, *H. contortus*, *B. malayi*, and *G. pallida* (McCoy *et al.*, 2014). A total of 325 *C. elegans* sequences were identified, with *A. suum* possessing 84% of *C. elegans flp* genes; in contrast *T. spiralis* had only 13%. The most highly conserved de-orphanized FLP-GPCRs were NPR-4, NPR-5, matched to FLP-18, and NPR-11, matched to FLP-21. Evidence suggested pan-phylum conservation of ligand/receptor.

An oxytocin/vasopressin-like peptide, nematocin, has been identified in *C. elegans*, encoded by *ntc-1*, together with two nematocin receptors, NTR-1 and NTR-2 (Garrison *et al.*, 2012). When their cDNAs were expressed in HEK293T cells, nematocin decreased intracellular cAMP, suggesting coupling to a pathway that antagonizes adenylyl cyclase. Nematocin facilitates experience-driven modulation of salt chemotaxis in *C. elegans* (Beets *et al.*, 2012). Subsequently, nematocin has been shown to occur in parasitic nematodes, including *N. americanus* and *A. caninum* (Beets *et al.*, 2013). Nematocin is also important in social interaction between adult and larval progeny of *C. elegans* (Scott *et al.*, 2017).

Many pharmacological studies involving the actions of neuropeptides have been investigated using isolated strips of body wall muscle and the ovipositor muscle from *A. suum* and the isolated pharyngeal muscle preparation from *C. elegans* (Cowden *et al.*, 1989; Franks *et al.*, 1994; Maule *et al.*, 1995; Moffett *et al.*, 2003). Peptides have also been injected into the body cavity of *A. suum* to determine their effects on the whole organism (Reinitz *et al.*, 2000). Some peptides—AF1, AF2, AF4, and PF3—contract isolated muscle strips of *A. suum*, while others—PF1 (SDPNFLRFamide), PF2 (SADPNFLRFamide), and PF4, isolated from *Panagrellus redivivus* (Geary *et al.*, 1992b)—are inhibitory, relaxing the muscle. The inhibitory effect can be complex, involving up to at least four different mechanisms, depending on whether the ventral or dorsal nerve cord is present and on

whether the preparation is innervated, including a role for NO (Bowman *et al.*, 1995; Maule *et al.*, 1995). The excitatory effect of AF1 and AF2 is preceded by an inhibitory phase (Bowman *et al.*, 1996). Using a combination of body wall muscle strip and intracellular recordings from *A. suum* muscle, evidence suggests that the inhibitory effect of PF1 is a postsynaptic action directly on the muscle rather than inhibiting the presynaptic release of ACh or activating the release of GABA (Franks *et al.*, 1994). The reversal potentials for the inhibitory effects of PF1 and GABA were different. In a second paper, the same group showed that PF1 and PF2 also had a presynaptic action by inhibiting cholinergic excitatory junction potentials (ejps) in the muscle following stimulation of motoneurons in the nerve cord (Holden-Dye *et al.*, 1995). PF4 also hyperpolarizes *A. suum* body wall muscle, EC_{50} of 98 nM, compared with GABA, EC_{50} of 59 μ M (Holden-Dye *et al.*, 1997). Ivermectin blocked the GABA-induced increase in membrane conductance while the response to PF4 was unaffected.

The effects of a series of FLPs have been examined on the terminal bulb of isolated pharynxes from *C. elegans* using intracellular electrodes to record spike activity (Rogers *et al.*, 2001). PF1 and PF2 inhibited spike frequency, threshold 100 nM, while APEASPFIRFamide (FLP13A) inhibited with a threshold of 0.1 nM and an EC_{50} of 43 nM. AF1, AF2, and AF8 all excited spike frequency, threshold 100 nM. This study was extended to over 30 FLPs encoded by *flp* genes on pharyngeal pumping in *C. elegans* (Papaioannou *et al.*, 2005). Five FLPs were particularly potent: the inhibitory peptides 13A and FLP11A (AMRNALVRFamide) and the excitatory peptides FLP17A (KSAFLRFamide), FLP17B (KSQYIRFamide), and FLP8 (AF1). *flp-13* and *flp-18* both encode seven peptides, all inhibited pumping. The potent action of FLPs on pharyngeal pumping and their widespread occurrence in pharyngeal neurons suggests that they play a key role in the modulation of pumping activity in *C. elegans* and probably in parasitic nematodes.

The astonishing number of neuropeptides and their receptors identified in nematodes, together with their widespread distribution, clearly shows that, as in other phyla, they play key roles in all aspects of the physiology of nematodes (Jékely *et al.*, 2018). The same neuropeptides have been shown to occur in different

nematode species, both parasitic and free-living, indicating that they probably have similar functions throughout the phylum.

***C. elegans* and Drug Discovery**

Currently, there is a considerable number of high-throughput screening approaches conducted using diverse cell culture models. However, many important biological processes underpinning aging, cancer or neurodegenerative diseases can only be comprehensively studied in multicellular organisms. In this sense *C. elegans* is an attractive organism for screens, as the nematodes are easy to manipulate and culture, and are amenable to imaging because of their transparency. Their genetic tractability provides the opportunity to engineer the biological scenario against which to screen for drug action. The conservation of the proteome and genetic pathways with humans has prompted the development of screens for drug discovery in human medicine but equally this opportunity has been exploited in the quest for novel anthelmintics and nematocides. In recent years it has been used in an increasing number of pharmacological studies searching for nematocides, compounds with anti-aging properties or therapeutic drug target, although arguably the fruits of these efforts have yet to be realized.

In chemical genetic screens, molecules are tested for the ability to elicit specific phenotypes and the subsequent molecular targets are identified. To achieve these goals, frequently the animals tested are mutagenized and re-screened against the compound that elicited the phenotype. Resistant and/or hypersensitive mutants are isolated for genotyping and in this way the genetic basis of the drug action may be elucidated, including the identification of the drug target. This approach allows the elucidation of the molecular mode of action of compounds as well as accelerating the discovery of molecular targets (Burns *et al.*, 2015).

The earliest drug-screening approaches were carried out incorporating the drugs for testing into the solid agar medium (Brenner, 1974) with visual inspection but this procedure is labor intensive and not best suited to high-throughput experiments. To speed up this process, there have been advances in automated image-based microscopy platforms

coupled with high-throughput liquid workflows facilitating large-scale screens using multicellular model organisms such as *C. elegans* to identify therapeutic drugs or disease modifiers. Thus, the first automated large-scale screens emerged in 2006 (for review, see O'Reilly *et al.*, 2014). In one of those studies an automated worm transfer system was introduced, the Complex Object Parametric Analyzer and Sorter (COPAS™ BIOSORT), coupled to a semi-automated image digitalization whilst scoring phenotypes visually (Kwok *et al.*, 2006). In addition, a 96-well plate format with *C. elegans* cultured in liquid was developed for an RNAi high-throughput screen which was facilitated by robot handling and in-wells imaging (Lehner *et al.*, 2006). Following this experimental design, other studies have been established to screen thousands of compounds identifying bacteriostatic or life-extending activities (Moy *et al.*, 2006; Petrascheck *et al.*, 2009).

The first fully integrated high-throughput screen study, including automated worm transfer, image acquisition, and analysis (Gosai *et al.*, 2010), used COPAS™ BIOSORT and 1280 compounds were screened. The screen deployed *C. elegans* expressing the human α 1-antitrypsin fused with GFP, which results in protein aggregates that can be detected through an increase in fluorescence. It delivered a total of 33 compounds that enhanced the clearance of intracellular accumulation of the misfolded protein aggregates. The introduction of fluorescence-based approaches using *C. elegans* as a screening platform led to liquid ultra-high throughput screens using 384 or 1536 multi-well formats, thus increasing the capacity of drug testing. In these designs, the use of fluorescence plate readers, to assess changes in signal intensity, allows faster automatization of read-outs (Leung *et al.*, 2013). More refined and advanced automated platforms have been designed combining flow cytometry with high-throughput image processing, incorporating image segmentation protocols for a faster and accurate analysis of fluorescence signal-employing tools such as Developer Toolbox (GE Healthcare) and CellProfiler software. In 2018, one study used this advanced automatization to establish a high-throughput screen to identify balanced lethal mutations employing *C. elegans* transgenic strains carrying "GFP stress reporters". This was useful to identify genetic interactors, with human orthologs, inducing the mitochondrial unfolded protein response (Hernando-Rodríguez *et al.*, 2018).

The development of microfluidic device techniques for *C. elegans*, incorporating multi-channels, layers, and valves, allows controlled immobilization as well as a precise administration of chemical compounds. These micro-devices have been used for genetic and chemical screens in *C. elegans*, focused on aging, neurodegeneration, or metabolic disorders, among others (Midkiff and San-Miguel, 2019). Carr *et al.* (2011) designed a device for screening nematocides compounds against parasitic nematodes. Behavioral patterns such as swimming or thrashing in single worms placed in individual droplets can be monitored and automatically analyzed based on velocity and position algorithms. This design allows the quantification of the drug responsiveness in real time comparing parameters pre- and post-exposure. A lab-on-a-chip for high-resolution imaging analysing levels of polyglutamine aggregation, with a capability of high throughput of about 100,000 worms with a library of different chemical compounds, has also been deployed (Mondal *et al.*, 2016). This microfluidic platform has the ability for imaging over 4000 worms in about 15 min.

Ding *et al.* (2017) used a microfluidic chamber to test combinatorial effects of anthelmintic drugs on the survival of *C. elegans* as a model for identifying drugs that can eliminate parasitic nematodes. Worms are placed in an open chamber where they are free to swim. Software measures the centroid velocity and track curvature of each worm in response to different combinations of four drugs that are fed to the device. The device relies on a feedback system control (FSC) scheme which uses iterative data from previously measured drug concentrations to modify and identify the most effective concentrations and drug combinations. In the case of anthelmintic drugs, the most effective combination yields the lowest centroid velocity with high track curvature. This platform can be used to test combinations of drugs with other non-anthelmintic functions and can be used with parasitic nematodes as well.

Outlook

From the above it can be seen that the pharmacology of nematodes is both the same, and different, compared with mammalian systems. It is the same in that there is an overall conservation

of the classes of neurotransmitter and the receptors that they signal through. However, like other invertebrates, and unlike mammals, nematodes utilize octopamine instead of noradrenaline. There are more remarkable differences in the ligand-gated ion channel family where nematodes express a larger subset of Cys-loop channels not found in mammals. The best-studied of these is the glutamate-gated chloride channel that is the target for ivermectin, but there are many others that require further characterization. These differences between the pharmacological properties of the nematode and mammalian systems are of considerable future interest with respect to the development of novel, resistance-breaking anthelmintics and nematicides with an excellent profile of selective toxicity.

The similarity of receptor signaling between nematodes and mammals has been important, as this has provided a route to improve fundamental understanding of the molecular determinants of the pharmacology of neurotransmitter receptor systems. An excellent exemplar of this is a genetic suppressor screen in *C. elegans* that identified an auxiliary subunit that regulates ionotropic glutamate receptors (Walker *et al.*, 2006). There is little doubt that the use of the powerful approach of forward

genetics in *C. elegans* will continue to deliver new pharmacological insights.

The experimental tools that are available now, and continuing to develop, to investigate the pharmacology of nematode systems provide the opportunity for an expansion in this important knowledge base. Genetic tools such as RNAi gene interference and CRISPR-cas9 gene editing are being deployed to good effect (McCoy *et al.*, 2015; Gang *et al.*, 2017). Moreover, the ability to “humanise” *C. elegans* by CRISPR-cas9 gene editing has the capability to provide *C. elegans* for drug screening that have been engineered to have optimal relevance to the therapeutic goals of the study (Rawsthorne *et al.*, 2021).

Arguably, the pharmacological topic that has attracted least attention in nematodes is that of pharmacokinetics. Insight into the biological processes that regulate drug absorption, transport, distribution, metabolism, and excretion is relatively sparse, although there is information on ABC transporter systems and their role in drug detoxification mechanisms (Ardelli, 2013). In the future, the pharmacokinetics of nematodes merits greater attention in order to underpin insight into anthelmintic action, to facilitate the use of chemical nematicides, and also to optimize the use of *C. elegans* for drug discovery.

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7 Nematodes as Ecological and Environmental Indicators

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Introduction

Aquatic and soil ecosystems throughout the world are seriously threatened by multiple forms of anthropogenic stress, such as chemical contamination, waste pollution, and climate change. The negative effects on living organisms, especially the decrease of species diversity, in turn impact the ecosystems they inhabit, making them dysfunctional and less resilient, with a limited ability to provide important services such as nutrient cycling, drinking water, and sustainable crop production. In addition, the accumulation of pollutants in aquatic and terrestrial food webs poses risks to human health via contaminated food and water.

Recognizing the threats arising from the pollution of water resources and soils, scientists and regulators have developed numerous methods for assessing the ecotoxicological risks to aquatic and soil ecosystems at all organizational (from molecules to whole organisms) and ecological (from single species to food webs) levels. Important regulations for evaluating the risk of chemicals in the environment, both pro- and retrospectively, have been adopted within the European Union (e.g., European Chemical Agency, European Food Safety Authority, EU-Water Framework Directive), by the USA (e.g., US Clean Water Act), and at the international

level (OECD). However, despite the effort invested to develop appropriate tools for risk and quality assessments, the protection of our environment remains a major challenge. To date, the goal to achieve a good ecological and chemical status of all surface waters in Europe (European Community, 2000) has been reached only for about 40% of European surface water bodies, with rivers generally having a lower status than lakes and coastal waters (European Environment Agency, 2018).

In this chapter, we present tools to assess the risk of chemical pollution in freshwater and soil ecosystems using nematodes as either indicators or models in ecotoxicology. In the last section, we further highlight the potential role of nematodes as ecological models in the meta-community context. Those analyses are key to understand how anthropogenic stressors affect not only local communities but also the regional species pool (metacommunities) and biodiversity, despite the role of spatial and other environmental factors.

Toxicity Testing

Tools to assess the risk of chemical pollution in the environment must be able to detect its impact, including on biodiversity loss and climate

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change (Landis *et al.*, 2013). In the case of contaminants released into aquatic and terrestrial environments, whether by agricultural, municipal, or industrial emissions, a variety of ecotoxicological approaches is required to fully document the extent of the stress. For example, biological methods can be employed to estimate the bioavailability of pollutants and their toxicity to the biota. Along with chemical analyses, biologically based approaches are an indispensable component of environmental risk assessments in water, sediment, and soil, both for single chemicals and for chemical mixtures (European Commission, 2003; ECHA, 2008; EFSA, 2013). Since the late 1960s, ecotoxicological methods have been continuously refined to encompass various habitats (water, sediment, soil), organisms (bacteria, plants, animals), trophic levels (decomposers, producers, consumers), organizational levels (from molecules to food webs), and effect mediators (e.g., specific receptors). Two major strategies have to be distinguished:

1. Prospective risk assessments are aimed at chemical authorization and restrict the types of chemicals that can be released into the environment (e.g., plant protection products) (EFSA, 2013).

2. Retrospective risk assessments target environmental samples suspected to have been contaminated with chemical substances (e.g., dredged sediments) (Manz *et al.*, 2007).

In prospective risk assessment, *in vitro* and *in vivo* toxicity tests are conducted to investigate mechanisms of toxicity and to set accurate toxicity thresholds (European Commission, 2003; EFSA, 2013). With higher-tier testing, toxicity thresholds can be refined so as to reduce safety risks (Boxall *et al.*, 2002). More realistic toxicity testing can be achieved using: (i) chronic toxicity endpoints such as reproduction; (ii) realistic exposure scenarios (for example, sediments for scarcely soluble substances); or (iii) ecologically more relevant, multi-species test systems (model ecosystems), such as micro- or mesocosms.

In retrospective risk assessments, the ecological status or quality of an ecosystem is determined by evaluating the *in situ* fauna or flora (EU-WFD) (European Community, 2000). In those studies, the application of indicator systems can help to identify habitats with poor ecological status and to link this status either to the

presence of chemical pollution or to other types of stress, including hydro-morphological modifications and climate change (e.g., Von der Ohe *et al.*, 2007; Höss *et al.*, 2011c; Von der Ohe and Goedkoop, 2013). However, in the case of efficient water management aimed at improving the ecological status of ecosystems (as mandated by the EU-WFD), methods are needed to unravel the causes of the deleterious effects of chemical pollutants on ecosystems (De Zwart *et al.*, 2009). Here, toxicity tests can be used to detect direct toxicity of chemicals in environmental samples (e.g., Tuikka *et al.*, 2011; Feiler *et al.*, 2013) and of the contaminated environmental samples themselves, such as dredged sediments (Manz *et al.*, 2007).

The many different ecotoxicological and ecological approaches for assessing the risk posed by chemical pollution in the environment are supported by a multitude of nematode-based tools. A comprehensive overview of these tools can be found in *Nematodes as Environmental Indicators* (Wilson and Kakouli-Duarte, 2009) and in several review articles (e.g., Haegerbaeumer *et al.*, 2015).

Single-species toxicity testing

As shown in Table 7.1, many different nematode species, apart from *Caenorhabditis elegans*, have already been used in single-species toxicity testing (Haegerbaeumer *et al.*, 2015).

However, the nematode species most commonly used in ecotoxicological studies remains *C. elegans*, as many of its features are advantageous for experiments, not only in ecotoxicology but also in genetics, medical sciences, and other life science disciplines. These features include the following.

- **Easy culturing.** If food is lacking, *C. elegans* is able to form dauer larvae that can be easily maintained for several months. If test organisms are needed, providing the dauer larvae with food initiates the continuation of their normal development.
- **Short generation cycle.** Within 3 days, *C. elegans* develops from the first juvenile stage (J1) to reproducing adult worms. This allows the nematode's use in ecologically relevant, sublethal toxicity endpoints, such

Table 7.1. Nematode species (other than *Caenorhabditis elegans*) used in toxicity testing.

Species	Toxicity endpoint	Reference
Soil and freshwater		
<i>Acrobeloides nanus</i> ; <i>A. sp.</i>	DEB modeling, development, population growth, reproduction, survival	Alda Álvarez <i>et al.</i> (2006); Hao <i>et al.</i> (2010); Mueller <i>et al.</i> (2020a); Wei <i>et al.</i> (2003)
<i>Aphelenchus avenae</i>	Growth, survival	Igonoffo and Dropkin (1977)
<i>Bursilla monhystera</i>	Chemotaxis, survival	Höckelmann <i>et al.</i> (2004); Monteiro <i>et al.</i> (2018)
<i>Caenorhabditis briggsae</i>	Activity, development, growth, reproduction	Bottjer <i>et al.</i> (1985); Briggs-Gochner and McCoy (1954)
<i>Heterocephalobus pauciannulata</i>	Life cycle traits	Kammenga and Riksen (1996)
Multi-species	Survival	Bongers <i>et al.</i> (2001); Haegerbaeumer <i>et al.</i> (2018b); Kammenga <i>et al.</i> (1994)
<i>Panagrellus redivivus</i> ; <i>P. silusiae</i>	Development, feeding, growth, gonad development, maturation, molting, movement, reproduction, survival	Boroditsky and Samoiloff (1973); Samoiloff <i>et al.</i> (1980); Bottjer <i>et al.</i> (1985); Boyd and Williams (2003); Debus and Niemann (1994); Haight <i>et al.</i> (1982); Igonoffo and Dropkin (1977); Monteiro <i>et al.</i> (2018); Mudry <i>et al.</i> (1982); Ongley <i>et al.</i> (1988); Ross and Henebry (1989); Samoiloff <i>et al.</i> (1983); Sherry <i>et al.</i> (1997)
<i>Panagrolaimus cf thienemanni</i>	Growth, reproduction	Brinke <i>et al.</i> (2011)
<i>Paroigolaimella bernensis</i>	Survival	Monteiro <i>et al.</i> (2018)
<i>Plectus acuminatus</i> ; <i>P. aquatilis</i>	Reproduction, life cycle traits, population growth; survival	Kammenga <i>et al.</i> (1996); Monteiro <i>et al.</i> (2018); Mueller <i>et al.</i> (2020a)
<i>Poikilolaimus sp.</i>	Survival	Monteiro <i>et al.</i> (2018)
<i>Pristionchus pacificus</i>	Development, egg hatching, fecundity, feeding, growth, lifespan, morphology, movement, reproduction, survival	Boyd and Williams (2003); Meyer <i>et al.</i> (2009); Rae <i>et al.</i> (2010); Rudel <i>et al.</i> (2013); Wei <i>et al.</i> (2003); Zhang <i>et al.</i> (2013)
Marine		
<i>Diploilaimella meylli</i> ; <i>D. oschei</i> ; <i>D. sp.</i>	Development; survival	Monteiro <i>et al.</i> (2018); Vranken and Heip (1986)
<i>Litoditis marina</i>	Survival	Monteiro <i>et al.</i> (2018)
<i>Enoplus brevis</i> ; <i>E. communis</i>	Survival	Howell (1984)
<i>Monhystera disjuncta</i> ; <i>M. microphthalmia</i>	Development, fecundity, survival	Vranken <i>et al.</i> (1985, 1988, 1989, 1991)
<i>Rhabditis (Pellioditis) marina</i>	Egg-hatching, population development, survival	Derycke <i>et al.</i> (2007); Lira <i>et al.</i> (2011); Meyer <i>et al.</i> (2009); Vranken <i>et al.</i> (1985)
Parasitic		
<i>Meloidogyne incognita</i>	ACHe/ATPase inhibition, chemotaxis, egg hatching, survival, root infection	Faske and Hurd (2015); Nasr (2015); Shivakumara <i>et al.</i> (2018); Silva <i>et al.</i> (2017)
<i>Rotylenchulus reniformis</i>	Survival, root infection	Faske and Hurd (2015)
<i>Steinernema feltiae</i>	Behavior, survival	Barua <i>et al.</i> (2020)

ACHe: acetylcholinesterase; ATPase: adenosine triphosphatase; DEB: dynamic-energy-budget

as reproduction, and in multi-generation testing. The life cycle of *C. elegans* is described in Muschiol *et al.* (2009).

- **Transparent body.** The transparency of the *C. elegans* body allows microscopic observations of tissues and internal structures (gonads, intestine) in the living organism without the need for histological processing. For example, labeled substances and the reactions of reporter genes (e.g., *gfp*) can be followed.
- **Maintenance in aqueous and solid substrates.** *C. elegans* can be maintained on agar, gelrite, water, sediment, and soil; this flexibility allows toxicity tests to be performed in all kinds of environmental samples.

The use of *C. elegans* as a model organism in environmental science has been reviewed in several publications (e.g., Leung *et al.*, 2008; Höss and Williams, 2009; Meyer and Williams, 2014; Haegerbaeumer *et al.*, 2015; Tejeda-Benitez and Olivero-Verbel, 2016; Hunt, 2017; Chen *et al.*, 2019; Höss, 2021). Moreover, *C. elegans*, although mainly found in terrestrial habitats, turned out to be well representative for many freshwater species in terms of their sensitivity to toxic chemicals (Haegerbaeumer *et al.*, 2018a). Many groups of chemicals (metals, pesticides, endocrine disruptors, nanoparticles, microplastics) have been tested in various substrates (water, sediment, soil) using many different toxicity endpoints, such as survival, growth, reproduction, and behavior, as well as a wide range of biomarkers, especially gene and heat shock protein expression. Mechanisms of toxicity have been investigated using hypersensitive or resistant mutant strains. The standardization of a toxicity test based on *C. elegans* led to an international standard guideline (ISO 10872:2020) (ISO, 2020) and an ASTM guideline (ASTM E2172-01) (ASTM, 2014), both of which allowed the testing of chemical toxicity in water, sediment, and soil samples.

In this section, we discuss the suitability of *C. elegans* as a model organism in the field of ecotoxicology, drawing upon examples spanning a large range of applications.

Chemical and material testing

Nematodes, and especially *C. elegans*, have already been used to assess the toxicity of many types of environmental chemicals, including

pesticides (Meyer and Williams, 2014; Neury-Ormanni *et al.*, 2019), endocrine disrupting chemicals (Novillo *et al.*, 2005; Höss and Weltje, 2007), polycyclic aromatic hydrocarbons (PAHs) (Sese *et al.*, 2009; Fischer *et al.*, 2016), metals (Williams and Dusenbery, 1990; Boyd *et al.*, 2003; Höss *et al.*, 2011b), nanoparticles (Gorka *et al.*, 2015; Höss *et al.*, 2015a; Hanna *et al.*, 2016), microplastics (Lei *et al.*, 2018; Mueller *et al.*, 2020b), and natural toxins (Wei *et al.*, 2003; Höss *et al.*, 2013). Moreover, toxicity tests have been conducted in different substrates, such as water, sediment, and soil, with toxicity endpoints ranging from molecular biomarkers to population growth.

Nano- and microplastic toxicity testing is a comparatively new discipline in ecotoxicology that combines the challenges of particle and chemical testing, as organisms are exposed not only to particles of different sizes and shapes, but also, potentially, to chemicals that leach from the particles into the dissolved phase (Duis and Coors, 2016; Wang *et al.*, 2018; Haegerbaeumer *et al.*, 2019a; Kögel *et al.*, 2020). Therefore, particle-feeding sediment- and soil-inhabiting organisms such as nematodes are well suited as test organisms to determine the impacts of nano- and microplastics. In their critical review of ecotoxicity test methods for nanomaterials, Handy *et al.* (2012) explicitly cited *C. elegans* as a promising test organism in assessments of the toxicity of nanoparticles, especially in complex substrates such as soils. In fact, the use of *C. elegans* in nano-toxicity research, and therefore knowledge on nano-related toxicity mechanisms, is steadily increasing (Wu *et al.*, 2019).

Recently, Fueser *et al.* (2019) examined the ability of nematodes to ingest polystyrene (PS) microbeads and found a simple relationship between the size of the beads and that of the buccal cavity of the studied nematode species. For *C. elegans*, its bacterial food is similar in size to 1 μm beads, such that both are quickly taken up in large quantities (Fueser *et al.*, 2019, 2020). In a subsequent study, Mueller *et al.* (2020b) investigated the potential toxicity of PS beads for *C. elegans* by setting up toxicity thresholds for beads of various sizes. Based on PS concentrations (mg PS/ml), size-related effects were determined for the beads (Fig. 7.1A) and they increased with decreasing bead diameter (EC_{50} : 6.3 mg/ml for 10 μm beads and 0.077 mg/ml for 0.1 μm

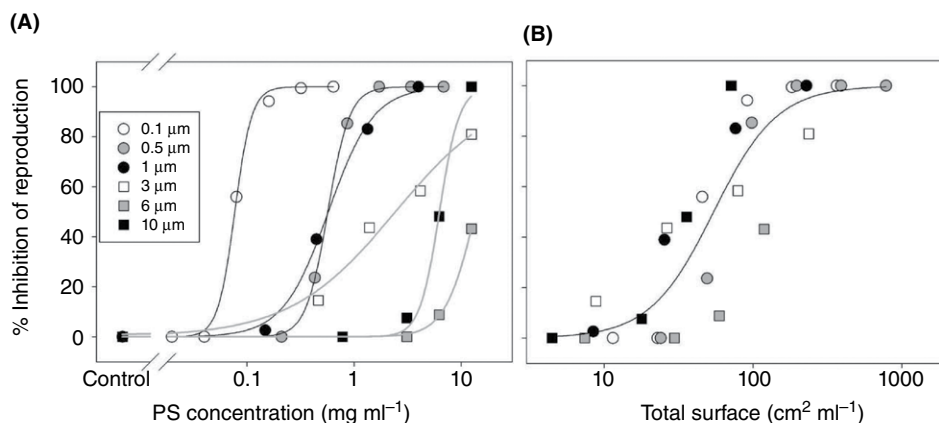


Fig. 7.1. Dose–response relationship for *C. elegans* exposed to PS beads of different diameters (0.1–10 µm). **(A)** Dose–response curves for each bead size based on the PS concentration. **(B)** Bead-size-independent dose–response curve based on the total surface area of all beads. Reprinted with permission from Mueller *et al.* (2020b), ©American Chemical Society (2020).

beads). By relating those effects to the total surface area of all beads present in the test system, the authors showed that, independent of bead size, a 50% inhibition of reproduction occurred at a total bead surface area of approximately 50 cm²/ml (Fig. 7.1B). Interestingly, this was true also for beads with a diameter > 3 µm, which cannot be ingested by *C. elegans*. Thus, external mechanisms caused the observed effects on *C. elegans*. Toxic effects of leached styrene (the monomer of PS) could be excluded by comparing styrene toxicity thresholds with measured concentrations in the test medium. Instead, indirect food effects, such as a reduced availability of food bacteria, were considered to explain the decrease in nematode reproduction.

Hanna *et al.* (2016) performed a “cause–effect” analysis of nanoparticles and *C. elegans* based on ISO 10872. In the discussion, the authors considered the factors that specifically influence nanoparticle testing and thus lead to a larger variability than occurs in toxicity testing of dissolved chemicals (Fig. 7.2A). Beside the density of food bacteria, these included the type of test medium, as demonstrated in tests of positively charged amine-coated PS nanoparticles (Hanna *et al.*, 2016). In a subsequent study, positively charged nanoparticles and the food bacteria supplied in the *C. elegans* toxicity test were shown to form hetero-agglomerations that subsequently induced indirect inhibitory effects on nematode growth (Hanna *et al.*, 2018) (Fig. 7.2B). With

negatively charged nano- and microparticles, agglomeration was not observed (Hanna *et al.*, 2018; Mueller *et al.*, 2020b). These studies demonstrated the importance of considering the test conditions in the interpretation of toxicity test results for nano- and microparticles and therefore also the need for standardized test methods.

Standardization

The standardization of toxicity tests is a prerequisite for obtaining reliable and reproducible test results. This is especially the case in tests of environmental samples and other complex substrates, such as aquatic sediments and soils, in which not all factors can be kept constant, and when sensitive toxicity endpoints, such as reproduction, are used (Traunspurger *et al.*, 1997) that are impacted not only by chemical pollution but also by other properties of the tested matrix, such as grain size composition, organic matter content, and pH. For *C. elegans*, the percentage of fine particles (clay and silt) was shown to correlate negatively with growth and reproduction (Höss *et al.*, 1999, 2010), probably due to the lower food availability in fine sediments. Therefore, the amount of food in the test system is among the crucial factors that have to be standardized (ISO, 2020). Ring tests have shown that *C. elegans* toxicity testing according to ISO 10872 is a reliable tool for assessing the toxicity of contaminated sediments and soils (Fig. 7.3) (Höss *et al.*, 2012; ISO, 2020).

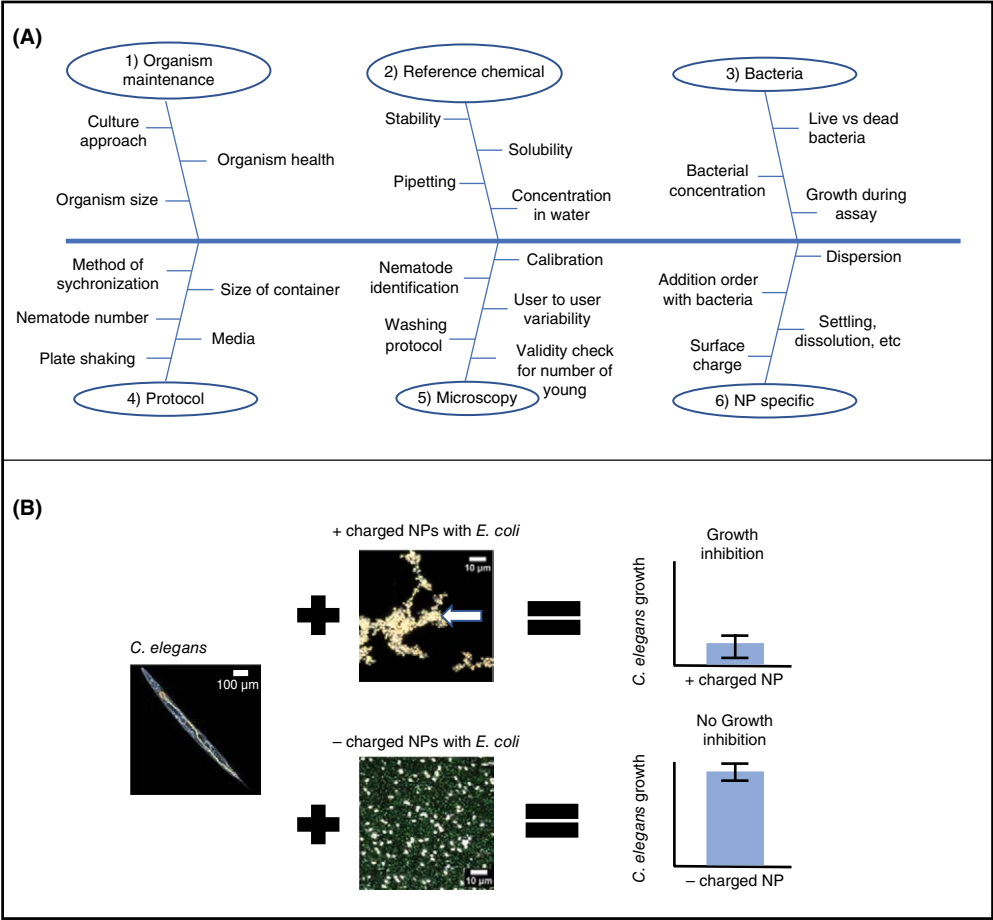


Fig. 7.2. (A) Cause–effect analysis of the major factors that lead to variability in the results of nano-toxicity testing with *C. elegans* based on ISO 10872; reprinted from Hanna *et al.* (2016) with permission of the Royal Society of Chemistry. **(B)** Influence of the surface charge of nanoparticles on the agglomeration of food bacteria and the consequences for toxic effects; adapted with permission from Hanna *et al.* (2018), ©American Chemical Society (2018).

To better classify sediments and soils according to their toxicity, thresholds have been established to account for the “natural” variability in the toxicity endpoints that occurs even in tests of sediments and soils with background levels of pollutants, i.e., without the added impact of toxic chemicals (Höss *et al.*, 2009, 2010). These thresholds facilitate decision making in risk assessments and help to avoid false-positive results (i.e., significant effects caused by factors other than the chemicals of interest). Studies in reference sediments and soils have revealed considerably less variability in the growth rate than in reproduction. Moreover, as for both endpoints there is less

variability in soils than in sediments, the threshold values for growth and reproduction in these two media differ. For sediments, inhibition of growth and reproduction compared with the control sediment must exceed 25% and 50%, respectively, before the effect can be regarded as toxic (Höss *et al.*, 2010). For soil, however, the thresholds for growth and reproduction are 10% and 40% inhibition, respectively (Höss *et al.*, 2009).

Testing of environmental samples

Despite the reliability and sensitivity of *C. elegans* in assessments of the toxicity of chemicals in

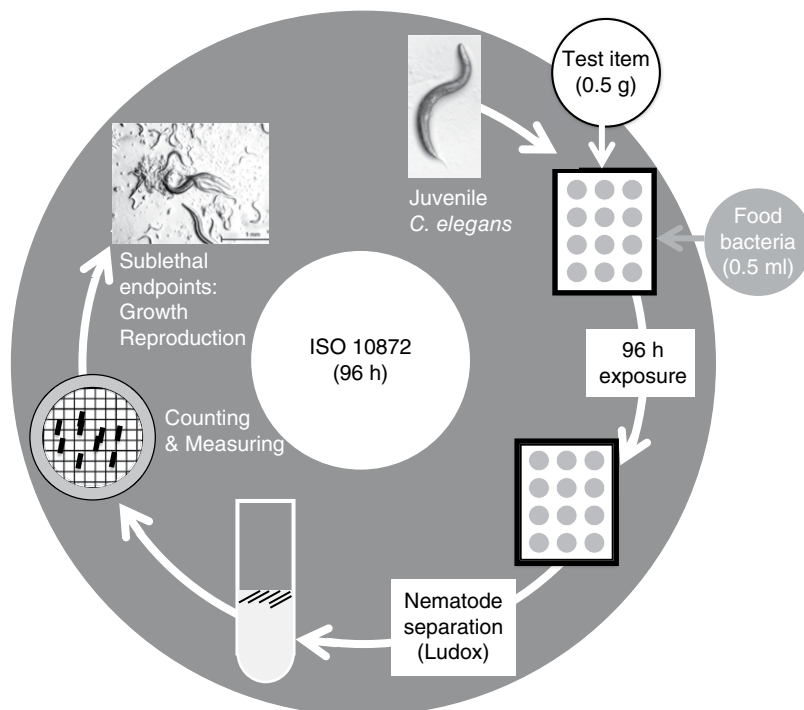


Fig. 7.3. Test scheme for toxicity testing with *C. elegans* (ISO 10872).

sediments and soils, there are few reports on the use of this test system. Instead, most toxicity tests with sediment are based on other soil invertebrates (for example, for sediment: the chironomid *Chironomus riparius*, the oligochaete *Lumbriculus variegatus*, the amphipod *Hyalella azteca*; and for soil: the oligochaetes *Eisenia fetida* and *Lumbricus terrestris*, the collembolan *Folsomia candida*). In the few studies that assessed toxicity in environmental samples using *C. elegans*, the nematode was used alone (Menzel *et al.*, 2009; Tejeda-Benitez *et al.*, 2016) but more often in combination with other test systems (Höss *et al.*, 2009; Tuikka *et al.*, 2011; Feiler *et al.*, 2013; Schertzinger *et al.*, 2019).

Feiler *et al.* (2013) conducted a battery of sediment contact tests to assess the toxicity of various river sediments using test organisms from various trophic and organizational levels (bacteria, yeast, higher plants, nematodes, oligochaetes, and fish embryos) and found a gradient of chemical contamination ranging from low to high. That study took advantage of both the various exposure routes of sediment- and pore-water-associated chemicals in the different test

organisms and the different susceptibilities of the organisms toward the chemicals. Using the non-redundant information provided by the various tests and applying test-specific toxicity thresholds, the authors were able to reliably rank the river sediments according to their toxicity and to link sediment-associated effects with the toxic potential calculated from the sediment quality guidelines provided by MacDonald *et al.* (2000). Within the test battery, nematodes belonged to the most sensitive test species and their response could be well related to the toxic potential of the measured organic chemicals (maximal TEC-Q) (Fig. 7.4A).

Similar results were obtained in toxicity assessments of soil and waste in which the performance of *C. elegans* was compared with that of routine soil test organisms, such as *Folsomia candida* (Collembola), *Eisenia andrei* (Oligochaeta), *Brassica rapa* (higher plants), and *Arthrobacter globiformis* (bacteria) (Höss *et al.*, 2009; Höss and Römbke, 2019). In those studies, as well, *C. elegans* provided non-redundant information regarding the overall toxicity of contaminated soils (Fig. 7.4B) and of waste that had been

mixed with standard soil (Fig. 7.4C). In contaminated soils, *C. elegans* detected toxicity in soils highly contaminated with PAHs, while the other test systems better demonstrated metal contamination (Höss *et al.*, 2009) (Fig. 7.4B). For the ecotoxicological assessment of waste, although *C. elegans* was less sensitive than collembolans earthworms and plants, it added information useful to the overall risk assessment (Höss and Römbke, 2019; Fig. 7.4C).

Eco(toxico)logical modeling

The short life cycle of *C. elegans* enables studies that use ecologically relevant toxicity endpoints, such as population growth rate, in combination with ecological modeling to obtain detailed insights into toxicodynamic and toxicokinetic processes. With the use of the appropriate techniques, the life cycle parameters and

population growth rates of *C. elegans* can be very accurately determined. Muschiol and Traunspurger (2007) carried out life cycle experiments in a very small but well-defined experimental set-up, consisting of 10 µl drops of a semi-fluid gellan gum (gelrite; comparable to 0.4% agar), that provided constant conditions for various species of nematodes throughout the duration of the experiment (up to 60 days). Muschiol *et al.* (2009) then used this same set-up to compare the life-history traits of two strains of *C. elegans*, the N2-strain (in continuous laboratory culture since the 1940s) and a more recently isolated strain (MY6; Münster, Germany). Although isolated 700 km and 60 years apart from each other, the two strains barely differed in their life cycle parameters, showing that the N2-strain still represents the natural population of *C. elegans* in terms of its life history traits (Fig. 7.5).

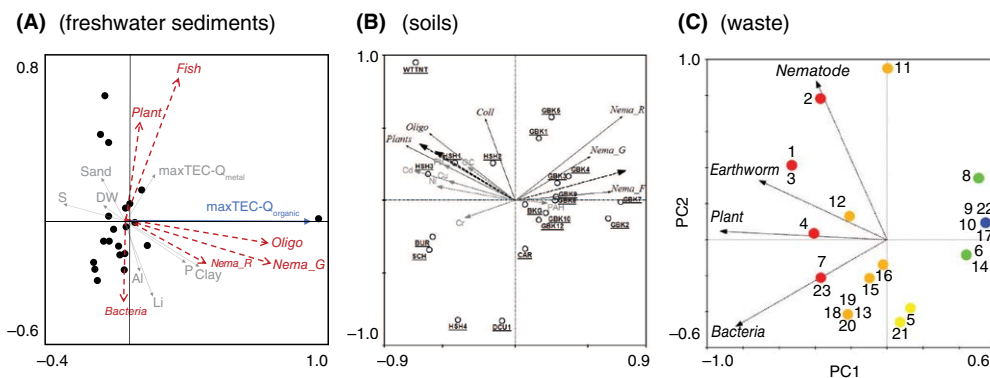


Fig. 7.4. Multivariate analysis based on the performance of *C. elegans* compared with that of routine test organisms in toxicity assessments of contaminated freshwater sediments **(A)**, contaminated soils **(B)**, and waste **(C)**. **(A)** Redundancy analysis (RDA) of sediment contact tests: Fish = mortality of *Danio rerio* embryos; Plant = inhibition of the growth rate of *Myriophyllum aquaticum*, ISO 16191; Bacteria = inhibition of the enzyme activity of *Arthrobacter globiformis*; Nema_R/G = inhibition of *C. elegans* reproduction/growth, ISO 10872; Oligo = inhibition of *Lumbriculus variegatus* reproduction, OECD 225; DW = sediment dry weight; P = total phosphorus; S = total sulfur; Al = aluminum; Li = lithium; max-TEC-Q_{metal/organic} = maximal quotients of the measured concentrations of metals and organic pollutants divided by those defined in sediment quality guidelines (threshold effect concentrations) according to MacDonald *et al.* (2000); modified from Feiler *et al.* (2013) with permission of John Wiley and Sons. **(B)** RDA of the toxicity tests performed with soils characterized by a pollution gradient. Plants = *Brassica rapa* shoot length; Coll = *Folsomia candida* reproduction; Nema_F/G/R = *C. elegans* fertility, growth, reproduction; Oligo = *Eisenia andrei* reproduction; PAH = polycyclic aromatic hydrocarbons. From Höss *et al.* (2009) with permission of Elsevier. **(C)** Principal component analysis (PCA) based on LID (lowest ineffective dilution) values of waste samples determined in four different soil toxicity test systems: bacteria (*Arthrobacter globiformis*); plant (*Brassica napus*); earthworm (*Eisenia fetida*); and nematode (*C. elegans*). Colors represent the hazard classes (blue = no hazard; green = low; yellow = moderate; orange = high; red = very high). From Höss and Römbke (2019) with permission of Springer Nature.

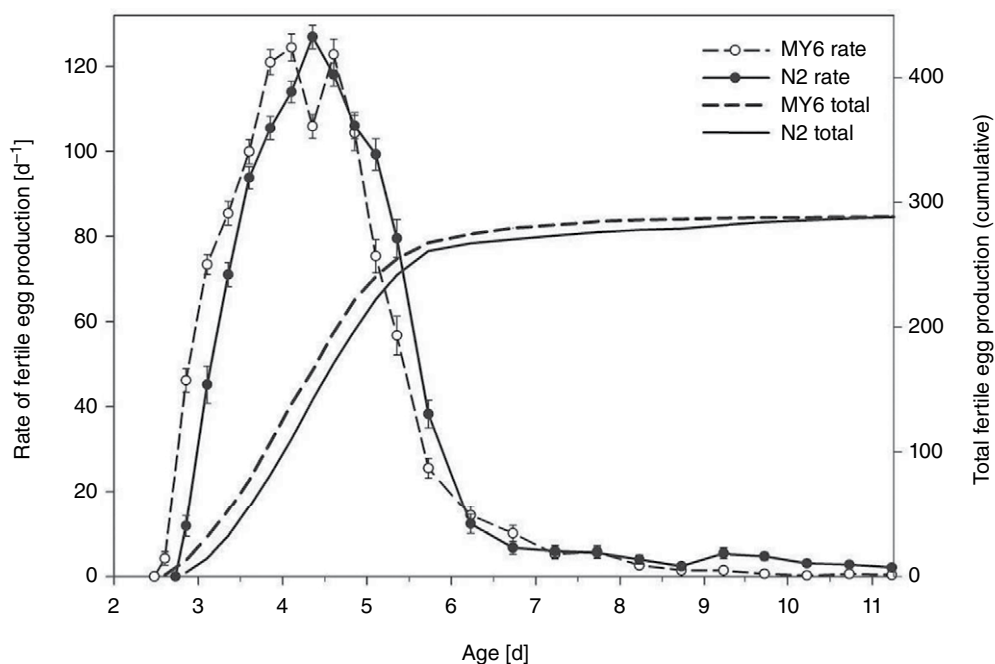


Fig. 7.5. Fecundities of *C. elegans* strains MY6 and N2. Population sizes of $n = 72$ (MY6) and $n = 69$ (N2) until age 5.7 days (d), and $n = 36$ (MY6 and N2) thereafter; bars indicate the standard error. Reprinted from Muschiol *et al.* (2009).

Using dynamic energy budget (DEB) modeling, a powerful tool that allows the extrapolation of toxicity data from the individual to the population level (Jager *et al.*, 2014a), Jager *et al.* (2014b) analyzed the effects on *C. elegans* of cadmium (Cd) and fluoranthene (FA) in both single and mixed exposures and then compared the modeling results with more traditional methods consisting of dose-response curves at single time points (Fig. 7.6). Both approaches indicated an antagonistic interaction of the two compounds in mixtures. However, DEB modeling provided greater insights into the dynamic processes underlying the toxic effects (Jager *et al.*, 2014b). A better fit of the model and a possible explanation for the antagonistic effect were achieved by assuming that, in the mixture, Cd caused a decrease in the elimination rate of FA in *C. elegans* (Fig. 7.6).

Toxicokinetic modeling

An understanding of toxicokinetic processes in organisms can improve assessments of the ecological risk of chemicals but requires knowledge

of the uptake and fate of those chemicals (including their absorption, distribution, metabolism, and elimination) in an organism over time (ADME studies) (Gupta, 2016). As an organism's response to chemicals is dependent on their environmental bioavailability, toxicokinetic data provide information that facilitates interpretation of ecotoxicological results.

Spann *et al.* (2015) investigated the bioaccumulation of phenanthrene in *C. elegans*, focusing on the uptake route of this hydrophobic PAH (i.e., via water or food ingestion). Their study was in part motivated by the unclear role of food-bound versus dissolved chemicals in the toxicity of chemicals to *C. elegans* (Offermann *et al.*, 2009; Höss *et al.*, 2011b; Yu *et al.*, 2012). A two-compartment model involving the partitioning of phenanthrene from a central to a peripheral compartment within *C. elegans* (Fig. 7.7A) best explained the experimental bioaccumulation data (Fig. 7.7B) (Spann *et al.*, 2015). Phenanthrene was taken up predominantly as a dissolved compound, even in the presence of food, which contributed only 9% to phenanthrene accumulation in the tissue. Nevertheless, an influence of

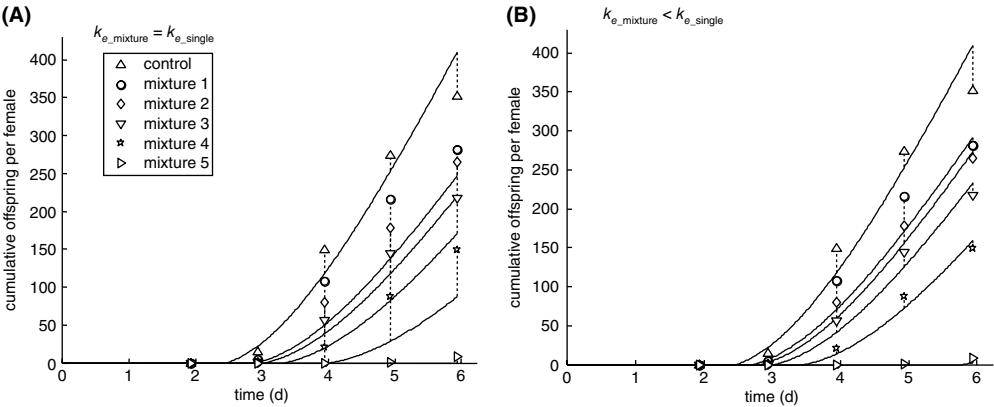


Fig. 7.6. Reproduction of *C. elegans* exposed to five different mixtures of cadmium (Cd) and fluoranthene (FA) and analysis of the data using a simplified DEB-model (DEBkiss), (A) without parameter fitting (parameters were fixed to the controls and the single-chemical fits), and (B) assuming a lower elimination rate of the Cd–FA mixture ($k_{e,mixture}$) than of either chemical alone ($k_{e,single}$). Adapted with permission from Jager *et al.* (2014b) (from Fig. 4 and S5), ©American Chemical Society (2014).

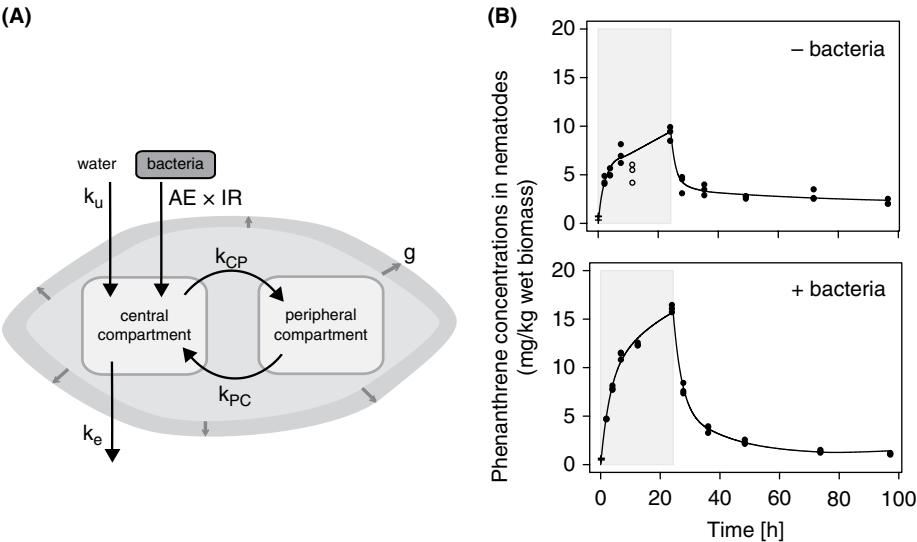


Fig. 7.7. (A) Schematic representation of the two-compartment model used to model phenanthrene accumulation in *C. elegans* (k_u : uptake rate constant; k_e : elimination rate constant; $k_{CP/PC}$: rate constants for transfer from the central to the peripheral compartment and vice versa; AE: assimilation efficiency; IR: ingestion rate; g : growth rate). (B) Toxicokinetics of phenanthrene in *C. elegans* exposed to the chemical in aqueous medium without (upper panel) and with (lower panel) food bacteria. Adapted with permission from Spann *et al.*, 2015 (from Figs 1 and 2a,b), ©American Chemical Society (2015).

food on the toxicokinetics of phenanthrene was still apparent, because in the absence of food the (starved) nematodes depleted their lipid reserves, which altered the toxicokinetic parameters such that the elimination rate of phenanthrene was

higher and its concentration in nematode tissues accordingly lower. These results point to the crucial role played by storage lipids and, more generally, by the nutritional state of the test organism in toxicokinetic processes.

Multi-species toxicity testing (model ecosystems)

Single-species toxicity testing is a necessary and reliable tool in environmental risk assessments conducted for chemicals, but extrapolating the results from short-term laboratory experiments to possible effects in the field is challenging (Forbes and Calow, 2002). A partial solution is offered by micro- and mesocosms. These small, well-defined representatives of ecosystems can be used as experimental units (model ecosystems) for a defined period of time. They are designed to study natural communities under controlled conditions and to provide sufficient replication and precise control over relevant experimental variables. The structure of the studied communities reflects their response to environmental conditions over the course of the experiment. Furthermore, micro/mesocosms allow the inclusion of all matrix and food web effects and thereby realistic exposure scenarios (Van den Brink *et al.*, 2005). These model ecosystems can thus be regarded as community-level bioassays able to yield insights into the biotic responses of natural assemblages and therefore as suitable tools to assess the potential hazard of contamination on fauna. As such, model ecosystems offer an ecologically more relevant approach to risk assessment than laboratory bioassays with single species while avoiding the complexity of field testing or large-scale outdoor studies (Wilson and Kakouli-Duarte, 2009; Haegerbaeumer *et al.*, 2015; Höss, 2021).

Nematodes are well suited for inclusion in microcosm experiments, as they reach high abundances and species richness in sediments and soils and can thus be tested in the laboratory using small volumes (0.01–10 l) of the test substrate (sediment, soil). In fact, the effect of chemicals on nematode communities has been assessed in microcosms containing sediments (freshwater and marine) and soils (for an overview see Haegerbaeumer *et al.*, 2015). In the following, we provide a few examples that illustrate the suitability of nematodes in microcosm-based assessments of chemical contaminants.

Brinke *et al.* (2010) used microcosms (glass jars, 1.7 l, inner diameter 12 cm) to examine the effects of the veterinary pharmaceutical ivermectin on nematode communities over a period of 224 days. The microcosms contained sandy

sediment collected from the river Ems (Germany) and then spiked with three concentrations of ivermectin (0.6, 6.2, and 31 µg/kg dry sediment; analyzed at the beginning of the experiment). Nematodes were extracted from the sediment at seven time points (0, 7, 14, 28, 56, 112, and 224 days) using density separation with colloidal silica (Ludox) and then taxonomically identified to the species level. Multivariate analysis of the nematode species composition revealed dose-dependent effects of ivermectin on the nematode community, with first significant effects occurring at a concentration of 6.2 µg/kg dry weight (dw) (see Fig. 7.8A); the no-observed-effect concentration (NOEC_{Community}) was 0.6 µg/kg dw. Interestingly, the maturity index (MI), developed to identify disturbances of soil nematodes (Bongers, 1990), did not reflect ivermectin-induced stress for freshwater nematodes (Brinke *et al.*, 2010).

Using the more recently developed Nema-SPEAR[%]-index (see also separate section below), a sensitive indicator of chemically induced disturbances in freshwater nematodes, Höss *et al.* (2017) re-analyzed the dataset of Brinke *et al.* (2010) and found clear dose-dependent effects of ivermectin on the nematode community (Fig. 7.8B). The results confirmed those of a large microcosm-based risk assessment of ivermectin, in which nematode communities were found to be the most sensitive organisms within the sediment compartment (Liebig *et al.*, 2010). In that study the risk quotient for ivermectin was 36, indicative of the considerable risk posed by ivermectin to benthic organisms.

A comparison of the NOEC_{Community}, defined for ivermectin and the nematodes in microcosms (0.6 µg/kg dry sediment), and the lower-tier NOEC based on toxicity data for the nematode *C. elegans* showed that the former differed by two orders of magnitude (100 µg/kg dry sediment; see Liebig *et al.*, 2010), evidence of the importance of long-term, multi-species testing in environmental risk assessments. In another study, in which the toxicity of the fungicide fludioxonil on nematodes was examined in single-species and microcosm tests, lower-tier testing with *C. elegans* was sufficiently protective of nematode communities, based on a safety factor of 10 (Höss *et al.*, 2020). With an NOEC of 81 mg/kg dry sediment (corresponding to 0.29 mg/l porewater),

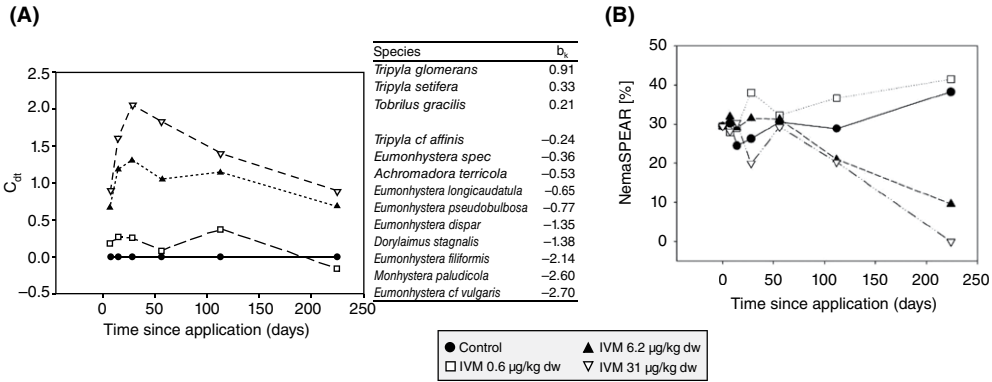


Fig. 7.8. (A) Multivariate analysis (principal response curves) (PRCs) of the nematode species composition in microcosms spiked with ivermectin (IVM); the distance of the canonical coefficients (C_{dt}) to the control line ($C_{dt} = 0$) is related to the dissimilarity of the species composition compared with the controls. The species scores (b_k) indicate how species contribute to the overall course of the PRCs. Adapted from Brinke *et al.* (2010) with permission of Elsevier. **(B)** NemaSPEAR[%]-index for nematode communities in freshwater microcosms spiked with IVM. Adapted from Höss *et al.* (2017) with permission of Elsevier.

the toxicity of fludioxonil detected by *C. elegans* was only twofold lower than that determined in a multi-species test system conducted in microcosms (NOEC_{Community}: 39.9 mg/kg dry sediment; corresponding to 0.39 µg/l porewater). Overall, nematodes were less sensitive than macrobenthic organisms to fludioxonil (Yin *et al.*, 2018).

A major advantage of using nematodes in environmental risk assessments is their analogous applicability in terrestrial and aquatic systems (Wilson and Kakouli-Duarte, 2009; Haegerbaeumer *et al.*, 2015). For the fungicide fludioxonil, Haegerbaeumer *et al.* (2019b) carried out a soil microcosm study using a small-scale test design consisting of 50 ml test tubes containing 30 g of fresh soil. A previous study demonstrated the suitability of this cost-effective laboratory test system in analyzing the effects of chemicals on soil nematode communities (Höss *et al.*, 2014). The authors were able to directly examine the effects of fludioxonil on both freshwater and soil nematode communities. In the soil microcosms, fludioxonil at a concentration of 62.4 mg/kg dry soil significantly affected the nematode community after 56 days of exposure, determined using a variant of the maturity index, the MI2-5, as the toxicity endpoint (Haegerbaeumer *et al.*, 2019b). The corresponding NOEC_{MI2-5} was 14.2 mg/kg dry soil. The study demonstrated the utility of trait-based indices, such as the MI and the enrichment index, in

detecting chemically induced changes in soil nematode communities. Compared with the NOEC_{Community} derived for nematodes in freshwater sediments (39.9 mg/kg dry sediment; Höss *et al.*, 2020), the soil NOEC was three times lower.

The outputs of microcosm studies can vary considerably if different types of soil are used, possibly due to the different susceptibilities of the indigenous nematode communities, but also to differences in soil properties and therefore in the concentrations of bioavailable chemicals. Moser *et al.* (2004) investigated the effect of the fungicide carbendazim on the nematode communities of four different soils, from Germany (Flörsheim), The Netherlands (Amsterdam), Wales (Bangor), and Portugal (Coimbra), within an interlaboratory ring test that included field tests and terrestrial model ecosystems (TMEs). The response (for example, the MI (Fig. 7.9)) of the nematode communities to carbendazim varied considerably depending on the soil type, leading to NOECs ranging from 3.24 to ≥ 87.5 mg carbendazim/ha. However, a comparison of the results of the TMEs to the respective field validation study yielded good agreement between the NOEC and EC₅₀ values of the respective soils. Moreover, the reproducibility between the various laboratories of the ring test was acceptable, despite the fact that different soils from different sites were used (Moser *et al.*, 2004).

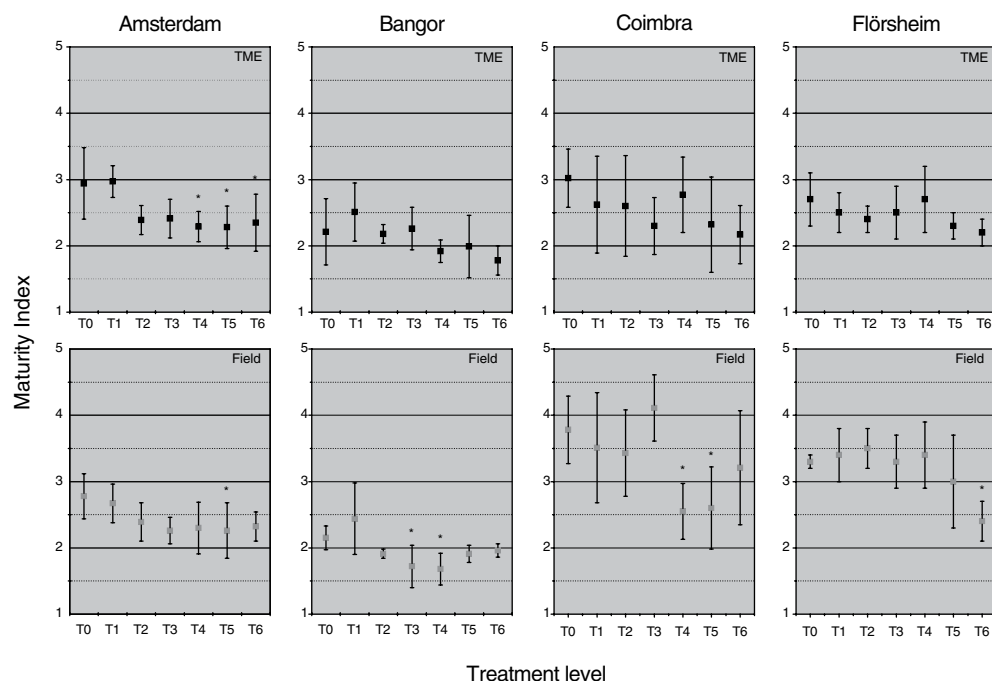


Fig. 7.9. Effect of carbendazim on the maturity index of nematodes in soils from Amsterdam, Bangor, Coimbra, and Flörsheim after 16 weeks of drug exposure. Data are from the ring test using terrestrial model ecosystems (TME) and from the field-validation study. Significant (Dunnett *t*-test, *l*-sided; $p \leq 0.05$) differences compared with the control are indicated by an asterisk. Treatment levels: T0 = control, T1 = 0.36, T2 = 1.08, T3 = 3.24, T4 = 9.72, T5 = 29.16, T6 = 87.48 kg active ingredient/ha. Reprinted from Moser *et al.* (2004) with permission of Springer.

In situ bioindication

Together with single- and multi-species toxicity tests, *in situ* bioindication is an essential component of environmental risk assessments, including those aimed at determining the ecological status of aquatic ecosystems (e.g., European Community, 2000). Weight-of-evidence concepts such as the triad approach have been developed for sediments (Chapman, 1986; Chapman and Anderson, 2005) and soils (Mesman *et al.*, 2006) and are based on a designation of the *in situ* fauna or flora as ecological quality elements. For aquatic ecosystems, macroinvertebrates are mainly used for bioindication (Hering *et al.*, 2004), although for many important compartments, such as fine sediments, meiofaunal organisms (e.g., nematodes) are often more abundant and species-rich than macroinvertebrates. By contrast, the exceptional ecological relevance of

nematodes in the soil food web is well established (Bongers and Ferris, 1999; Van den Hoogen *et al.*, 2019) such that nematodes are included in soil frameworks designed to assess the threats to soil-derived ecosystem services (Jensen *et al.*, 2006).

However, in both aquatic sediment (Dano-varo *et al.*, 2009; Höss *et al.*, 2011c) and soils (Ferris *et al.*, 2001; Neher, 2001; Nagy, 2009), nematodes have proven to be very suitable bio-indicators of certain types of stress (Bongers, 1990; Höss *et al.*, 2011c; Zhao and Neher, 2013). Stress-specific indices are very useful for identifying the causes of a poor ecological status and for setting thresholds for classifying potentially polluted sites in terms of their ecological status. In the following we briefly present the most promising approaches that rely on nematode-based indices to identify chemically induced stress in freshwater sediments and soils.

The NemaSPEAR[%]-index for freshwater systems

Drawing on a large dataset comprising nematode species data, geo-physicochemical data, and chemical data for priority pollutants based on > 200 sediments sampled from > 100 sites of rivers and lakes, Höss *et al.* (2011c) developed the NemaSPEAR[%]-index using a co-occurrence approach. Nematode species were classified according to their occurrence along a pollution gradient as species at risk (NemaSPEAR) and species not at risk (NemaSPEnotAR). NemaSPEAR were mainly found in the sediments of reference sites with low-level pollution but only rarely in polluted sediments (Fig. 7.10A). In contrast to analogous indices for macroinvertebrates (SPEAR-indices) (Liess and Von der Ohe, 2005; Schäfer and Liess, 2013), the NemaSPEAR[%]-index is based solely on empirical data for nematode communities rather than on trait-based data for single species. The index is calculated as follows:

$$\text{NemaSPEAR}[\%] = 100 \times \frac{\sum \log \% \text{NemaSPEAR}}{\sum \log \% \text{All species}}$$

This index was validated with 85 sediment samples from 73 different sites, and its robustness in detecting sediment contamination (Höss *et al.*, 2017) (Fig. 7.10B) and seasonal variations within nematode communities (Brüchner-Hüttemann *et al.*, 2021) was demonstrated (see also Höss and Traunsperger, 2021). Based on the variation of the NemaSPEAR[%] within reference sites with low-level pollution, thresholds could be defined for classifying sediments or sites according to their ecological status (Höss *et al.*, 2017) (Fig. 7.10B).

As nematodes are a challenging group regarding their morphological taxonomy, especially at the species level, experts in nematode taxonomy are rare. Therefore, in toxicity assessments, approaches had to be found that were applicable also at higher taxonomic levels (such as genus) or that relied on non-morphological methods. The former includes the NemaSPEAR[%]_{genus} index developed by Höss *et al.* (2017), which can be applied on the genus level and is responsive to the toxic potential of the sediments. As a method independent of taxonomic expertise, the DNA-based metabarcoding framework of Schenk *et al.* (2017, 2019) reveals the taxonomic composition of freshwater nematode communities, based on

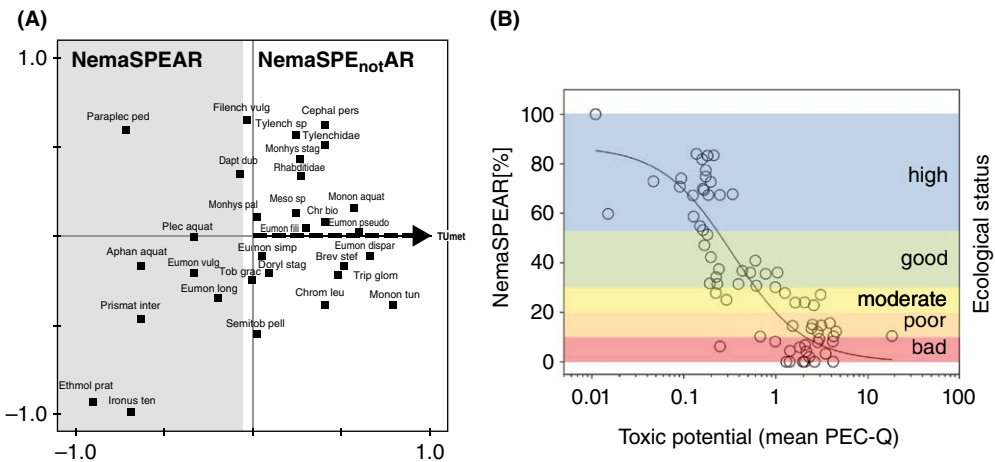


Fig. 7.10. (A) Classification of nematode species into species at risk (NemaSPEAR) and not at risk (NemaSPEnotAR) using canonical correspondence analysis (CCA), based on the nematode species composition in 155 sediment samples along a pollution gradient (here expressed as toxic units for metals in porewater: TUmets); species at the left side (gray field) mainly occurred at sites with low-level contamination. Reprinted from Höss *et al.* (2011c) with permission of Elsevier. **(B)** Response of NemaSPEAR[%]-index to the toxic potential of sediment (mean quotient of measured sediment concentrations divided by sediment quality criteria). PEC = [probable effect concentration] defined by De Deckere *et al.* (2011) with thresholds for classes of ecological status: high, NemaSPEAR[%] ≥ 54; good, 30 to < 54; moderate, 20 to < 30; poor, 10 to < 20; bad, < 10). Modified from Höss *et al.* (2017).

the finding that the relative read abundances for two primer sets (18S and 28S markers) correlate positively and almost perfectly with the relative biomass of the species. The quantitative species data generated by this method are a prerequisite for calculating the NemaSPEAR[%]-index. In subsequent work, Schenk *et al.* (2020a,b) applied their metabarcoding approach to analyze the taxonomic composition of nematode communities along a pollution gradient represented by sediments of different rivers and then compared the results with those obtained by morphological taxonomy. At the species level, the overlap between the morphological taxonomy and the DNA-based taxonomy in the taxonomic assignment of the 25 most dominant species was < 30%. On the genus level, the agreement between microscopic and molecular techniques increased considerably, with an overlap of 40% (18S) and 48% (28S) for the 25 most dominant genera. Nevertheless, the community analysis based on multivariate techniques (non-metric multidimensional scaling (nMDS) ordination) yielded comparable results for the morphological and DNA-based data, especially regarding the 28S marker, as similar clusters were identified for the nematode communities at the

various sites (for genus composition, see Fig. 7.11A). The NemaSPEAR[%]_{genus} calculated based on the molecular dataset correlated well with the morphologically derived index (Fig. 7.11B; Schenk *et al.*, 2020a,b). At the species level, the morphological and molecular approaches also resulted in a similar ranking of the examined sites according to their ecological status, with the three least contaminated sites having a good or high (NemaSPEAR[%] > 30) ecological status and the two most contaminated sites a moderate to bad (NemaSPEAR[%] < 30) ecological status. These results demonstrated that the NemaSPEAR[%]-index is a suitable and generally applicable measure for assessing the ecological status of freshwater sediments and changes therein. Indeed, recent studies have shown that the NemaSPEAR[%] can add valuable information to the macroinvertebrate-based indices used in quality assessments of contaminated river sites (Sonne *et al.*, 2018; Bighiu *et al.*, 2020; Brüchner-Hüttemann *et al.*, 2021).

Trait-based indices for soil systems

In the assessment of soil quality, trait-based indices can be used to link changes in the structure

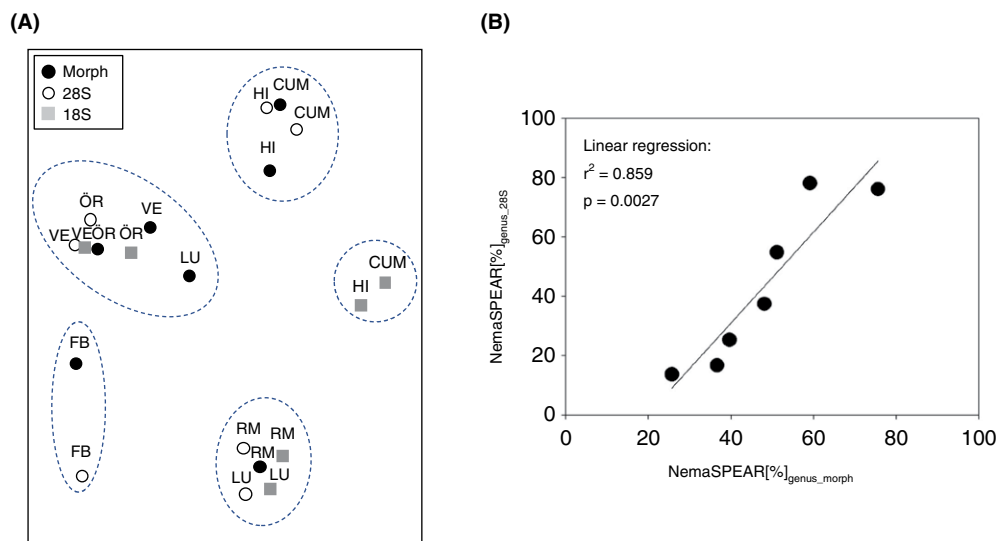


Fig. 7.11. (A) nMDS comparing the nematode genus composition in river sediments sampled from seven sites (CUM = Cumlosen, HI = Hitzacker, RM = Mischmühle, LU = Luppe, ÖR = Örtze, VE = Veerse, FB = Furlbach); the analyses used the Bray-Curtis similarities of the untransformed data based on biomass; hatched lines = significantly different clusters at 20% similarity level (SIMPROF; $p < 0.05$); nMDS stress: 0.16. (B) Correlation of the NemaSPEAR[%]_{genus} calculated from morphologically and DNA-based (28S) data for nematode genus composition. Reprinted from Schenk *et al.* (2020a) with permission of Elsevier.

of nematode communities to anthropogenic stress factors, such as chemical pollution, organic enrichment, and tillage. The use of life history traits (maturity indices) (Bongers, 1990; Bongers and Ferris, 1999; Korthals *et al.*, 1996a,b) or functional guilds (combining life-history and feeding traits) (Ferris *et al.*, 2001) enables the classification of soils according to their disturbance, enrichment, and dominant decomposition pathways.

The MI, structure index (SI), and enrichment index (EI) (Ferris *et al.*, 2001) have often been used to assess effects of various stressors on soil nematodes in the field. Those studies have included stressors such as heavy metals (Georgieva *et al.*, 2002; Pen-Mouratov *et al.*, 2008; Martinez *et al.*, 2018), pesticides (Fiscus and Neher, 2002; Pen-Mouratov *et al.*, 2010; Gupta *et al.*, 2019), soil management practices (Fiscus and Neher, 2002; Hu and Qi, 2010; Ney *et al.*, 2019), and insecticide-producing genetically modified maize (for example, Bt-maize: Höss *et al.*, 2011a, 2015b; Neher *et al.*, 2014).

In a field experiment, Georgieva *et al.* (2002) treated soil with sewage sludge containing various levels of copper (Cu), nickel (Ni), or zinc (Zn) and analyzed the nematode community in the treated soils. Consistent with the theory of the MI, they showed that chemical disturbance shifts the ratio of persisters to colonizers (*K*- to *r*-strategists) to the benefit of the more stress-tolerant colonizers, evident as a decrease of the

MI. This was well explained by the soil concentrations of Cu and Zn, because in those treatments large reductions in the density of cp3–5 nematodes were observed (Fig. 7.12A) (Georgieva *et al.*, 2002), as this group is particularly sensitive to chemical pollution. The MI2–5 omits the group of colonizers indicative of organic enrichment (cp1: enrichment opportunists) (Ettema and Bongers, 1993) and thus reflects the tolerance of cp2 nematodes (general opportunists) to chemical disturbance. It is therefore more specific in detecting the presence of chemical toxicity in nematode communities (Korthals *et al.*, 1996a). Georgieva *et al.* (2002) showed that the MI2–5 correlates well with the levels of Cu and Zn pollution in soils (Fig. 7.12B), with a slightly better correlation coefficient ($r^2 = 0.82$; see Fig. 7.12B) than achieved with the full MI ($r^2 = 0.76$; correlation with data taken from Tables 1 and 3 of Georgieva *et al.*, 2002).

DNA-based taxonomic approaches have also been developed for soil nematodes, thus opening up nematode ecology research to a wider community that includes non-taxonomists (Griffiths *et al.*, 2006; Holterman *et al.*, 2008; Porazinska *et al.*, 2009). A recent study compared taxonomic data for soil nematodes from steppe and forest ecotones based on morphological identification and high-throughput DNA-sequencing (HTS) (18S rDNA) in terms of the agreement between these methods in describing the respective community structure

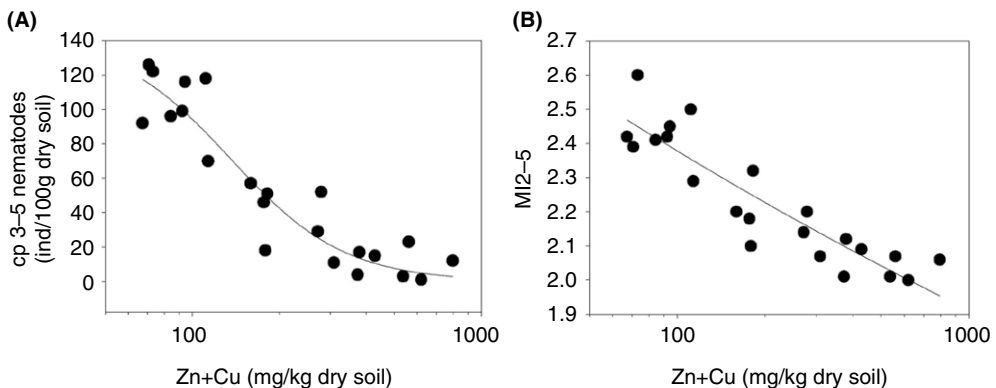


Fig. 7.12. (A) Densities of nematodes classified as persisters (cp3–5) and (B) the maturity index (omitting cp1 nematodes; MI2–5) calculated for soil nematode assemblages in fields amended with sewage sludge containing various mixtures of different metals; ind = individuals. Data taken from Tables 1 and 3 in Georgieva *et al.* (2002); dose–response curves were fitted to the data by non-linear regression using a logistic function (A: $r^2 = 0.88$, $p < 0.001$; B: $r^2 = 0.82$, $p < 0.001$).

(Du *et al.*, 2020). Despite considerable differences in the genus-level composition determined by morphological and HTS-based taxonomies (Fig. 7.13A), the results of the two methods were similar at the family level and thus also in their MI, EI, and SI values (Fig. 7.13B,C). Soils from the different ecotones were ranked similarly, with significantly higher MI and SI values but a lower EI value for steppe soils than for forest soils.

Nematodes in Ecological Metacommunity Models

The previous sections demonstrated the utility of nematodes both as a model and as indicator organisms in ecotoxicology. Inherent to the respective analytical approaches is their analysis of nematode assemblages in the context of community ecology. However, recent studies (De Mendoza *et al.*, 2017; Gansfort and Traunspurger, 2019) have shown that nematodes are also useful as ecological models at the scale of metacommunities, a regional set of communities potentially connected by dispersal (Wilson, 1992; Leibold *et al.*, 2004).

Metacommunity ecology considers the role of environmental (local, niche-based) and spatial (regional, dispersal-related) processes (Cottenie, 2005; Logue *et al.*, 2011), as well as their deterministic and stochastic components (Vellend *et al.*, 2014). The importance of environmental- and dispersal-related processes shifts with dispersal intensity and the degree of environmental heterogeneity within and among local communities (Fig. 7.14). As a result, the importance

of environmentally related factors increases with the increasing environmental heterogeneity of a metacommunity and the degree of specialization of its species. However, the influence of dispersal-related (spatial) factors on (meta-) community structure peaks under very low and very high dispersal intensities, as both lead to a spatial structure within the metacommunity that is independent of the environmental circumstances (Fig. 7.14). The latter is a result of the dispersal ability of a species, the connectedness of the physical habitat, and the spatial extent of the studied area.

While interest in the metacommunity concept grew rapidly after the publications of Leibold *et al.* (2004) and Holyoak *et al.* (2005), its first application to include nematodes was reported about 10 years later (Gansfort and Traunspurger, 2019; Gansfort *et al.*, 2020). Those studies defined a large set of characteristics that favored the use of these meiofaunal-sized organisms in metacommunity studies, as follows.

- **Species richness.** Nematodes usually exhibit a high diversity even in small-scale investigations (for example, > 100 species in one lake) (Traunspurger *et al.*, 2020; Traunspurger and Majdi, 2021). Therefore, field studies provide a wealth of data for use in ecological modeling at the species level within a single taxonomic group.
- **Small size and high abundances.** These features enable metacommunity studies to be conducted also within a relatively small spatial extent (for example, a few square meters of lake sediment).

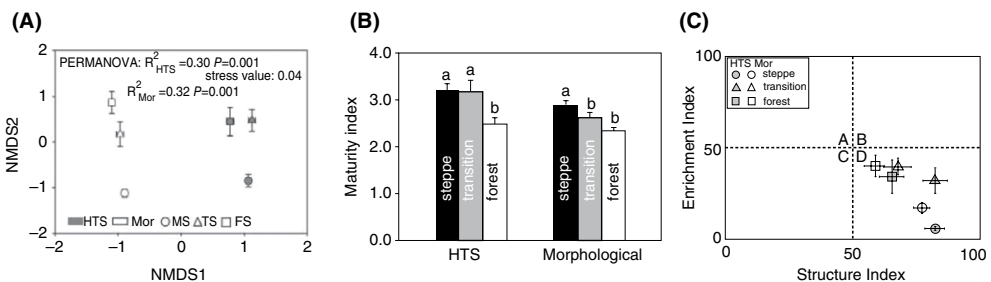


Fig. 7.13. (A) nMDS ordination based on the Bray-Curtis similarities of nematode genus composition in steppe, transition, and forest soils. (B) Maturity Index (MI) for the three soil types. (C) Nematode faunal profile for the three soil types based on high-throughput sequencing (HTS) (gray symbols) and morphological (white symbols). Modified from Du *et al.* (2020) with permission of Elsevier.

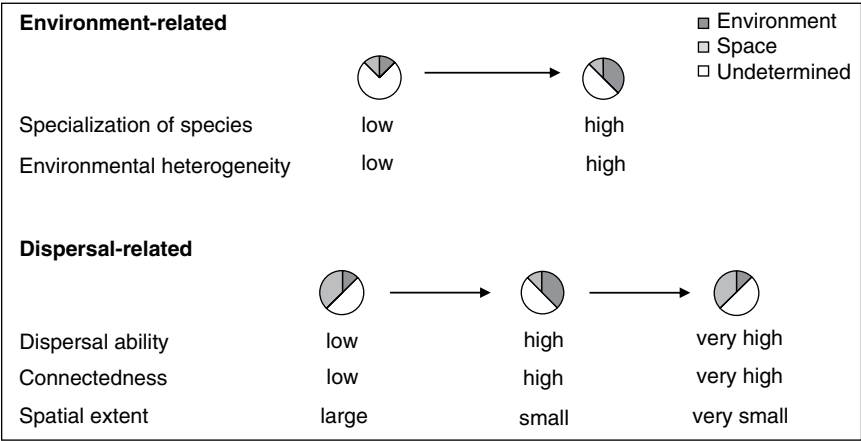


Fig. 7.14. The relative roles of environmental processes (dark gray), dispersal-related processes (light gray), and stochastic components (white) on the metacommunity structure of biological communities. Modified from Gansfort *et al.* (2020).

- **Multiple habitats.** Nematodes are present in almost every habitat on earth. Moreover, their assemblages in different habitats are subjected to different degrees of isolation (for example, a patch of moss on a tree is a more isolated habitat than a sediment patch in a stream network), which allows comparisons of dispersal-based processes among these habitats.
- **Dispersal modes.** Both active and passive processes contribute to nematode dispersal, and dispersal abilities differ between species (see review by Ptatscheck and Traunspurger, 2020). Thus, nematodes can serve as models to test the influence of dispersal-related traits on metacommunity structure.

The use of nematodes, as well as other meiobenthic organisms, as models in metacommunity ecology studies was reviewed by Gansfort *et al.* (2020). Since 2004, when the term metacommunity became widely established (see above), only five studies have focused on nematode metacommunities within freshwater ecosystems (reviewed in Gansfort *et al.*, 2020; e.g., Gansfort and Traunspurger, 2019; Ptatscheck *et al.*, 2020). However, their results clearly demonstrated the ecological knowledge that can be gained from nematodes and from comparisons of their distribution with that of other less ubiquitous animals (De Mendoza *et al.*, 2017). Two examples are presented below.

Model of metacommunity structure

A metacommunity framework can be useful in disentangling the roles of different (usually environmental versus spatial) variables in metacommunity structure. This was the aim of the nematode metacommunity analysis conducted by Gansfort and Traunspurger (2019) using a large dataset of nematode assemblages in German river networks. Specifically, a model of nematode community similarities in 115 sediment samples within the Elbe river network was calculated by incorporating different predictor groups. Thus, the relative importance of several factors for the nematode community was evaluated: (i) the sediment content of different elements; (ii) physical variables (grain size fractions and river kilometer); (iii) the ecotoxicological potential (combined as environmental factors); (iv) spatial characteristics (geographical position of sites and their positions within the river network); and (v) temporal factors (sampling date). The model was then further tested for its ability to predict community similarities among 59 samples of the Rhine river network.

The results showed that environmental variables, in this case mostly the nitrogen content of the sediment, had the strongest influence on the community assembly of sediment-dwelling nematodes in the Elbe (Fig. 7.15). The second most important environmental factor was the spatial arrangement of sites along the river

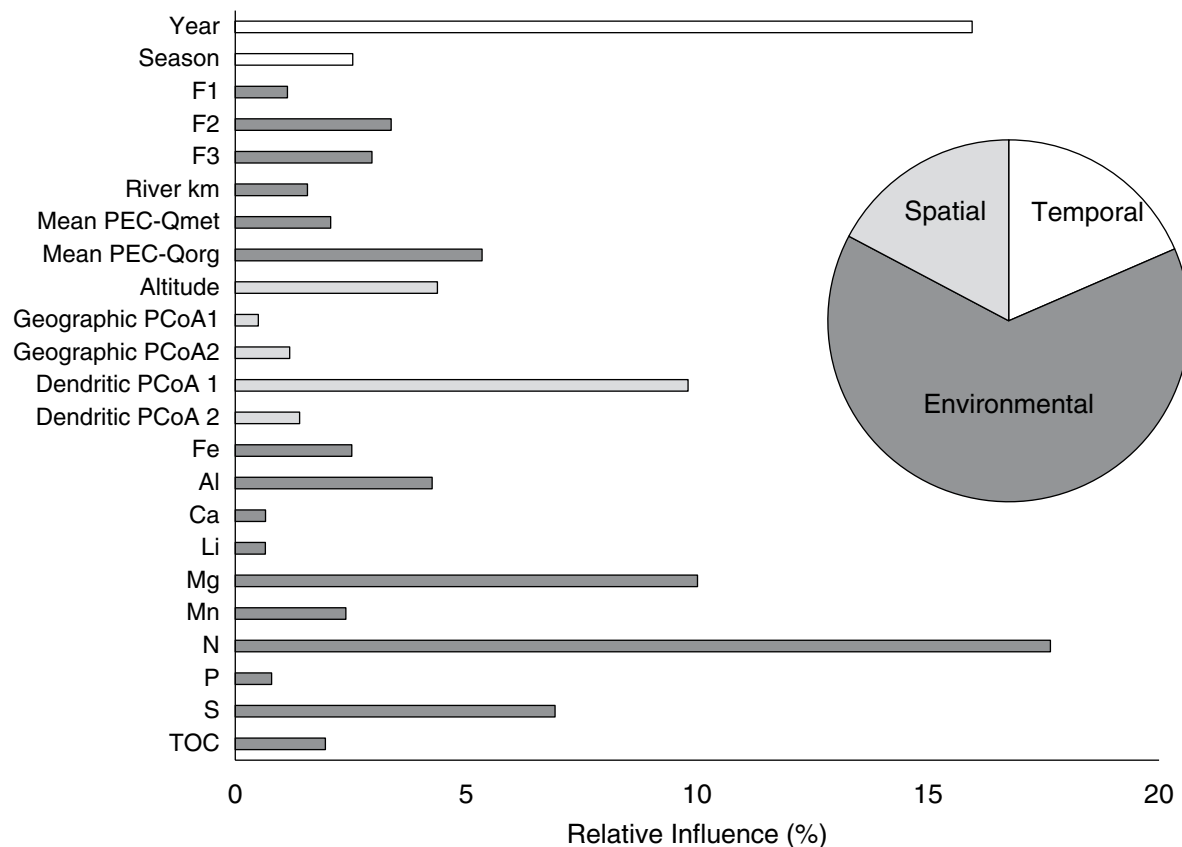


Fig. 7.15. The relative influences of several predictors on nematode community similarities among 115 samples from 47 sampling sites of the Elbe river system, based on abundance data and determined using boosted regression tree models. The variables were assigned to three groups: environmental (dark gray), spatial (light gray), and temporal (white). Variables: F1–F3, grain size fractions from < 20 µm to > 2000 µm; River km, distance of sampling site measured from the spring; Mean PEC-Q met/org, mean quotient of probable effect concentrations (De Deckere *et al.*, 2011) and real contaminant organic and metal contaminants; Geographic and Dendritic PCoAs, axes of the principal coordinate analyses with distance matrices based on the overland and watercourse distances of the sampling sites; Fe, Al, Ca, Li, Mg, Mn, N, P, S, TOC (total organic carbon), content of these elements with respect to all chemical compounds in the sediment. Modified from Figure 3b in Gansfort and Traunsburger (2019).

network (i.e., along the watercourse's dispersal pathway) (Fig. 7.15). This example therefore confirmed the following two major findings of other nematode metacommunity studies.

1. Environmental parameters are significant variables shaping nematode community assemblies, with resource availability playing a dominant role within metacommunity studies (significance of trophic state index) (Dümmer *et al.*, 2016); no significant environmental influence in the absence of a productivity gradient (De Mendoza *et al.*, 2017; Ptatscheck *et al.*, 2020).
2. Dispersal-related processes and thus spatial variables strongly influence nematode metacommunities, with their impact depending especially on the spatial scale of the field study (Fig. 7.15) (Dümmer *et al.*, 2016; Ptatscheck *et al.*, 2020).

The latter result fueled discussions on how nematodes, given their ubiquity (Zullini, 2018), nonetheless show a spatial signal also at smaller than continental or global scales. However, as mentioned above, spatial structure can be due to dispersal limitations (i.e., species cannot reach every site in a region where they could, based on environmental conditions, persist) or dispersal surplus (i.e., species may persist even in suboptimal habitats, due to constant immigration from suitable habitats). As nematodes may be one of the few organismal groups in which the latter commonly occurs, they provide a valuable opportunity to study this metacommunity type (Leibold and Chase, 2018; Gansfort and Traunspurger, 2019; Ptatscheck *et al.*, 2020).

A limitation of the example provided by the study of Gansfort and Traunspurger (2019) is that the role of stochastic factors on metacommunity structure was not explicitly considered, even though stochastic factors are often the most prominent "influential variable". However, their importance in nematode metacommunities has been demonstrated in studies showing that nematodes are passive dispersers and are thus more prone to stochastic distribution than active dispersers (Ptatscheck *et al.*, 2015, 2020). The degree of stochasticity will also depend on the size, isolation, and heterogeneity of the studied habitats (Fukami, 2010). The benefit of studies investigating the role of stochasticity in structuring nematode metacommunities is that they are found in habitats ranging from small, isolated tree-holes (Ptatscheck *et al.*, 2015) to

large connected sites such as river networks (Gansfort and Traunspurger, 2019). Regardless of the stochastic component of nematode metacommunity structures, the similarity between nematode communities along sites of a river network can be predicted using spatial and environmental variables. Thus, predictions based on the model trained with the Elbe data significantly correlated with real data from the Rhine samples, with a coefficient > 0.5 (Gansfort and Traunspurger, 2019). However, the results suggest that presence/absence assemblies are better predicted than abundance data.

A further special characteristic of the study of Gansfort and Traunspurger (2019) is that it explicitly evaluated the role of temporal dynamics, which in metacommunities is largely ignored as sampling is conducted in the same season and/or within a short (maximum of 2 years) time period (Jacobson and Peres-Neto, 2010). Thus, temporal dynamics are often a source of unexplained variation within metacommunities. By contrast, using a dataset collected over 13 years, Gansfort and Traunspurger (2019) showed that time has a large effect on community structures; in their study, its influence was as strong as that of nitrogen (Fig. 7.15). The nematode metacommunity model presented by the authors indicated a strong historical contingency of ecological communities. Such temporal shifts could be due to temporal presences, such as those reflecting the seasonal dynamics of dispersal (Ptatscheck *et al.*, 2018), or to the absences of species, such as through extinctions due to extreme environmental events.

Models of biodiversity

As the above-described example demonstrates, nematodes can be used to model metacommunity structure with respect to many different variable groups. However, nematodes are also valuable in studies contributing to investigations of the relationship between biodiversity and factors related to the environment and to dispersal. Given the difficulty of accurately measuring dispersal rates in the field and the even greater difficulty in manipulating them, such studies are usually conducted in the laboratory (Matthiessen *et al.*, 2010; Pedruski and Arnott, 2011), where the use of nematodes in micro- and mesocosms

allows natural communities to be observed and manipulated also over long periods of time (Ristau *et al.*, 2012; Haegerbaeumer *et al.*, 2018b). Indeed, nematodes are particularly well suited as model organisms in ecology studies aimed at testing theoretical assumptions, such as the relation between dispersal intensity and biodiversity as established by Mouquet and Loreau (2003). The latter was tested in a mesocosm experiment (Gansfort *et al.*, 2021, Fig. 7.16) in which nematode communities were captured under homogeneous or heterogeneous environmental conditions and the dispersal rates between communities were manually manipulated to varying extents by mixing the sediments of different local communities from each meta-community at different time intervals.

In general, the results of the experiment were in accordance with the theoretical assumptions based on the calculations of Mouquet and Loreau (2003) (Fig. 7.16). However, it was also determined that the interaction between the influence of dispersal intensity on alpha- and gamma-diversity and the degree of environmental heterogeneity differed depending on the regional environment. Specifically, higher dispersal rates led to a decline in diversity in the homogeneous regional environment but had no effect on, or even increased, diversity in the heterogeneous regional environment (Fig. 7.16). An interesting finding of the experiment was that different nematode species differed in their responses to the dispersal treatments. For example, *Daptonema dubium* greatly benefited from

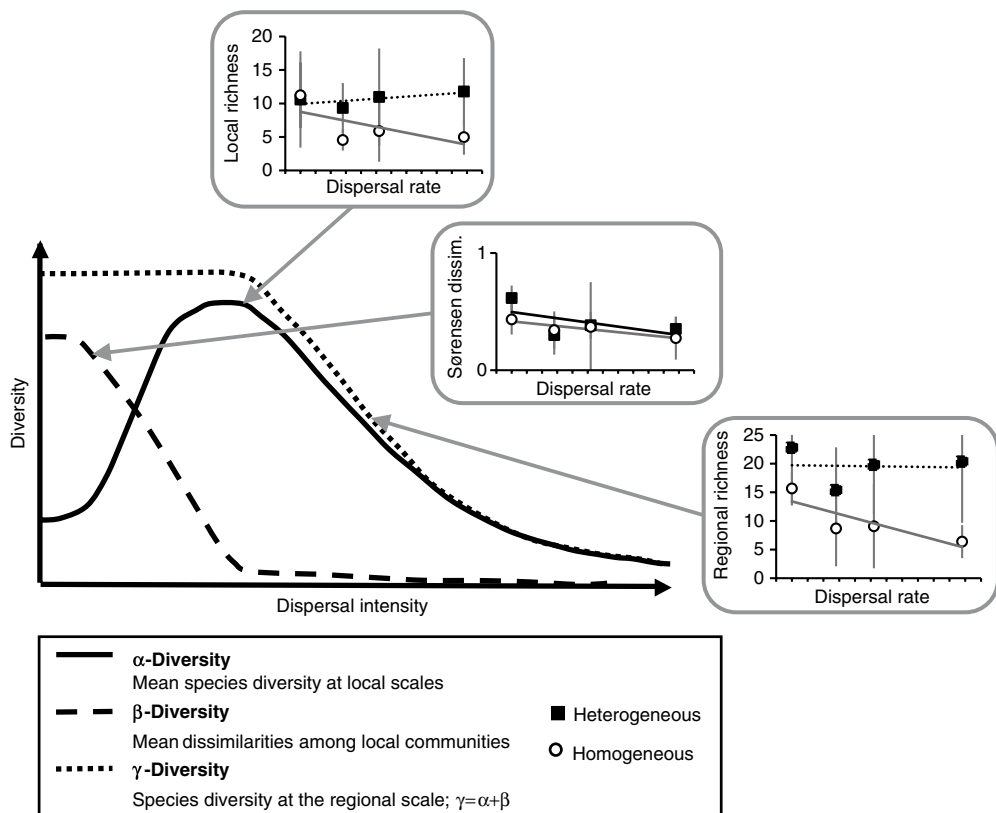


Fig. 7.16. Theoretical model of the relationship between biodiversity and dispersal intensity calculated by Mouquet and Loreau (2003) and evaluated in a mesocosm-based laboratory experiment using nematode communities (Gansfort *et al.*, 2021). The results are shown in gray boxes. Solid lines indicate significant relationships and with respect to environmentally related processes (under heterogeneous and homogeneous conditions). Modified from Mouquet and Loreau (2003) and Gansfort *et al.* (2021).

the strong connectivity of local communities whereas for many other species, such as *Eumonhystera vulgaris* and *Monhystera paludicola*, population dispersal was more effective when local communities were completely isolated from each other. This wide range of dispersal–colonization abilities among nematode species can be exploited by researchers to test general theoretical concepts of (metacommunity) ecology.

Conclusions

Nematodes are the most abundant metazoans on earth. They are characterized by a very high diversity of species and functional traits, explaining their key role in freshwater, marine, and soil food webs and their versatility as model organisms in ecology and ecotoxicology. Since there are many nematode-based tools for assessing changes in ecosystems, from the DNA to the metacommunity level, from the laboratory to the field scale, it is remarkable that nematodes are still underrepresented in regulatory ecotoxicology, as well as in ecological research.

With *C. elegans*, there exists an excellent model organism for assessing the risk of chemicals, chemical mixtures, nanomaterials, microplastics, and waste for aquatic and terrestrial ecosystems. The multifaceted applicability of *C. elegans* as a model for unraveling toxicity mechanisms is complemented by highly standardized tools for a reliable and reproducible risk assessment (ISO 10872, ASTM E2172-01). On the other hand, powerful measures are available, enabling *in situ* indication in complex substrates, such as sediments and soils, based on the species

and trait composition in nematode communities (e.g. NemaSPEAR[%]-index; Maturity Index). Combined with index systems using other organism groups (macroinvertebrates, plants, microorganisms), nematode-based indices can contribute considerably to effect-based biomonitoring, allowing the unraveling of cause–effect relationships and the facilitation of decision making in water and soil management. Bridging the gap between single-species toxicity testing and field investigations, multi-species test systems with nematodes have been developed, using small-scale model ecosystems (microcosms). These intermediate-tier test systems can be set up in the laboratory, but also upscaled to outdoor test systems, allowing researchers to validate effect extrapolations from the laboratory to the field.

Although nematodes were seldom the focus of metacommunity analyses, in recent years they have been proven to be valuable organisms to build and test models in metacommunity ecology in both laboratory and field studies. Their large species number, occurring in such a wide range of different habitats, strongly varying dispersal–colonization abilities, and degrees of specialization allow exploration of many predictions that are made by metacommunity theory. However, we are only just beginning to discover the use of nematodes as ecological models. So far, they have been suggested to be useful to demonstrate a “spatial null distribution” (De Mendoza *et al.*, 2017), or a rare example of “mass effects” (Gansfort and Traunspurger, 2019; Ptatscheck *et al.*, 2020). When the body of literature on nematode metacommunity studies grows, it will become clear whether those applications are of general interest.

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8 Use in Soil Agro-Ecology

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Introduction: Soil as Live Resource

Soil is a complex matrix, comprising unconsolidated mineral material, organic matter, air, and water as well as a myriad of organisms that develop their whole lifespan, or just a part of it (or in-part), associated with this matrix. The characteristics of a soil are determined by the material from which it is derived (bedrock and surrounding areas linked by different edaphic-morphological processes), and the multiple physical, chemical, biological, and morphological processes that occur in time and space. Hence, soils are dynamic, because they evolve and vary in their properties across time. However, because the generation of soil is measured on a geological scale, the soil is considered as a non-renewable resource in the human perspective, with high socio-economic and natural value. Several human activities generate severe impacts and irreversible degradations in this ecosystem, such as intensive agriculture, industrial activity, and urban development. On the one hand, agricultural management has great influence on the activity and biodiversity of soil organisms. Apart from the excessive use of agrochemicals that degrade the environment, agriculture modifies the ecosystem by reducing the diversity of root systems of the plant species, thus limiting the variety of habitats and

resources for soil organisms, and deeply alters soil physical structure by tillage operations, among other actions. Therefore, preserving the soil in agricultural systems is a matter of food security, since it has a direct impact on the health and resilience of crops. On the other hand, the industry has produced an extreme disequilibrium in the soil ecosystem by polluting the soils with chemical compounds and heavy metals. Hence, these soils will remain degraded in the long term and would require specific actions to minimize these impacts on the environment. Finally, due to urban development, natural soils are displaced by different kinds of construction (buildings, roads, etc.) and hence are lost as providers of ecosystem services.

Soil biodiversity is huge, since it gathers a quarter of the planet's known species (Decaëns, 2010). Soil communities are extremely complex and diverse, including organisms from microscopic size (archaea, bacteria, oomycetes, fungi), to others of medium and large size (nematodes, mites, collembolan, earthworms, snails, moles, etc.). In addition, plants are linked with the soil through the roots, producing a special area of interaction with the soil matrix, denominated the rhizosphere, which is essential for their development and survival. Compared with the above-ground system, knowledge of soil biodiversity is quite limited as well as the understanding of the

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interactions, often forgotten or hidden. Nowadays, it is well established that soil biodiversity (organisms and their activities) provides a wide range of key ecosystem services, such as the maintenance of soil structure, aeration, and regulation of hydrological processes, and the decomposition of organic matter by directly intervening in the cycles of nutrients (Wall, 2012; Bardgett and van der Putten, 2014). Hence, it is urgent to expand knowledge of soil inhabitants and their role in the ecosystem to prevent their loss and to enhance their beneficial services.

Among soil organisms, nematodes are considered a key component of the below-ground community. Indeed, they are present in almost every habitat on earth, representing four-fifths of all animals in the soil (Platt, 1994; Bongers and Bongers, 1998; Hodda *et al.*, 2009). Soil nematodes are complex organisms and are taxonomically diverse; they might be classified into multiple trophic and functional groups with varying functional roles (Yeates, 1979; Ettema, 1998; Ferris *et al.*, 2001). Such functional diversity led to the development of several ecological indices to evaluate the condition of soil health and quality (Bongers, 1990; Ferris, 2010). Hence, the study of nematode assemblages in the soil ecosystem can serve as a model to understand the impact of human activities, such as agricultural management or industrial activity (Sánchez-Moreno *et al.*, 2015, 2018; Gutiérrez *et al.*, 2016) in the soil. Moreover, there are many nematodes that are reared under laboratory conditions and hence can be explored as models for basic and applied research in soil ecology. The perspective will differ, depending on the soil nematode type and the ecological question. For example, some free-living nematodes (FLNs) are often used as models for understanding general concepts in neurobiology, behavior, development, cell biology or evolution, with *Caenorhabditis elegans* as one of the best-known models (see <http://www.wormbook.org/>, accessed 20 October 2020). Certain FLNs can also be employed in soil ecology questions, such as evaluation of the degree of pollution in a soil, or to understand the plasticity of feeding structures upon interaction with environmental conditions (Anbalagan *et al.*, 2012; Sommer *et al.*, 2017). Other nematodes, described as plant-parasitic nematodes (PPNs) are investigated in the context of the development of control measures

to minimize crop yield losses (for example, *Meloidogyne* spp. are among the most dangerous nematodes for many crops worldwide) (Saucet *et al.*, 2016). Also, the group of entomopathogenic nematodes (EPNs) in the genera *Steinernema* and *Heterorhabditis* are beneficial for growers, because they help to control arthropod pests (Campos-Herrera, 2015). In summary, nematodes are excellent ecological models at varying scales, from cellular to community level. Here, we review the current status of nematode ecology, discuss the traditional and new molecular methods employed for their study, and provide various examples in which nematodes are used as model organisms in different ecological scenarios, addressing both fundamental and applied scientific questions.

Nematodes as Soil Organisms

Soil nematodes are among the most common metazoans on earth, virtually occupying all ecological niches from the bottom of the oceans to the highest elevations of mountain ranges. In terrestrial ecosystems, soil nematode abundance ranges from a few dozen to a few thousand individuals per 100 g of soil, with highest abundances in boreal and sub-boreal ecosystems (van den Hoogen *et al.*, 2019), and they are, for example, the most common metazoans in Antarctic soils (Zawierucha *et al.*, 2019). In spite of presenting a relatively simple body plan, soil nematodes are diverse, complex, and highly specialized (De Ley, 2006).

Soil nematodes are extraordinarily diverse. Dozens of genera and an undetermined number of species may inhabit a given soil, belonging to five main trophic groups: bacterivores, fungivores, herbivores, omnivores, and predators (Yeates *et al.*, 1993) (Fig. 8.1). In the soil, bacterivorous nematodes are active and fast-moving, often the most abundant nematode trophic group in the soil nematode community, which feed by sucking soil bacteria (such as Rhabditidae, Cephalobidae, Panagrolaimidae, Plectidae, or Monhysteridae). Since their abundance is coupled with soil bacteria, they commonly increase their abundance after the bloom of bacterial populations that occurs when the soil is enriched in nutrients or organic matter (Milkereit *et al.*, 2021), and their abundance is associated with the content of labile



Fig. 8.1. Example of various nematode shapes and functional groups. **(A)** Order Dorylaimida, functional group “omnivorous”; **(B)** Family Mononchidae, functional group “predatory”; **(C)** Family Paratylenchidae, functional group “herbivore”; **(D)** Family Cephalobidae, functional group “bacterivore,” attacked by a trapping nematophagous fungus.

organic matter in the soil. They present tubular or funnel-shaped mouths with sometimes modified lips to graze on decomposing organic matter. Fungivorous nematodes, on the other hand, commonly feed on hyphal fungi by piercing the cell wall with a delicate, thin stylet (such as Aphelenchidae and Aphelenchoididae), although some feed on unicellular fungi and yeasts. They are associated with the fungal-mediated decomposition channel, and thus with the slower decomposition rates associated with fungal decomposition of recalcitrant organic matter (Margenot and Hodson, 2016).

Herbivorous nematodes are probably the best-known nematode functional group, due to the relevant damages and losses associated with nematode infestations in agricultural crops worldwide (Atolani and Fabiyi, 2020). Herbivorous nematodes present a wide range of feeding strategies; although specificities exist, many feed on wide ranges of plant species (see, for example, Fanning *et al.*, 2018, and Sikandar *et al.*, 2020). All herbivorous nematodes present a hollow stylet used to pierce the cell wall and suck the

internal cell content, commonly in the root system. As a consequence, damaged roots lose functionality and plants show general decay and loss of vigor, producing unspecific symptoms of decay in the plant. Herbivorous nematodes may act as ectoparasites (as most Dolichodoridae), feeding externally by piercing root cells from the root surface; or as semi-endoparasites (such as Tylenchulidae), inserting part of their body into the root tissue to establish a permanent feeding site; or as endoparasites, introducing themselves into the roots and migrating through the root tissue, destructively feeding on root cells (migratory endoparasites such as nematodes in the family Paratylenchidae) or establishing a permanent feeding site and inducing the formation of galls or cysts (sedentary endoparasites such as Meloidogynidae and Heteroderidae).

Finally, omnivorous and predatory nematodes may feed on various food sources, including other nematodes, algae, and fungi. Commonly, omnivorous nematodes (such as Dorylaimidae and Qudsianematidae) present short and strong stylets that pierce their prey and suck their internal

content, while predators are often larger and present strong stylets and modified lips to attach to their prey, or wide stomas with one or multiple teeth (such as Aporcelaimidae, Discolaimidae, Tripylidae, and Mononchidae). Both omnivores and predators are commonly sensitive to both chemical and physical soil perturbation, and, as K-strategists, quickly disappear from the community when the soil is heavily perturbed.

Besides trophic groups, other functional classifications may be applied to the nematode community. Bongers (1990) proposed the colonizer/persister (c-p) scale, which classifies nematode families into five c-p groups, from c-p1 to c-p5. Briefly, c-p groups classify nematodes according to their life cycle characteristics. Those in lower groups (c-p1 and c-p2) are active nematodes with short life cycles, lay large numbers of eggs, are resistant to soil perturbations, and are typically microbial feeders. By contrast, nematodes in upper c-p groups (c-p4 and c-p5) are larger, sensitive to soil disruption, lay few eggs, and have long life cycles. The intermediate group (c-p3) presents intermediate characteristics, and, although including taxa from different trophic groups, many are plant-parasites. While nematodes in lower groups are *r*-strategists considered as colonizers (able to colonize perturbed soils), families in upper groups are *K*-strategists and are defined as persisters (which persist in stable habitats).

Other nematodes live in the soil in lower numbers. Animal parasites, including parasites of invertebrates and vertebrates, often present soil-inhabiting stages, but appear in extremely low numbers in most soils. Among them, EPNs are especially relevant, in terms of abundance and in terms of commercial interest, since they are used as biological control agents and sold as formulations to be applied in agroecosystems. Their role in ecosystems as biological control agents is described in the section "Use of nematodes as model system in plant protection" later in this chapter.

Evaluation of the Nematodes in the Soil for Ecological Studies

Isolation of nematodes

Soil nematodes actually inhabit the water film that surrounds soil particles, and depend on

water to perform their vital functions. Nematodes do not survive fast desiccation, due to their permeable cuticle (although they may survive slow desiccation through anhydrobiosis). Soil nematode extraction is always performed in fresh soils and is based either on wet-sieving or on flotation and centrifugation techniques. While wet-sieving extractions require a final step in which nematodes actively pass through a sieve (active methods), the efficiency of the method relies on nematode activity, and may be inappropriate for slow-moving nematodes. Centrifugation and flotation-based techniques do not require active nematode movements (passive methods) and may be efficient for extracting immobile individuals.

The most commonly used wet-sieving extraction technique is with Baermann funnels (Baermann, 1917; Barker, 1985). Although many variations exist, and each laboratory may use its own protocol, the Baermann funnel extraction is commonly performed in two steps. First, one or several wet-sieving steps are done to remove coarser soil fractions, so that the soil volume used in the second step of the extraction is reduced. Second, the sample is deposited on a fine sieve (30–40 μm) that is put on the top of a funnel filled with water. Active nematodes pass through the sieve and are then collected from the funnel stem. Alternatively, the fresh soil may be dispersed over a large sieve with no previous sieving (Whitehead and Hemming, 1965).

Passive extraction methods include Oostenbrink's elutriation and centrifugation on concentration gradients. The Oostenbrink's elutriator is a device that passes a continuous water flow through a wet soil sample so that nematodes are dragged out of the sample and collected in a vial. Sucrose centrifugation (Caveness and Jenses, 1955), on the other hand, is used after wet-sieving of soil or plant material. Plant roots, previously disaggregated in a blender, or fresh soil are wet-sieved to remove coarse soil fractions or plant debris. Then the resulting suspension is centrifuged in a sucrose concentration with a specific gravity that retains nematodes in the supernatant while the mineral soil fraction is sedimented, and nematodes are collected from the supernatant afterwards.

Other specific techniques are available for specific nematodes, including ultrasonic extraction of plant-parasitic nematodes (Tangchitsomkid

et al., 2015), specific techniques for root-knot or gall and cyst nematodes (Riggs *et al.*, 1997; Ruan *et al.*, 2012; den Nijs and van den Berg, 2013) or insect bait-technique for EPNs (Bedding and Akhurst, 1975).

In summary, good extraction techniques should be able to extract as many nematodes as possible irrespective of the soil type, environmental conditions or nematode(s) of interest (McSorley and Parrado, 1987). However, since no technique is completely efficient, selecting the right one depends on the circumstances, and is a necessary first step to develop further nematode studies. Efficiency under different criteria may be consulted in available literature (Nagy, 1996; Verschoor and de Goede, 2000; McSorley and Frederick, 2004; Blauel *et al.*, 2016).

Method for identification and quantification: from visual to molecular approaches

Once the nematodes have been extracted from the soil, the next step is the identification and quantification of the different members that comprise the nematode community. The most intuitive approach is to perform a visual identification based on the study of microscopic observations of nematode morphology. The advantage of this method is its feasibility in terms of equipment and installations, since only a good-resolution microscope and minor material for establishing the permanent or semi-permanent slides are *a priori* required for the study (Seinhorst, 1959, 1966). However, it is also a time-consuming and labor-intensive process that requires specialized personnel.

Nowadays, almost all DNA-based identification and quantification of the nematode community's procedures are based on polymerase chain reaction (PCR) protocols, and their implementation for addressing soil ecology questions is gaining interest. Briefly, a PCR produces a considerably high amount of target DNA from a small amount of material (Hoelzel and Green, 1992). The reaction only requires: (i) the deoxyribonucleotides; (ii) the primers that flank the target region of interest; (iii) the buffer to allow the reaction; and (iv) the enzyme that will synthesize the new sequences. Then, the mixture is subjected to a cycling of denaturation–annealing–synthesis until the desired quantity is synthesized (Mullis

and Faloona, 1987; Mullis, 1990). The simplicity of PCR reactions, and the accessibility of the minor equipment needed for their implementation in an institution, has promoted their incorporation as a routine method in many laboratories.

In most of the applications, the key step is the design and selection of the most appropriate set of primers. However, the study of randomly amplified polymorphic DNA (RAPD) or amplified fragment length polymorphism (AFLP) does not include any previous information about the target area of study. These approaches are used for nematode identification and for measuring diversity in a group (Lu *et al.*, 1998; Lopes *et al.*, 2019). DNA sequencing, a pioneering method developed by Sanger *et al.* (1977), is linked to PCR-based approaches. In particular, DNA barcoding is used in nematology to obtain full sequences of the target area of the genome (defined by the primers) where the presence of single nucleotide polymorphisms (SNPs), considered the highest possible resolution of genetic differences, can be explored (Morin *et al.*, 1999). Then, the obtained sequences are compared with other sequences available in different international databases. For example, nowadays, GenBank (available at <https://www.ncbi.nlm.nih.gov/genbank/>, accessed 30 December 2021), a reference genetic sequence database with an annotated collection of publicly available DNA, contains more than 4.3 million entries from more than 13,000 nematode species.

Advanced approaches employ the identification of SNPs for screening differences of nematode composition in soil samples without the necessity of sequencing the whole sample. For example, the technique of denaturing gradient gel electrophoresis (DGGE), although it has less power to resolve differences than direct sequencing, has the advantage of being able to process various samples at once. In this case, PCR employs specific primers for a selected region, and the resulting fragment will migrate in a polyacrylamide gel in a buffer with an increasing gradient of the specific compound (for example, formamide and urea). The denaturation point will be characteristic of each sample and, hence, the migration stops at different points of the gel, producing a pattern that can be compared among samples. However, one of the limitations is the possibility to compare between different runs. Some examples of use of this

method are evaluation of a novel fumigation agent for controlling PPNs associated with banana crops (Su *et al.*, 2015), and the study of nematode variation in the genus *Globodera* (Nuaima *et al.*, 2019). Another technique that can also provide information on the variability of nematode composition among multiple samples without the necessity of whole sequencing is PCR-based terminal restriction fragment-length polymorphism (PCR T-RFLP). This approach has revealed changes in assemblage composition due to different organic amendments, providing accurate values of different trophic groups (Donn *et al.*, 2012). In addition, a recent study using this approach to study nematode and microbial communities has shown the connectivity between both soil biotic components in grassland soil (Neilson *et al.*, 2020), highlighting the huge potential of combining these approaches with various soil organism guilds.

During the past decade, more sophisticated molecular approaches have been displacing the use of conventional PCR in favor of the use of quantitative real-time PCR (qPCR) and next-generation sequencing (NGS) approaches for soil nematode identification and quantification, from species to community level. On the one hand, qPCR uses the same elements as a conventional PCR but with the addition of linking fluorescent products (SYBR Green® for double-stranded DNA or TaqMan® probes designed to be specific also for the primer-amplified sequence). The fluorescence signal is produced in each cycle only in the case of presence of selected target and this signal will be proportional to the initial amount of the target. Hence, it is possible to establish the amount of DNA of the target species in the sample and, if samples of known concentrations are run in parallel, it is possible to perform the absolute quantification of the target organisms (Campos-Herrera and Lacey, 2018). This technique has been employed for many soil nematodes, in particular those of economic relevance for yield loss or those subjected to quarantine, such as nematodes in the genera *Meloidogyne*, *Globodera* or *Pratylenchus* (see review in Campos-Herrera *et al.*, 2010). In some cases, multiplex reactions (i.e., targeting two or more species in the same reaction by combining various specific sets of primers) have been proposed; however, the fact that the accuracy of detection can be affected by the varying numbers of the selected species (Zijlstra

and van Hoof, 2006) means that the single-plex reaction is used more often.

The use of qPCR approaches allows exploration of the nematodes' assemblage with other soil organisms or even reveals the structure of the soil community. For example, the qPCR system has been employed to study the EPN soil food-web assemblage in various habitats (citrus orchards, vineyards, annual crops, and natural areas) from different biomes (temperate, subtropical, and Mediterranean) (Campos-Herrera *et al.*, 2011a,b, 2013, 2015a,b, 2019a; Blanco-Pérez *et al.*, 2020). Similarly, qPCR approaches have been implemented to study trophic groups and various ecological indexes in various agro-nomical scenarios (Vervoort *et al.*, 2012; Quist *et al.*, 2016, 2017). More sophisticated methodologies such as digital qPCR (dPCR) (Hindson *et al.*, 2011) and microfluidic systems (also microfluidic droplet PCR) (Zhu *et al.*, 2012; Chang *et al.*, 2013) are novel methods that allow DNA amplification in a compartmentalized reaction in a minimal volume. However, these have not yet been employed in the study of soil nematode ecology.

Finally, NGS (also called high-throughput sequencing) is replacing the traditional clone-sequencing system by the direct sequencing of areas flanked by selected primers, with substantial reduction in costs and simplification of the procedure (Pompanon *et al.*, 2012; van der Heijden and Wagg, 2013). NGS is a technique used when the main objective is to describe the maximum biodiversity in a sample. This metagenomics approach implies dealing with thousands of sequences generated; it requires standardization of the units to molecular operational taxonomic units (MOTUs), and the performance of multiple alignments and classification, involving hierarchic associations within samples to establish similar sequences belonging to the same species or target group (Blaxter *et al.*, 2005). A seminal study made it possible to compare MOTUs with artificially generated nematode communities (called "mock communities", with specific numbers of individuals of certain species) (Porazinska *et al.*, 2010). This study revealed some of the shortcomings related to the study of nematode communities by NGS approaches: (i) under- or overestimation of species richness caused by failure to detect a number of nematode species or caused by intraspecific sequence variants

increasing the number of MOTUs or “molecular” species; (ii) flaws in quantification; and (iii) poor ability to link molecular species with real biological species. Current studies are advancing to solve these limitations. For example, Geisen *et al.* (2018) showed that there is a high positive correlation between the values provided by NGS, qPCR, and traditional morphometric studies, but in the case of NGS records, it provides a higher degree of taxonomic resolution. In a recent study using nematode mock communities, the selection of the DNA extraction method was revealed as a key factor to success in the accurate description of nematode composition using NGS. Different protocols have variable tissue lysis capacity, which can lead to significant differences in species richness (Waeyenberge *et al.*, 2019). Likewise, the selection of the primers that will amplify the specific region for the sequence is also decisive (Waeyenberge *et al.*, 2019). In addition to these methodological limitations, another key factor is the knowledge of the possible species that by biogeography can be found in an unknown sample and the need to have a valid sequence available to be compared with the data provided by the MOTUs. Hence, although the NGS approach is a powerful method for biodiversity studies and the analysis of soil nematode communities as bioindicators of the soil disturbance, additional work is needed to validate the protocols, harmonize results, and make them compatible with the traditional records.

Functional diversity and the employment of indexes for ecological approaches

Based on either morphological or molecular identifications, the adscription of soil nematode taxa to functional groups (trophic and c-p groups) permits the calculation of several families of diversity indices and ecological indicators of soil health based on the composition of the nematode community. Three different families of indices have been proposed, and are extensively used to assess the effects of agricultural soil management, soil chemical pollution, physical soil disturbance, construction and mining activities, forest management, and climate change on soil food-web structure and overall soil functioning.

Maturity indices were proposed as indicators of (agro-)ecosystem conditions during ecological succession (Bongers, 1990; Yeates, 1994; Bongers and Bongers, 1998). The original Maturity Index (MI) (Bongers, 1990) is based on the proportion of different nematode c-p groups; it ranges from 1 to 5 and is indicative of the development of the community. It only includes free-living nematodes (its calculation excludes plant parasites), since herbivore nematodes often bloom in agroecosystems due to anthropogenic causes, and thus do not represent a “natural” soil food-web condition. To assess the complexity and development of herbivore nematode assemblages, the Plant-Parasitic Index (PPI), similar to the MI but including only plant-parasitic taxa, was proposed at the same time (Bongers, 1990). The inclusion of herbivore nematodes in the MI (Σ MI) was proposed by Yeates (1994), arguing that the appearance of vegetation is a natural step in ecosystem development and so excluding herbivore nematodes from the study of soil food-web structure would be inconsistent. Other modifications of the original MI include the exclusion of c-p1 nematodes (MI2–5 and Σ MI2–5) (Korthals *et al.*, 1996; Neher and Campbell, 1996), which are enrichment-opportunistic nematodes that bloom shortly after soil nutrient enrichment and thus are transitory components of the community.

The joint classification of nematode families into trophic and c-p groups results in the description of 16 functional guilds (bacterivores c-p1, bacterivores c-p2, bacterivores c-p3, fungivores c-p2, and so on, although not all combinations exist) that respond differently to soil enrichment and perturbation (Ferris *et al.*, 2001). Using the percentage contribution of each functional guild to the community, which is weighted by its importance on the enrichment (response to nutrients) and structure (food-web complexity) components of the soil food-web, four soil food-web indices are calculated (Ferris *et al.*, 2001). Briefly, the Enrichment Index (EI) weights the contribution of bacterial-feeding, opportunistic nematodes to the community, and is used as an indicator of soil fertility, nutrient availability, and fast organic matter decomposition mediated by bacteria. The Channel Index (CI) indicates slow decomposition of recalcitrant organic matter mediated by fungi by weighting the importance

of fungivores in relation to bacterivores. The Structure Index (SI) is based on the abundance of omnivores and predators in high soil food-web levels, thus indicating soil food-web complexity. Finally, the Basal Index (BI) weights the prevalence of generalist nematodes resistant to environmental perturbation, and is thus an indicator of soil disturbance (Ferris *et al.*, 2001).

The last family of nematode-based indicators was proposed by Ferris (2010) as a development of the soil food-web analysis concept by soil food-web indices. Based on the previous conceptual framework and including the estimated average biomass of each nematode taxa provided by the morphometric data present in the taxa original description, Nematode Metabolic Footprints were proposed as metrics of the magnitude of ecosystem services provided by trophic groups and functional guilds (Ferris, 2010). The Structure, Enrichment, and Bacterivore, Fungivore, Herbivore, Omnivore, and Predator Metabolic Footprints are thus indicators of the metabolic activity of such trophic and functional guilds in the specific food-web carbon channel in which they participate.

Examples of Use of Nematodes as Model System in Soil Ecology Approaches

Use of nematodes as model system in plant protection

The current plant protection paradigm must face the challenge of reducing agrochemicals by providing alternative management strategies that respect the environment. One of the most popular alternatives is the search and use of biological control agents, such as the nematodes in the trophic guild of entomopathogens, the EPNs. However, over decades, there are records of successes and failures on their use in several crops and against numerous target pests (reviewed by Dolinski *et al.*, 2012, and Lacey and Georgis, 2012). The relevance of the understanding of the biotic and abiotic factors that modulate their activity is now recognized (Stuart *et al.*, 2015), so the better we understand how and when EPNs are active,

the better will be their success in integrated pest management (IPM) and organic management programs. Indeed, this information is critical for both augmentation and conservation biological control strategies. Associated with citrus groves in Florida, there are various studies illustrating this critical approach. The citrus weevil *Diaprepes abbreviatus* (Coleoptera: Curculionidae) is highly polyphagous and is considered as a silent pest because the main damage is caused in the roots. The larvae can directly damage by feeding in the roots, but at the same time, the wounds they create are natural entrances for *Phytophthora* spp. (Graham *et al.*, 2003), and both together can produce the decline of a grove in just 1–2 years (Campos-Herrera *et al.*, 2015c). Although there are chemical and biological tools for managing the eggs and adults present in the citrus canopy, there are almost no alternatives for the management of the larvae in the soil. The biological control potential of the EPNs has been evaluated since the 1980s, but there are success and failure records (Beavers *et al.*, 1983; McCoy and Boucias, 1989; Duncan *et al.*, 2003). The seminal study by Duncan *et al.* (2007) highlighted the relevance of the integration of the study of EPN natural enemies, such as nematophagous fungi (NF), free-living nematodes (FLNs), and ectoparasitic bacteria (El-Borai *et al.*, 2005; Campos-Herrera *et al.*, 2011b, 2012; Pathak *et al.*, 2012). Duncan *et al.* (2007) observed that after an EPN augmentation, the rest of the soil organisms associated with EPNs can respond in a way that can substantially decrease the EPN efficacy against the pest. Subsequent studies showed that the soil can modulate the EPN soil food web and their possible contribution to management of *Diaprepes*. Campos-Herrera *et al.* (2013) showed that soil properties related to water content (groundwater depth, available water capacity, clay and organic matter content) defined the EPN soil food-web assemblage in two eco-regions in Florida. Those variables directly or indirectly throughout the natural enemy's modulation could be the drivers of the weevil differential presence in those regions (Campos-Herrera *et al.*, 2013; Pathak *et al.*, 2017). The vegetation and soil properties linked to water content were consistent as key drivers of the EPN soil food-web assemblage in other

regions and habitats, both in natural and in agricultural lands (Campos-Herrera *et al.*, 2016a, 2019a; Jaffuel *et al.*, 2018). Hence, in conservative approaches, the characterization of the soil properties and their link with key biotic component are crucial to establish the possibility of EPNs in an area. In addition, the crop and associated soil could be managed in a way that can provide conducive areas for enhancing the EPN presence and limiting natural enemies. For example, Duncan *et al.* (2013) showed that EPN presence was higher in areas where citrus planting holes were replaced with sand than in citrus planted in native soil. Similarly, the use of spontaneous cover crops in vineyards can favor the presence and activity of EPNs while decreasing the presence of their natural enemies (FLN, NF, and ectoparasitic bacteria) compared with traditional tillage strategy soil management (Blanco-Pérez *et al.*, 2020) (Fig. 8.2).

Beside their practical usefulness as biological controls, the study of the EPN soil food web can be used as model system in ecological questions about subjects such as predator–prey or interaction with other beneficial soil

organisms. For example, the abundance of three EPN species, seven NF species, one FLN, and an ectoparasite bacterium were monitored for almost 2 years in four Florida citrus orchards to evaluate the temporal distribution in the soil profile (Campos-Herrera *et al.*, 2019b). In this study it was shown that the NF species *Drechslerella dactyloides* exhibited evidence of top-down regulation, being inversely related to population dynamic of *Heterorhabditis indica* at two of three sites. Moreover, the ectoparasitic bacterium *Paenibacillus* sp. showed significant phase–space, predator–prey dynamics at two of three sites with changes in the population of the EPN *S. diaprepesi*, a species-specific host of the bacterium (Fig. 8.3). This study highlights the necessity of a better understanding of the temporal patterns of EPNs and their natural enemies to introduce accurate and successful practices that favor their activity.

Finally, from a plant protection approach, the interaction of nematodes with other beneficial soil organisms or other biological controls can show the potential of combined use. For example, in Swiss wheat crops, three beneficial soil organisms were inoculated alone or

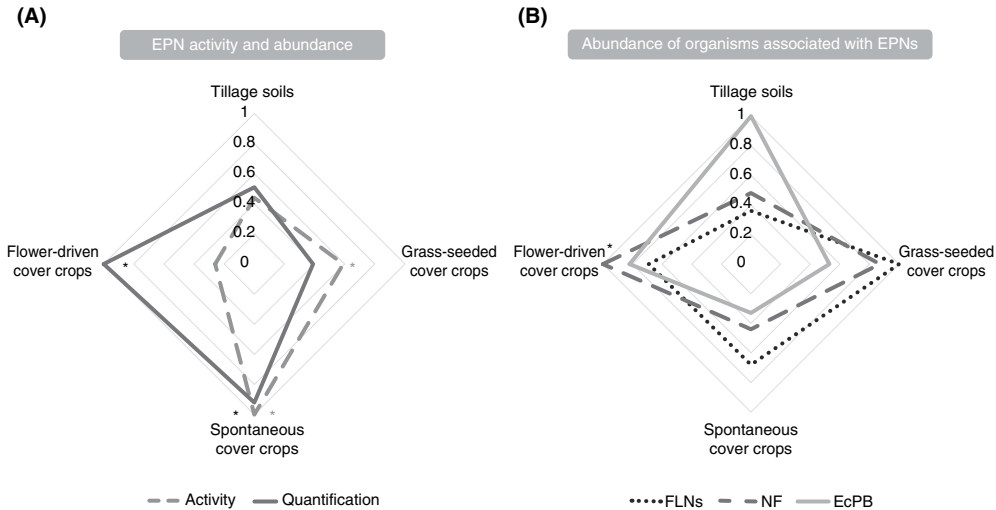


Fig. 8.2. Radar chart of the effect of the use of the cover crops grass-seeded, flower-driven, and spontaneous on the entomopathogenic nematode (EPN) soil food web compared to tilled soils. **(A)** EPNs as number of infective juveniles (IJs) and frequency of occurrence. **(B)** Natural enemies of EPNs: free-living nematodes, nematophagous fungi, and ectoparasitic bacteria. Asterisks indicate significant differences from generalized linear mixed models testing within pair-treatment comparisons of tillage management and each cover crop at $*P < 0.05$ and n.s. (not significant). Values are least-square means. Data from Blanco-Pérez *et al.* (2020), modified.

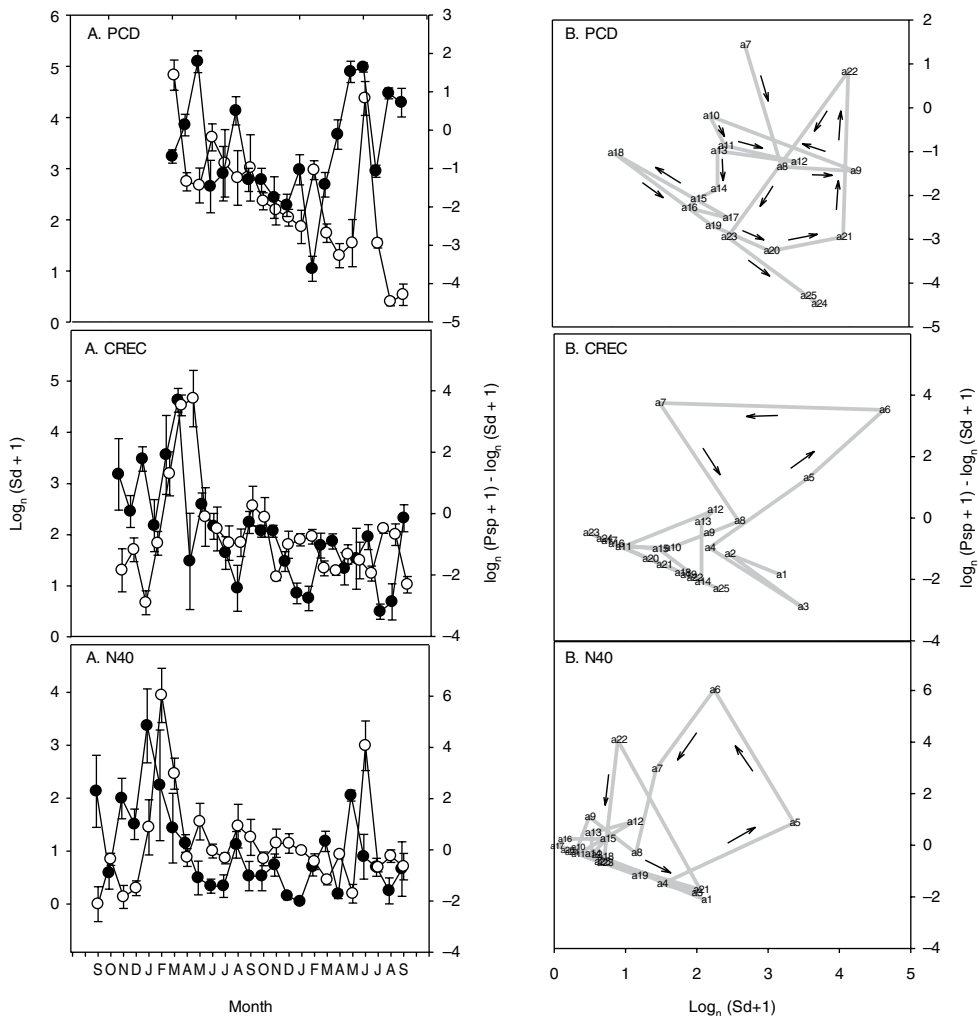


Fig. 8.3. Temporal study on the abundance of *Steinernema diaprepesi* (Sd, left panels, black) and *Paenibacillus* sp. endospore infestation rate (Psp, left panels, white) at three sites in Florida (USA). The phase–space diagrams show the sequential direction of monthly flux of *Paenibacillus* sp. endospore infestation rate compared with *S. diaprepesi* (Sd) abundance (right panels) of the means. Reprinted from Campos-Herrera *et al.* (2019b) with permission, licence number 4932991455889.

combined at seeding: plant-beneficial *Pseudomonas* bacteria, arbuscular mycorrhizal fungi (AMF), and EPNs (Imperiali *et al.*, 2017). During three independent trials (2014–2015) it was observed that the beneficial soil organisms were compatible when combined, with similar presence rates as in single application treatments. In addition, various *Pseudomonas*, mycorrhiza, and nematode treatments improved plant health and productivity, although additive

or synergistic effect were not recorded. The beneficial impacts in the crop (plant growth, yield) were most evident for plants under biotic stress and for certain individual treatments. A subsequent study was performed in Missouri (USA), applying the three beneficial soil organisms in a maize field in the presence or absence of the western corn rootworm (WCR), *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) (Jaffuel *et al.*, 2019).

During three consecutive years (2015–2017), the three organisms were applied alone or combined, in plots infested and not infested with WCR. Overall, in two of the three experiments, in the plots treated with EPNs and/or *Pseudomonas* bacteria, the roots were less damaged than the roots of plants in control plots. Also, in one of the trials, these two treatments enhanced yield measured as total grain weight. Again, as in the Swiss wheat field experiments, the effects of the treatments varied considerably during the years and the treatments but, in general, they were shown to have a positive impact on plant health and performance. Hence, a finetuning of the species, application time, and rate is proposed to achieve a combined improvement of plant protection using various beneficial soil organisms, including nematodes.

Use of nematodes as model system in community changes due to disturbance

Soil nematode communities respond differently to various types of soil disturbance. Most of the research done on nematode-based indicators was primarily applied to agricultural systems, although the focus has greatly extended in recent years. Agricultural management is one of the most relevant soil disturbance factors worldwide, and both physical (tillage, compaction, erosion) and chemical (fertilization, pesticide application, salinization) disturbances are common in most agricultural systems. The use of nematode-based indicators has greatly helped in understanding the effects of such disturbance in soil biological communities.

The effects of fertilizers on the nematode community might be direct (toxicity of nitrogen compounds) or indirect (mediated by the microbial community). Toxic nitrogen solutions may affect the most sensitive nematodes (those in high c-p groups) (Tenuta and Ferris, 2004), which may cause declines in the complexity of the soil food web as indicated by the Structure Index (Forge *et al.*, 2020). On the contrary, the addition of mineral fertilizers and the incorporation of organic matter (manure,

compost) into the soil enhances the abundance of bacterivorous and fungivorous nematodes, with subsequent increases of the Enrichment Index and the Bacterivore, Fungivore, and Enrichment Footprints (Pan *et al.*, 2020). The quality of the amendments also plays a critical role, since organic amendments tend to increase the diversity and complexity of the nematode community and the Structure Index (Liu *et al.*, 2016) while mineral fertilizers reduce the Maturity Index and the Channel Index (Song *et al.*, 2016).

Tillage is a common and recurrent physical perturbation in most cropping systems. The disruption of soil aggregates and the inversion of soil layers primarily affect large, sensitive omnivorous and predatory nematodes, reducing the complexity of the soil food web, with significant reductions in the Maturity Index and the Structure Index (Bongiorno *et al.*, 2019). In no-till systems, the nematode community presents greater complexity (Leslie *et al.*, 2017). Herbivorous nematodes, on the other hand, also respond to crop management in various ways. While monoculture commonly increases the presence of herbivorous and PPNs, adequate crop rotation may reduce nematode infestation (Kratochvil *et al.*, 2004). Standard tillage also reduces PPNs by damaging plant roots and thus eliminating their food source (Neher *et al.*, 2019). Organic amendments may also reduce PPNs by a number of different mechanisms, including the promotion of antagonists and biological control agents, releasing nematocidal compounds, and increasing plant resistance or tolerance (Oka, 2010). PPNs and other soil pests are often controlled with chemical treatments, which, together with herbicides, might be also directly toxic to other FLNs (Ruess *et al.*, 2002; Carrascosa *et al.*, 2015; Sánchez-Moreno *et al.*, 2015; Haegerbaeumer *et al.*, 2019).

Another important soil disturbance is soil chemical pollution due to human activities, such as metal extractions, the presence of chemical industries or soil contamination with waste residues. Several studies have investigated the impact of these activities on the soil nematode community. For example, Navas *et al.* (2010) investigated the impact of the rupture of a mining reservoir containing

mineral residues in 1998 in southern Spain. This accidental spill polluted a large section of the Guadianamar River Basin with heavy metals, including lead (Pb), nickel (Ni), and copper (Cu). The analysis of soil nematode communities in the polluted area revealed that nematode taxa abundance and frequency, as well as diversity and Maturity Indices, were all negatively affected by the spill. In particular, Ni and Cu were shown to be the metals with the most toxic effect on the nematode community. Other studies have investigated the impact of long-term heavy-metal presence, such as that derived from a mining district. For example, Rodríguez-Martín *et al.* (2014) showed that the diversity, abundance, and metabolic footprints of the nematode community were significantly reduced in a highly polluted area related to an ancient exploitation of Pb in Murcia (Southern Spain), compared with natural and agricultural areas in the vicinity. In this area, predatory nematodes were sensitive to mine and agricultural activities, while the presence of omnivorous nematodes was reduced only in the agricultural lands. Surprisingly, the presence of EPNs in this polluted region was not significantly different than in the natural and agricultural areas, being present in areas with high Pb content (Campos-Herrera *et al.*, 2016b) (Fig. 8.4). Similarly, the presence of pharmaceutically active compounds and personal care products (PPCPs) in the environment is considered ubiquitous (Fernández *et al.*, 2010; Chen *et al.*, 2013). Concern about the ecological effects of PPCPs and their effect on human and wildlife health (Stuart *et al.*, 2012) is encouraging the quantification of these impacts. Gutiérrez *et al.* (2016) explored the impact of these new pollutants on the nematode community. Nematodes were shown to respond to these emerging contaminants, resulting in highly altered diversity and structure community indices. In addition, the nematodes characterized as K-strategists were negatively affected by certain pollutants. Interestingly, certain nematodes seem to be adapted to soils with high levels of heavy metals, but this trend was not observed for soils with emerging pollutants like PPCPs.

Conclusions and Future Directions

The ubiquitous presence of nematodes in both natural and agricultural soils, and the fact that biotic and abiotic factors can affect nematode species in different manners, make them excellent models in soil ecology studies. In this chapter we have presented several examples of the use of nematodes to address different soil ecology questions, both from fundamental and from applied perspectives. This non-exhaustive presentation seeks to illustrate the potential of the use of nematodes in these approaches, with the aim of stimulating further studies on the applications of these concepts. Advances in new molecular tools are speeding up the production of novel data, but this process should be critically revised to ensure that the new findings are valid, maximizing their compatibility with previous records using traditional methods and, in all the cases, with caution, revealing new paradigms in nematode ecology and soil science. In addition, the integration of other disciplines such as chemical ecology can reveal the behavioral responses of nematodes, for example revealing how nematodes react to certain stimuli produced by plants under attack (Rasman *et al.*, 2005) or how the same stimuli can be a signal to other soil organisms integrated in trophic cascades (Ali *et al.*, 2012, 2013). Overall, soil ecology is still a dark but passionate “black-box” where nematodes are being revealed to be critical to the understanding of the physical and chemical processes and of the interactions among soil organisms. Future research integrating various disciplines such as soil science, molecular biology, geostatistics, and chemical ecology will provide new and exciting paradigms where nematodes are called to be in a priority position.

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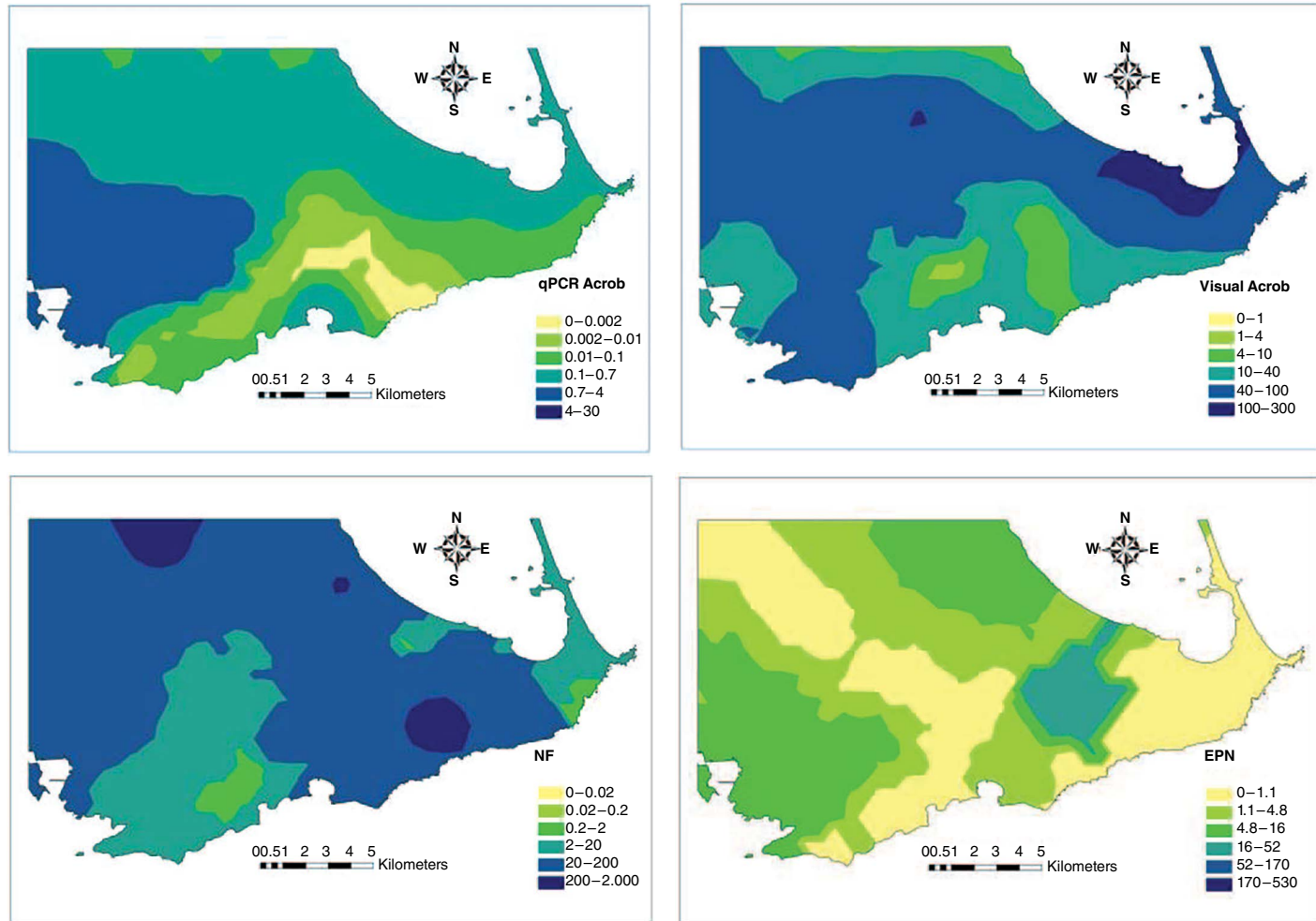


Fig. 8.4. Spatial distribution of entomopathogenic nematodes (EPNs), nematophagous fungi (NF), and free-living nematodes *Acrobeloides* (qPCR and Visual) in Cartagena–La Unión (Murcia, Spain). Values of the organisms detected by qPCR were magnified by one or more order of magnitude to obtain numbers to be used in the statistical procedure. Experimental semivariograms fitted as spherical model and mapped by ordinary kriging (ISATIS V10.0, the Geostatistical Analyst extension for ArcGIS 9.3). Reprinted from Campos-Herrera *et al.* (2016b) with permission, licence number 4933000182819.

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9 Stress and Survival Mechanisms

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Introduction

Nematodes are among the most abundant organisms on earth, filling all trophic levels in the soil food web, as they exist in almost every possible habitat and ecosystem (Bernard, 1992; Ettema, 1998; De Ley, 2006; Powers *et al.*, 2009; van den Hoogen *et al.*, 2019). Indeed, these organisms can be found in aquatic (marine and freshwater) and terrestrial ecosystems ranging from the tropics to the poles and from the highest to the lowest of elevations (van den Hoogen *et al.*, 2019). Furthermore, nematodes have exploited a wide range of ecological niches encompassing free-living and parasitic species. These diverse ecological niches are characterized by temporal variation of temperatures, salinity, moisture, and oxygen concentrations. As these animals evolved in the different environments, they developed unique and fascinating mechanisms to acclimatize to these conditions. For example, the first plant-parasitic nematodes were recorded in 1743 by the English biologist John Needham, who recovered resistant juveniles of the wheat nematode *Anguina tritici* from dry, diseased wheat kernels (Poinar, 1983). He described them as “fibrous objects in water, which after time come to life”. Since then, the ability of the nematodes to withstand harsh environments

has been demonstrated widely. These unique abilities attracted the attention of many scientists who characterized the incredible capability to withstand extreme conditions such as complete desiccation, freezing, or high salinity; these scientists also attempted to understand the physiological and molecular mechanisms of such phenomena.

This chapter describes the survival mechanisms and implications for other biological systems. For further information related to these issues see the following reviews: general characterization of nematode survival (McSorley, 2003; Cooper and Van Gundy, 1971; Perry and Wharton, 2011); desiccation (Burnell and Tunacliffe, 2011; Glazer, 2002); heat tolerance (Grewal *et al.*, 2011); freezing (Wharton, 2011); and osmotic tolerance (Wharton and Perry, 2011).

Nematode Life Cycle in Relation to Survival

The most generalized life cycle of a nematode involves an egg, four juvenile stages (referred to as J_1 to J_4), and the adult (Poinar, 1983). In many species, the appearances of juveniles and adults are similar, but great diversity exists in the life cycles of this large group (Wharton, 1986). The

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life cycle of some nematodes offers built-in opportunities for resisting environmental stresses. For example, the cereal nematode *Heterodera avenae* survives the dry season in a cyst form (Cooper and Van Gundy, 1971) and new J_2 nematodes emerge from the eggs as soon as environmental conditions (moisture and presence of host root) are favorable (Brown, 1984). 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 *Ditylenchus dipsaci* spend summer months in a form of dormancy (Cooper and Van Gundy, 1971; Norton, 1978). In the animal-parasitic Protostrongylidae nematodes (on small ruminants and goats), the J_1 survives harsh desert conditions in a desiccated state in the animal host's feces (Solomon *et al.*, 1997). Among several rhabditid families, environmental stress (lack of food, high population density, heat, etc.) induces 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 nematodes (EPNs) from the families Steinernematidae and Heterorhabditidae, the dauer stage is also the infective stage (also termed infective juvenile, or IJ). The IJ locates and invades insect hosts (Lewis *et al.*, 1992). This IJ 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 molt, which are retained to provide additional protection (Timper and Kaya, 1989; Campbell and Gaugler, 1991).

The Nematode Species Used for Survival Studies

There is no "single model" nematode that can be used as an example for the adaptations inherent in survival strategies, because various species show different combinations of adaptations. Although *Caenorhabditis elegans* has been used most widely as a biological model, it is not the best suited to study stress mechanisms. That is because it originates from a mild and moderate environment in England (Brenner, 1974) and its

capability to withstand extreme conditions such as desiccation or high temperature is limited (Burnell and Tunnacliffe, 2011). Nonetheless, due to its general suitability as a model organism with vast molecular and genetic information, *C. elegans* has been used to investigate the effect of stress on longevity (Johnson *et al.*, 2002) as well as recent studies on molecular and biochemical mechanisms of desiccation tolerance (Erkut and Kurzchalia, 2015), as will be further described.

The plant-parasitic nematode *D. dipsaci*, which reproduces abundantly on infected plants, has been widely used for physiological, biochemical, and ultrastructural studies (e.g., Perry, 1977, 1999; Barrett, 1991; Wharton and Lemmon, 1998). Another highly tolerant plant-parasitic nematode species is *Anguina tritici*, creating galls in grain seed. Each gall contains several thousand desiccated J_2 .

The free-living nematode *Aphelenchus avenae* can be cultured in large quantities in flasks of autoclaved wheat seeds inoculated with the fungus *Rhizoctonia solani*. The large volume of nematodes recovered makes *A. avenae* ideal for biochemical studies, but it takes about 4 weeks to obtain a harvestable culture; microscopic observation of the nematodes *in situ* is difficult, being obscured by the fungal mycelium; and *A. avenae* is not amenable to RNA interference (RNAi) by feeding (Reardon *et al.*, 2010) unless a suitable fungal vector system to deliver dsRNA can be developed.

The genus *Panagrolaimus* has been used extensively for stress tolerance studies, both for desiccation and freezing tolerance. *Panagrolaimus* feed on bacteria and occupy a variety of niches, ranging from Antarctic, temperate and semi-arid soils to terrestrial mosses. This genus includes both fast-dehydration and slow-dehydration anhydrobiotic strategies (Wharton and Barclay, 1993; Ricci and Pagani, 1997; Shannon *et al.*, 2005). The Antarctic species *Panagrolaimus davidi*, which has been used extensively by Wharton and colleagues (e.g., Wharton and Brown, 1991; Wharton *et al.*, 2003) to study the physiology of freezing tolerance, is also a slow-dehydration strategist anhydrobiote (Wharton and Barclay, 1993). Shannon *et al.* (2005) also found that *Panagrolaimus* is amenable to gene knockout by RNAi when fed on *Escherichia coli* expressing dsRNA for essential conserved genes.

Entomopathogenic nematodes from the families Steinernematidae or Heterorhabditidae are important biological control agents (Ehlers, 2001; Glazer, 2002; Grewal *et al.*, 2005). Although their third-stage IJs have moderate tolerance to environmental extremes (Glazer, 2002; Burnell and Tunnacliffe, 2011), extensive attention has been devoted to their survival capabilities in storage and under field conditions. These studies include evaluation of desiccation and osmotic stress tolerance (Glazer, 2002, 2015) as well as sensitivity to extreme temperature (Grewal *et al.*, 1994, 2011), implying that this may help to increase persistence and efficacy in the field as well as storage and shelf life of these biological control agents (Glazer, 2015; Sumaya *et al.*, 2017) (see also Chapter 1, this volume). Entomopathogenic nematodes can be readily cultured in large quantities in the laboratory and have been used as convenient models to study gene induction in response to mild dehydration stress (Gal *et al.*, 2003, 2005; Tyson *et al.*, 2007; Somvanshi *et al.*, 2008).

How Nematodes Encounter Extreme Conditions

Quiescence is a form of dormancy that involves physiological adaptations that enhance survival (Perry and Moens, 2011). Quiescence is a facultative response, involving a lowered metabolism in response to unfavorable environmental conditions, and is readily reversible when favorable conditions return. If the stress persists, some organisms can enter a state of cryptobiosis where there is no measurable metabolism. Unfavorable environmental conditions include absence of water, osmotic stress, extreme temperatures (heat or freezing), and lack of oxygen; the types of quiescence induced in organisms by these conditions are termed anhydrobiosis, osmobiosis, thermobiosis, cryobiosis, and anoxybiosis, respectively (Barrett, 1991). Under natural conditions these situations may intertwine and the organisms may face multi-stress situations, as will be discussed further. At this point the most important stress factors will be outlined in this chapter, and the response of certain nematodes to these conditions will be characterized. The chapter will also summarize current knowledge

on behavioral, physiological, genetic, and molecular adaptation to these stresses.

Desiccation tolerance

Plants can remain viable under dry conditions mainly in the form of seeds. Among invertebrates, a handful of animals, such as tardigrades, brine shrimps and nematodes, can overcome dry conditions (Cooper and Van Gundy, 1971; Wharton, 1986; Perry and Moens, 2011) even though some are aquatic organisms and need a film of water surrounding their body in order to move (Norton, 1978). Some nematodes can survive in a desiccated state for many years (Cooper and Van Gundy, 1971; Wharton, 1986). For example, the second-stage larva (L₂) of the wheat-parasitic *A. tritici* remained in flower galls for 32 years. The stem and bulb nematode *D. dipsaci* at the fourth-stage larva (L₄) showed similar survival ability as *A. tritici* in dried plant tissue (Evans and Womersley, 1980; Perry, 1977). The ability to survive under dry or freezing conditions for long periods, even years, fascinated scientists who desired to preserve live eukaryotes for space voyages and long-term preservation (Guo *et al.*, 2000; Puhlev *et al.*, 2001; Potts *et al.*, 2005).

The dauer larval stages of rhabditid nematodes are adapted for motile dispersal, longevity, reduced metabolic activity, and food finding. While they may have some desiccation resistance (depending on the species), no examples of fully anhydrobiotic rhabditid dauer larvae have been described. For example, exposure of the *C. elegans* dauer larvae to 97% relative humidity (RH) for 24 h resulted in 30% mortality of the treated worms (Gal *et al.*, 2004).

Entomopathogenic nematodes have also been subjected to intensive study for their ability to withstand desiccation. The motivation for these studies is due to the importance of EPN persistence in the environment during their use as biological control agents. EPNs can persist for 2–3 weeks in dry soil (Kaya, 1990; Kung and Gaugler, 1990). Most studies investigating the ability of steinernematid nematodes to survive desiccation have focused on *Steinernema carpocapsae* (e.g., Simons and Poinar, 1973; Ishibashi *et al.*, 1987; Womersley, 1990; Glazer, 1992). The general

finding has been that various strains of *S. carpocapsae* can survive for appreciable lengths of time under slow-drying conditions. However, differences in survival capabilities were observed between species of *Steinernema* (Kung *et al.*, 1990; Baur *et al.*, 1995; Solomon *et al.*, 2000).

As for heterorhabditids, Surrey and Wharton (1995) tested the desiccation survival of *Heterorhabditis zealandica*. Their experiments indicated that survival was poor once water had been lost from the substrate. Poor survival of *Heterorhabditis 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) showed substantial differences in survival ability 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.

In a broad comparison of EPN strains and species (10 species and 21 strains), *S. carpocapsae* exhibited the highest level of desiccation tolerance among species, followed by *Steinernema feltiae* and *Steinernema rarum* (Shapiro-Ilan *et al.*, 2014a). The heterorhabditid species exhibited the least desiccation tolerance, and *Steinernema riobrave* and *Steinernema glaseri* were intermediate. Intraspecific variation was not pronounced (unlike interspecific variation) (Shapiro-Ilan *et al.*, 2014a).

The rate of water loss from the body of the nematode is a key factor in its ability to survive extended periods without water in a state of anhydrobiosis (Crowe and Madin, 1975). Two broad categories of anhydrobiotic nematodes are recognized: fast- and slow-dehydration strategists (Womersley, 1987). Perry and Moens (2011) categorized these two groups as external dehydration strategists and innate dehydration strategists.

Fast-dehydration strategist nematodes inhabit environments exposed to frequent desiccation and they can survive immediate and prolonged exposure to rapid dehydration. These nematodes include *Panagrolaimus rigidus*, a nematode that inhabits extremely exposed environments on the surface of moss cushions (Ricci and Pagani, 1997), and the J_4 of *D. dipsaci*. Fast-dehydration strategists may be preadapted at a cellular level to survive desiccation, possibly

by constitutively expressing protective molecules required for anhydrobiosis. Alternatively, they may have behavioral or morphological adaptations which slow the rate of water loss (Perry, 1999). Womersley (1990) suggested that the sheath of infective juveniles of entomopathogenic nematodes might help to reduce the rate of water loss during desiccation. In species such as *Rotylenchulus reniformis* and *H. megidis*, the morphological adaptation of cuticle retention prolongs the time taken for the nematode's water content to reach levels that are lethal to these species (Perry and Moens, 2011).

Slow-dehydration strategist nematodes require preconditioning for a slow removal of water content from the organism prior to exposure to extreme desiccating conditions. This preconditioning period enables the nematodes to induce the necessary biochemical changes required to survive in an anhydrobiotic state. Such nematodes are found in soil habitats that experience slow rates of water loss. The majority of anhydrobiotic nematodes are believed to fall into this latter group (Womersley, 1987).

Coiling is a behavioral response to dehydration that reduces the surface area of the nematode exposed to drying conditions. When exposed to desiccation, the infective larvae of *Trichostrongylus colubriformis* form tight coils (Wharton, 1981). Coiling has been shown to reduce the rate of water loss of *Ditylenchus myceliophagus* (Womersley, 1987). Aggregation, or clumping, occurs in very few species. The L_4 of *D. dipsaci* can survive extreme desiccation for many years and thousands of L_4 can be found in aggregates, called eelworm wool, on the basal plate of infected narcissus bulbs or inside bean pods at the end of the growing season. The larvae on the periphery of the aggregations die and apparently provide a protective coat that enables survival of the L_4 in the center by slowing their rate of drying (Ellenby, 1968). As for EPNs, behavioral 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).

Successful entry into long-term anhydrobiosis depends on subsequent biochemical and molecular adaptations. The biochemical mechanisms involved in the induction of anhydrobiosis are not fully understood. Previous work on the biochemistry and molecular genetics of anhydrobiosis in nematodes has been focused primarily on the fungal-feeding soil nematode *A. avenae*, which is a slow-dehydration strategist (Crowe and Madin, 1975).

Tyson *et al.* (2012) analyzed the molecular mechanism governing desiccation tolerance in the anhydrobiotic nematode *Panagrolaimus superbus*. They suggested a gene-expression scheme that included kinases and phosphatases triggered by the detection and sensing of related genes. These enzymes activate transcription factors and microRNAs, which then induce the expression of an array of genes involved in cell protection under stress conditions.

Yaari *et al.* (2015) used 454 pyrosequencing to analyze transcriptome expression in *Steinernema* species that differ in their tolerance to desiccation and heat stress. Analyzing gene-expression patterns of the stress response showed a large fraction of downregulated genes in the desiccation-tolerant nematode *S. riobrave*, whereas a larger fraction of the genes in the susceptible *S. feltiae* strains were upregulated. This phenomenon was further observed in metabolic pathways and the expression of specific stress-related genes. Somvanshi *et al.* (2008) studied the regulation of four genes associated with stress tolerance in five EPNs with varying resistance to desiccation. Interestingly, all four genes showed the greatest degree of upregulation in the least desiccation-resistant nematode species; an inverse correlation between gene expression and the phenotype of desiccation tolerance in the examined EPNs was also observed.

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, *A. avenae*, lipid and glycogen levels declined while glycerol and trehalose accumulated during dehydration. Solomon *et al.* (1999) recorded increased trehalose levels of the EPN *S. feltiae*

as reported for other anhydrobiotic nematodes during the dehydration process (Womersley, 1987, 1990; Crowe and Crowe, 1992).

Trehalose accumulation has been associated with the onset of desiccation in several anhydrobiotic nematodes, including *A. avenae*, *Anguina tritici*, *D. dipsaci*, and several species of *Panagrolaimus* (Shannon *et al.*, 2005). Trehalose was found in high concentrations in a number of anhydrobiotic organisms (e.g., bacteria, yeast, nematodes, and brine shrimps) suggesting that the physical principles governing the stability of certain organisms under dry conditions may be universal (Crowe *et al.*, 1992). The mode of action of these non-reducing disaccharides—trehalose (in animals and fungi) and sucrose (in plants)—are thought to be involved as either water-replacement molecules or vitrification agents (Tunnacliffe and Lapinski, 2003). Other hypotheses, which are not incompatible with the above, have also been proposed (Bryant *et al.*, 2001; Teramoto *et al.*, 2008 for a review), and it seems likely that trehalose and similar molecules have multiple stabilizing functions within anhydrobiotes. The enzyme trehalose-6-phosphate synthase, which catalyzes the first step in the biosynthesis of trehalose, is encoded by two genes in *A. avenae*, and the expression of both genes is upregulated in response to desiccation stress (Goyal *et al.*, 2005b).

Late embryogenesis abundant (LEA) proteins have been associated with tolerance to desiccation in seeds, pollen, and desiccation-resistant plants. Solomon *et al.* (2000) was the first to identify the increase of this protein in the EPN *S. feltiae*. The genes encoding this desiccation-related protein were then reported in *A. avenae* as well as in *C. elegans* (Browne *et al.*, 2004; see also review by Gal *et al.*, 2004). Homologs of LEA genes were identified in the pinewood nematode *Bursaphelenchus xylophilus* (Kikuchi *et al.*, 2007). This finding led to further discovery of this group of proteins in other anhydrobiotic animals. In nematodes and other invertebrates, almost all LEA proteins characterized belong to group 3 (Pfam LEA_4) (Sharon *et al.*, 2009). Expression of LEA proteins has been associated with tolerance of water stress caused by desiccation, salt or low temperature in many studies (Burnell and Tunnacliffe, 2011). LEA proteins may protect cellular components against the effects of desiccation (Goyal *et al.*, 2005a).

Furthermore, reduction in expression of LEA protein genes by RNAi in *C. elegans* (Gal *et al.*, 2004), or by mutation in bacteria (Battista *et al.*, 2001), has been shown to decrease resistance to desiccation.

Membrane fluidity is affected by phospholipid composition and degree of fatty acid desaturation. The introduction of unsaturated bonds into the fatty acids of membrane phospholipids decreases the temperature for the transition from gel to liquid crystalline state (Los and Murata, 1998). Synthesis of transcripts encoding the fatty acid desaturase 2 gene (*fat-2*) was strongly upregulated in response to desiccation in *S. carpocapsae* (Tyson *et al.*, 2007) and *S. feltiae* (Yaari *et al.*, 2015).

Another group of molecules known to have a protective role under stress conditions is the heat shock proteins (HSPs), which is among the largest groups of molecular chaperones. Molecular chaperones and unfolded protein-response HSPs are essential for the correct folding and maturation of a great diversity of client proteins and for protection of proteins from stress-induced unfolding and aggregation (Mirimoto, 2008; Richter *et al.*, 2010). Eukaryotic HSP families contain multiple genes, which may be either constitutively expressed or induced by stress and targeted to specific cellular compartments (Kabani and Martineau, 2008; Vos *et al.*, 2008). The HSP-expression repertoire of an anhydrobiotic organism may thus be very important in maintaining the integrity of the proteome during the dehydration and recovery phases of anhydrobiosis (Sales *et al.*, 2000; Jonsson and Schill, 2007). The *Steinernema* dataset contains representatives of all HSP classes characteristic of nematodes, including four distinct small HSPs (sHSPs). These are the major “holding” chaperones, retaining unfolded proteins in a conformation suitable for subsequent refolding, thus preventing their irreversible aggregation (Eyles and Gierasch, 2010; Stengel *et al.*, 2010). In a proteomic analysis of desiccated *S. feltiae*, Chen *et al.* (2006) reported more than 400 protein spots identified by 2D-gel electrophoresis. Ten spots showed changes in abundance on desiccation, one of which was the chaperonin Hsp60 (Chen *et al.*, 2006). The evidence for involvement of classical protein homeostasis systems is also rather tentative in other anhydrobiotes. For example, tardigrades show upregulation of

Hsp70 during anhydrobiosis, although it seems to be more highly expressed during recovery from the dry state (Schill *et al.*, 2004).

Although *C. elegans* is sensitive to desiccation and osmotic stress, the molecular resources available for this model nematode have been utilized very skillfully to identify signaling pathways (Gal *et al.*, 2004; Solomon *et al.*, 2004; Lamitina *et al.*, 2006) and gene products required for survival during acute hypertonic stress (Choe and Strange, 2007, 2008). Similarly, RNAi has been used to demonstrate a role for *Ce-lea-1* (a gene encoding an LEA protein) in the ability of *C. elegans* dauer larvae to withstand desiccation, and osmotic and heat stress. *C. elegans* could also provide a suitable desiccation-sensitive reporter system to investigate the effects of transgenic expression of candidate anhydrobiotic genes from other nematodes.

Erkut *et al.* (2011) decided to use *C. elegans* for investigating molecular mechanisms of anhydrobiosis. The authors wrote that “this nematode species is a well-established model organism extensively used in genetics, biochemistry, and developmental biology”. They demonstrated that the *C. elegans* dauer larva is a true anhydrobiote and can survive even after losing 98% of its body water. This ability is correlated with a several-fold increase in the amount of trehalose. Mutants unable to synthesize trehalose cannot survive even mild dehydration. Light and electron microscopy indicate that one of the major functions of trehalose is the preservation of membrane organization. Fourier-transform infrared spectroscopy of whole worms suggests that this is achieved by preserving homogeneous and compact packing of lipid acyl chains. Erkut *et al.* (2013) used a systems approach to identify factors that are activated during this preconditioning of *C. elegans* dauer larvae. Using microarray analysis, proteomics, and bioinformatics, genes, proteins, and biochemical pathways that are upregulated during this process were identified. These pathways were validated via reverse genetics by testing the desiccation tolerances of mutants. Results indicated that the desiccation response is activated by hygro-sensation (sensing the desiccative environment) via head neurons. The desiccation response leads to elimination of reactive oxygen species and xenobiotics, expression of heat shock and intrinsically disordered proteins, polyamine utilization, and induction of

the fatty acid desaturation pathway. Remarkably, the response is specific and involves a small number of functional pathways, which represent the generic toolkit for anhydrobiosis in plants and animals.

Osmotic stress

Nematodes, as terrestrial organisms, may have to tolerate considerable variation in the concentrations of salts in the environment and thus are subject to osmotic stress (Evans and Perry, 1976; Wharton, 1986). Since water in nature has salts and other solutes dissolved in it, nematodes must regulate their internal ionic and osmotic composition to maintain their internal solutes at different concentrations to those in the surrounding medium. The degree to which this is necessary depends upon the habitat of the nematode. Marine nematodes are thought to be isosmotic to the surrounding seawater (Wright, 2004) and do not have to face much variation in the osmotic and ionic composition of their surroundings.

Nematodes rely on a high turgor pressure for locomotion (Harris and Crofton, 1957), and hyperosmotic regulation may be necessary to maintain a positive turgor pressure. If a nematode is hyposmotic to its surroundings, water would be lost, turgor pressure reduced and the nematode would become inactive. In *C. elegans*, body stiffness is affected more by the mechanical properties of the cuticle than by internal hydrostatic pressure (Park *et al.*, 2007). *P. davidi* maintains its internal osmotic concentration hyperosmotic to its surroundings across a range of external concentrations.

One way of dealing with osmotic stress is to avoid it by moving to a place where conditions are less stressful. This involves sensing and responding to changes in environmental conditions. *C. elegans* is attracted to low concentrations of salts, sugars, and other substances, but high concentrations produce an avoidance reaction (Choe and Strange, 2007). Infective larvae of the plant-parasitic nematode *Meloidogynе javanica* are repelled by high salinity (Prot, 1978), as are those of *M. incognita* (Le Saux and Quenec'hervé, 2002).

For nematodes living in soil, or in marine or freshwater sediments that are sometimes

exposed to the air, the osmotic and ionic composition of their medium will vary as the soil becomes saturated with water by the incoming tide, or rainfall. When the soil is drying out, the solutes become concentrated upon evaporation of the water. Animal- and plant-parasitic nematodes will face markedly different physiochemical conditions during their life cycle as they move from a parasitic to a free-living phase and back again. 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).

Some nematodes can tolerate very high osmotic stresses; nematodes have been found in Australian saline lakes at salinities up to the equivalent of 9.3% NaCl (Bayly and Williams, 1966; Deckker and Geddes, 1980; Hodda *et al.*, 2006). Some nematodes are found in terrestrial mineral springs. Antarctic saline lakes are of marine origin and contain nematodes at salinities up to 3.5% (Andrassy and Gibson, 2007).

There are three genera of nematodes on the mangrove plateau of the west coast of Zanzibar where hypersaline sediments reach over 10% salinity (Olafsson, 1995). Chinese salterns (where seawater is concentrated by evaporation to harvest salt) have been reported to contain the nematode *Prochromadora orleji* (Tarjan *et al.*, 1991).

D. dipsaci can survive exposure to 1.6 M NaCl for 24 h (Viglierchio *et al.*, 1969). This may be related to a low cuticular permeability that restricts water flux (Wharton *et al.*, 1988). *C. elegans* survives hyperosmotic stress and can retain activity in agar media containing up to 0.5 M NaCl. The ability to survive at higher osmotic concentrations (0.5 M NaCl) is enhanced by prior exposure to a lesser hyperosmotic stress (0.2 M NaCl) (Lamitina *et al.*, 2004). If first acclimated to 0.2–0.4 M NaCl, the nematodes swell and then shrink again upon exposure to hyposmotic stress (0.021 M NaCl).

The ability to enter an ametabolic state of osmobiosis due to osmotic pressure (Keilin, 1959) may be considered to be similar to anhydrobiosis

induced by water evaporation (Cooper and Van Gundy, 1971; see Perry and Moens, 2011). The mechanisms of osmoregulation among the EPN 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 IJs to a high temperature assay (45°C) resulted in a rapid reduction in viability, yet under the same assay viability of evaporatively and osmotically desiccated nematodes showed enhanced ability to withstand temperature stress. When IJs 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 such as glycerol at concentrations from 2.2 to 3.8 mol per liter, polyethylene glycol (PEG) 300 MW at concentrations of 1.2–1.6 mol per liter, and PEG 600 MW at 0.8 M did not reduce the viability of IJs, although all nematodes were found shrunk after 48 h. Furthermore, 72 h exposure to solutions of non-ionic solutes resulted in an increase in heat tolerance similar to that of evaporatively dehydrated nematodes. High viability was also recorded among nematodes that were stored for 72 days following a gradual increase in glycerol concentration.

Using PEG 600, Strauch *et al.* (2004) demonstrated induction of osmo/anhydrobiosis on IJs of *Heterorhabditis bacteriophora* following exposure to solutions that gave water activity (a_w) of 0.97 for 72 h. The treated IJs were then able to tolerate further exposure to a_w of 0.90. This led to further use of osmotic solution for genetic enhancement of desiccation tolerance of inbred lines of *H. bacteriophora* (Anbesse *et al.*, 2012, 2013) (see also Chapter 1, this volume).

Levy *et al.* (2020) utilized a similar approach of osmotic desiccation with PEG 600 to screen natural populations of *H. bacteriophora* for genetic diversity. Gene expression patterns and genomic variation were then studied following osmotic desiccation stress. Transcriptomic study enabled identification of genes and molecular markers as genetic selection tools for enhancement of entomopathogenic nematodes' ability to withstand environmental stress conditions.

When a nematode is immersed in distilled water, or other hyposmotic media, water will

enter and if not removed the nematode will swell and die. The behavior and structure of the excretory system in some nematodes suggests that it is involved in the removal of excess water. A variety of nematode structures have been suggested to have an excretory function (Wright and Newall, 1976; Wright, 2004) but water removal has been most closely associated with the tubular type of excretory system found in secernentean nematodes. This excretory system often has associated glands and is referred to as a secretory–excretory system (Wright, 2004).

As mentioned above, in some species it may be the intestine, rather than the excretory system, that is the site of water removal. Increased rates of filling and emptying of the intestine in distilled water have been observed in *Rhabditis terrestris* (Stephenson, 1942), and ligation of the anal region reduces the ability of *E. brevis* to regulate its volume in 50% seawater (Wright and Newall, 1976).

The cuticle is likely to be a major route of water flux under osmotic stress. Marine nematodes are usually isosmotic to seawater and have high cuticular water permeabilities. Soil nematodes, some plant-parasitic nematodes, and the free-living stages of animal-parasitic nematodes are more likely to be exposed to osmotic stress and have much lower cuticular permeability (Wright and Newall, 1976; Wharton *et al.*, 1988). Slowing down the rate of water flux allows time for osmoregulatory mechanisms to operate. Low cuticular permeability is associated with the ability to survive other environmental stresses, such as desiccation (Wharton *et al.*, 1988; see also Perry and Moens, 2011). The decreased ability of *Caenorhabditis briggsae* to withstand osmotic stress with age has been associated with changes in the epicuticle and an increase in permeability (Searcy *et al.*, 1976). Extracuticular lipid material covers the cuticle of some nematodes, forming an accessory layer that may be important in controlling water flux (Wharton *et al.*, 2008).

The decrease in osmotic tolerance due to aging is consistent with a general model of the response of animal cells to hyperosmotic stress (Strange, 2007; see also Barrett, 2011). Water is rapidly lost upon exposure to hyperosmotic stress and the cell shrinks. The cell responds by the activation of regulatory cell volume increase by salt uptake, involving $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporters or

Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchange mechanisms. Water follows the osmotic gradient thus established and cell volume returns to normal levels. The high ionic concentrations results in damaged cells, so the inorganic ions are replaced by compatible organic osmolytes, such as glycerol. The final process is the repair of molecular damage, including DNA breaks and protein denaturation (Strange, 2007).

Under hyperosmotic stress *C. elegans* shrinks, suggesting a loss of water from the body, but then returns to a more normal volume (Lamitina *et al.*, 2004; Strange, 2007). The nematodes accumulate glycerol in response to hyperosmotic stress (Lamitina *et al.*, 2004). The accumulation of glycerol in *C. elegans* is mediated by glycerol-3-phosphate dehydrogenase (GPD), which is involved in glycerol synthesis. This enzyme is encoded by two genes: *gpdh-1* and *gpdh-2*. Expression of *gpdh-1* is upregulated by hyperosmotic stress, whilst *gpdh-2* shows only a weak and transient increase in expression levels (Lamitina *et al.*, 2006).

Upon transfer from hyperosmotic to normal media, glycerol levels return to those of controls due to glycerol being excreted to the external medium, perhaps via the excretory system and/or intestine (Lamitina *et al.*, 2004). Double-knockout mutants (to *gpdh-1* and *gpdh-2*, in which the products of the genes are not produced) have greatly reduced glycerol levels when exposed to hyperosmotic stress (Lamitina *et al.*, 2006). Transgenic nematodes where *gpdh-1* and *gpdh-2* are tagged with a green fluorescent protein (GFP) reporter gene show that *gpdh-2* is constitutively (continually) expressed under normal conditions in the intestine, hypodermis, and excretory cell, whilst *gpdh-1* is expressed in the intestine and hypodermis only during hyperosmotic stress (Lamitina *et al.*, 2006).

Using the *gpdh-1*-GFP reporter system, Lamitina *et al.* (2006) performed a genome-wide RNAi feeding screen to look for genes that controlled *gpdh-1* expression. RNAi involves feeding an *Escherichia coli* clone expressing double-stranded RNA to the nematode, resulting in degradation of the corresponding mRNA and suppression of the gene in question (Ahringer, 2006). Increased levels of damaged or denatured proteins, as a result of the high ionic concentrations produced by hyperosmotic stress, may thus trigger

osmoprotective gene expression (*gpdh-1*) and the accumulation of glycerol.

Further evidence for this has come from a genome-wide RNAi screen of *C. elegans* that has identified 40 genes that are essential for hyperosmotic survival (Choe and Strange, 2008). Half of these genes encode proteins that are involved in the detection, transportation, and degradation of damaged proteins. Other genes that may be involved in, or regulate the response to, hyperosmotic stress in *C. elegans* include: collagen genes; genes encoding a germinal centre kinase-3 and a with-no-lysine (K) protein kinase (Choe and Strange, 2007); genes involved in insulin signaling pathways (Lamitina and Strange, 2005); *che-14* and other genes whose mutations produce phenotypes with defects in osmoregulation and sense organs (Liegeois *et al.*, 2007); *Ce-lea-1* encoding an LEA protein with roles in the response to water stress (Gal *et al.*, 2004); *cyk-3* encoding a ubiquitin C-terminal hydrolase in embryos (Kaitna *et al.*, 2002); and novel genes (*osm-7*, *osm-11*, *osr-1*) associated with hyperosmotic stress resistance, high glycerol levels, and long defecation cycles (Solomon *et al.*, 2004; Wheeler and Thomas, 2006).

The infective larvae of the EPN *S. carpocapsae* synthesize glycerol and trehalose during osmotic dehydration (Qiu and Bedding, 2002), whilst in several other nematode species glycerol synthesis is associated with anaerobic conditions (Barrett, 2011). The patterns of protein synthesis of osmotically dehydrated *S. feltiae* IJ (exposed to 24% glycerol) and unstressed controls have been compared using two-dimensional electrophoresis (Chen *et al.*, 2005). Ten novel proteins were produced and a further ten upregulated in response to osmotic dehydration. The proteins that could be identified by mass spectrometry included actin (filament formation and muscle fibers), proteasome regulatory particle (involved in protein degradation), and GroEL and GroES (members of the Hsp60 family of chaperones involved in protein folding). A further study compared the response to osmotic dehydration (24% glycerol) and evaporative dehydration (97% then 85% RH) in *S. feltiae* (Chen *et al.*, 2006). Many of the proteins identified were expressed in response to both osmotic and evaporative dehydration, including proteins known to be involved in stress responses, such as Hsp60, coenzyme Q (ubiquinone), biosynthesis

protein 4, inositol monophosphatase and fumarate lyase, and also proteins involved in the regulation of the cell cycle, gene transcription, and the organization of macromolecular structures.

Aquaporins (AQPs) are proteins that are embedded in cell membranes, forming channels that regulate water flow (Campbell *et al.*, 2008). Eight AQP genes have been identified in *C. elegans* (Kuwahara *et al.*, 2000), and a further three are present in the *C. elegans* genome (Mah *et al.*, 2007). The genes *aqp1*–*8* have been expressed in *Xenopus* oocytes to study the transport properties of the AQPs. Three of these AQPs transport water only, one AQP transports glycerol only, two AQPs transport both water and glycerol, and two AQPs did not transport any of the substances tested (Huang *et al.*, 2007). The only AQP that is exclusively expressed in the excretory system is *aqp-8*. The expression of *aqp-8* is regulated by CEH-6, which is a POU homeobox transcription factor (Mah *et al.*, 2007). Nematode aquaporins have also been reported from *Plectus murrayi* (Adhikari *et al.*, 2009).

The study of osmotic and ionic regulation in nematodes may seem to be of rather narrow academic interest. However, being relatively simple animals, nematodes can help us understand the processes that occur in more complex animals, including humans. Studies on the excretory system of *C. elegans* can be applied to the development of the human kidney and the study of renal diseases (Barr, 2005) and to the development of tubular systems in general (Buechner, 2002). Nematodes are the most numerous and diverse members of the meiofauna in marine, freshwater, and soil environments (Yeates *et al.*, 2009). Understanding their environmental physiology will thus contribute to our knowledge of how these ecosystems function.

Extreme temperatures

Cold tolerance

Nematodes inhabiting the soil are exposed to sub-zero temperatures in temperate, arctic and subarctic regions as well as at high altitudes. Nematodes are essentially aquatic organisms and, unless they are desiccated, face the risk of inoculative freezing by the ice in their surroundings, seeding (via body orifices) the freezing of

their body fluids. Wharton (2011) comprehensively reviewed the cold tolerance strategies of nematodes. There are a number of strategies by which nematodes may survive sub-zero temperatures. The two main cold tolerance strategies are freeze avoidance and freezing tolerance.

Freeze-avoiding animals reduce their risk of freezing at sub-zero temperatures, often by removing or masking ice nucleators. Ice nucleators are substances that cause ice nucleation, which is the initial process that results in the formation of an ice crystal. The nematodes survive in a supercooled state where their body fluids are still liquid at temperatures below their melting point, but they die if their body fluids freeze.

In some cases, a physical barrier, such as an eggshell or a sheath, protects the nematode against inoculative freezing and allows its body fluids to supercool to low temperatures, even though the animal is encased in ice. For example, infective larvae of the plant-parasitic nematodes *Globodera rostochiensis* and *Globodera pallida* are protected within an eggshell and a cyst wall (Wharton *et al.*, 1993; Wharton and Ramlöv, 1995; Devine, 2010). The sheath of *H. zealandica* prevents inoculative freezing, allowing extensive supercooling to -32°C , whereas exsheathed infective juveniles froze above -6°C and did not survive (Wharton and Allan, 1989; Wharton and Surrey, 1994). Additionally, in EPNs, the host cadaver was found to provide protection against freezing temperatures (Lewis and Shapiro-Ilan, 2002).

Where the nematode cannot prevent freezing and yet survives, it is considered to be freezing tolerant. Intracellular freezing is usually considered to be fatal to organisms (Mazur, 1984). However, *P. davidi* has been shown to survive extensive intracellular ice formation (Wharton and Ferns, 1995; Wharton *et al.*, 2005). This remains the only organism known to have this ability, although survival of intracellular freezing has been demonstrated in isolated tissues and cells of some other organisms (Lee *et al.*, 1993; Acker and McGann, 2002; Salinas-Flores *et al.*, 2008; Sinclair and Renault, 2010). *P. davidi*, which was isolated in east Antarctica, is the most-studied nematode from a cold tolerance perspective. This species is capable of using different strategies to survive sub-zero conditions (Wharton, 2011). The eggshell of *P. davidi* can resist inoculative freezing and allow

the enclosed embryo or first-stage larva to super-cool in the presence of ice (Wharton, 1994). If exposed to freezing in a situation where the propagation of ice in its surroundings is relatively rapid, inoculative freezing occurs and *P. davidi* is freezing tolerant (Wharton and Ferns, 1995; Wharton *et al.*, 2003). It survives both intracellular and extracellular ice formation (Wharton *et al.*, 2005). If freezing occurs at a high sub-zero temperature, ice formation is slow, there is no inoculative freezing, and it survives by cryoprotective dehydration (Wharton *et al.*, 2003, 2005). In an anhydrobiotic state it will survive exposure to -20°C or -80°C for at least 30 days (Wharton and Brown, 1991). These studies, as well as previous reports on desiccation and freezing tolerance of panagrolaimid species, particularly *P. davidi* (Wharton, 2011), made them great candidates to study survival mechanism.

Interest in cold tolerance of EPNs was related to their overwintering survival as well as efficacy at lower temperatures ($< 15^{\circ}\text{C}$). Steinernematids have been isolated in 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). Isolation of EPNs from such cold regions indicates that they are able to withstand sub-zero conditions. Brown and Gaugler (1997) demonstrated that *S. feltiae*, *Steinernema anomali* and *H. bacteriophora* were all freezing tolerant, with lower lethal temperatures of -22°C , -14°C , and -19°C , respectively. In a broad comparison of freezing tolerance among ten EPN species, poor freezing tolerance was observed in *Heterorhabditis indica*, and in *S. glaseri*, *S. rarum*, and *S. riobrave*, whereas *Heterorhabditis georgiana* and *S. feltiae* exhibited the highest freezing tolerance; significant intraspecies variation among strains was also observed (Shapiro-Ilan *et al.*, 2014a). Wharton and Surry (1994) showed that *H. zealandica* is freeze-avoiding. Cryopreservation studies (Curran *et al.*, 1992; Popiel and Vasquez, 1989) have demonstrated that EPNs can be stored indefinitely in liquid nitrogen.

Knowledge about the biochemical and molecular changes involved in cold tolerance has been accumulated in recent years. Trehalose appears to be the main cryoprotectant/antifreeze used by nematodes. An increase in trehalose

concentration during low-temperature acclimation has been shown in *P. davidi* (Wharton *et al.*, 2000), *Nematodirus battus* eggs (Ash and Atkinson, 1986), *Steinernema kushidai* (Ogura and Nakashima, 1997), *S. carpocapsae* (Qiu and Bedding, 1999), *S. feltiae*, *S. riobrave* (Grewal and Jagdale, 2002; Jagdale and Grewal, 2003) and *Heterodera glycines* (Yen *et al.*, 1996), and as a cold shock response in *Heterorhabditis bacteriophora* (Jagdale *et al.*, 2005). Increased trehalose concentration is associated with increased freezing survival or cold tolerance in *P. davidi*, *S. kushidai*, and *S. carpocapsae* but not in *S. feltiae* and *S. riobrave* (Ogura and Nakashima, 1997; Wharton *et al.*, 2000; Jagdale and Grewal, 2003).

The concentration of trehalose is mediated by trehalose-6-phosphate synthase (gene: *tps*). The presence of these enzymes has been demonstrated in a variety of nematodes (Behm, 1997). Cold shock increases the activity of trehalose-6-phosphate synthase and decreases the activity of trehalase in *H. bacteriophora*, resulting in an increase in trehalose concentration (Jagdale *et al.*, 2005). There is less evidence for the role of other organic solutes in nematode cold tolerance. Nematodes synthesize a variety of polyols, including glycerol, inositol, ribitol, and sorbitol (Barrett, 1991; Womersley, 1981).

Unsaturated fatty acids (UFAs) are more fluid than are saturated fatty acids, and hence an increase in the proportion of UFAs enables the organism's membranes to resist solidification at lower temperatures (Margesin *et al.*, 2007). The proportion of UFAs is increased at lower temperatures in *S. feltiae*, *S. carpocapsae*, and *S. riobrave* (Jagdale and Gordon, 1997), and in *C. elegans* (Tanaka *et al.*, 1996). Chilling-resistant *C. elegans* worms can be produced by culturing at 10°C . These will survive exposure to 0°C , whilst those cultured at 25°C do not survive (Murray *et al.*, 2007). Chilling-resistant worms have an increased proportion of UFAs compared with chilling-sensitive worms.

The HSPs are induced by cold exposure in some organisms (Gross, 2004; Zhang and Guy, 2006; Devaney, 2011). A temperature shift from 37°C to 4°C increases the production of Hsp70 in the parasitic first-stage larvae of *Trichinella spiralis*, *Trichinella nativa*, and *Trichinella nelsoni*, but levels of Hsp60 and Hsp90 decline or do not change (Martinez *et al.*, 2002). A 50 kDa protein that cross-reacts with Hsp90 antibody is

induced by cold and osmotic stress in *T. spiralis* (Martinez *et al.*, 2002).

LEA proteins are implicated in anhydrobiotic survival in nematodes and other organisms (Browne *et al.*, 2004; Burnell and Tunnacliffe, 2011) and may have cryoprotective functions (Honjoh *et al.*, 2000; Goyal *et al.*, 2005a).

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). Understanding of the nematodes response to heat comes largely from genetic and molecular studies using the free-living model nematode *C. elegans* (see review by Devaney, 2011).

“Temperature niche breadth” (Anderson and Coleman, 1982) is the term in which organisms develop under ranges of temperature. For example, the speed of development of *C. elegans* increases with increasing temperature over a particular range, i.e., development at 25°C is 2.1 times faster than at 16°C and 1.3 times faster at 20°C than at 16°C (Stiernagle, 2006). However, deleterious effects are observed at temperature extremes; for example, both the lifespan and fecundity of *C. elegans* N2 are significantly decreased at very high temperatures (> 35°C) (Klass, 1977). This was suggested to be related to the production of decreased numbers of viable sperm (Harvey and Viney, 2007). The optimal temperature for development varies from species to species within a genus and depends upon the normal growth habitat of the organism. Some species of *Caenorhabditis*, such as those found in close association with birds or mammals, thrive at much higher temperatures than does *C. elegans* (Kiontke and Sudhaus, 2006).

In *C. elegans*, temperature modulates the neuroendocrine signaling pathway that controls the decision between reproductive development and dauer formation (Ailion and Thomas, 2000; Edison, 2009). Temperatures > 20°C promote entry of wild-type strain (N2) of *C. elegans* into the dauer pathway as long as worms are exposed to elevated temperatures prior to the first larval molt (Golden and Riddle, 1984).

Laser ablation of specific neurons together with the availability of genetic mutants has allowed the neural circuits and downstream

signaling pathways involved in thermal sensing to be defined (reviewed in Mori *et al.*, 2007). *C. elegans* has the ability to “remember” the temperature at which it was cultivated, so that when worms cultured at a particular temperature are placed on a temperature gradient, they migrate back to their original growth temperature. This migratory behavior is known as thermotaxis (Hedgecock and Russell, 1975; Mori and Ohshima, 1995), and demonstrates that the worm has a mechanism for detecting and responding to changes in ambient temperature.

For parasitic nematodes the ability to move along a thermal gradient is likely to be an important aspect of host finding. For example, the behavior of the dog hookworm, *Ancylostoma caninum*, changes in response to warming and other stimuli associated with the presence of a mammalian host (Granzer and Haas, 1991). A laser ablation approach has been used with *A. caninum* (Ashton *et al.*, 1999). Ablation of the presumed AFD analogous neuron of the first-stage larva (L₁) of this parasitic nematode resulted in a significantly reduced thermotactic response (Bhopale *et al.*, 2001).

When exposed to elevated temperatures, most organisms respond with a heat shock response and nematodes are no exception. The heat shock response permits the maintenance of homeostasis in cells when exposed to conditions that compromise protein integrity and it involves the accumulation of misfolded or unfolded proteins, elevated levels of which are responsible for the induction of the heat shock response. This results in an expression of HSPs. These protect the cell by chaperoning misfolded or denatured proteins and targeting them for refolding or degradation. This is accomplished through the activity of the heat shock transcription factor, HSF-1. *C. elegans* expresses only one HSF, HSF-1 (Walker and Lithgow, 2003). Heat shock of *C. elegans* N2 worms results in the transcription of a full range of HSPs (Snutch and Baillie, 1983). Comparison of the sequence of *C. elegans* HSF-1 with other eukaryotic heat shock factors reveals a high degree of conservation over the DNA-binding domain.

It has long been known that exposure to a short burst of elevated temperature protects the cell from more extreme temperatures. This so-called “induced thermos tolerance” results in the synthesis of HSPs, and studies in *C. elegans*

demonstrated the role of ILS in this process (Lithgow *et al.*, 1995). DAF-16, the downstream target of the ILS pathway, is a forkhead transcription factor that functions in dauer formation and affects lifespan and reproduction. Ligation of DAF-2, the insulin-like receptor, initiates a signaling cascade resulting in the phosphorylation of DAF-16 and its sequestration in the cytoplasm, where it is inactive. Overexpression of *daf-16* results in worms that are long-lived and stress-resistant (Henderson and Johnson, 2001), as does overexpression of the small HSP, *Hsp16.2* (Walker and Lithgow, 2003).

Heat-shocked *C. elegans* display higher levels of resistance to infection with *Pseudomonas aeruginosa* (Singh and Aballay, 2006). Further, the role for Hsp90 and Hsp16 in immunity to infection was demonstrated. Long-lived ILS mutants showed an increased resistance to infection that was dependent on *hsf-1*, and animals overexpressing *hsf-1* were more resistant to infection than wild-type worms (Singh and Aballay, 2006). In a different pathogen system, infection with *Enterococcus faecalis* was shown to cause protein aggregation (a sign of damage) in the intestine, an effect that could be overcome by the expression of *hsf-1* in the cells of the intestine (Mohri-Shiomi and Garsin, 2008).

More than 40 single-gene mutants in *C. elegans* have been demonstrated to lead to increased lifespan of 20% or more; these are referred to collectively as "Age" mutants.

The first studies identifying long-lived genetic variants of the nematode *C. elegans* (Johnson and Wood, 1982) attracted extreme curiosity and interest of scientists and the general public, due to the expectation of prolonged life expectancy.

All age mutants, like *daf-16* and *old-1*, increase the ability of the worm to respond to several stresses, including heat, ultraviolet, and reactive oxidants (Johnson *et al.*, 2002). Johnson *et al.* (2002) showed that these genes were found to be epistatic to the long-life phenotype of most mutants and also yield overexpression strains that are stress-resistant and long-lived. They suggested that the role of the age genes in both longevity and stress resistance indicates that a major evolutionary determinant of longevity is the ability to respond to stress.

Since EPNs are used commercially in agriculture, high temperature can adversely affect their efficacy (Hiltpold, 2015). Intensive studies

had been devoted to understanding the EPN activity and persistence under high temperatures as well as their survival in storage and shipment in elevated temperatures. 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.* (1996) 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.

Grewal *et al.* (2004, 2006) reported that temperature adaptation of EPN species appears to be a continuum, with species representing both the cold and warm extremes. Measuring the thermal infection (host penetration and host death), establishment (development of the IJs to adults in the host following infection), and reproduction niche breadths of eight species, they concluded that *S. feltiae* represented the coldest extreme and *S. riobrave* represented the warmest extreme. EPN thermal niche breadths were progressively narrower for movement (4–40°C), host penetration (5–39°C), host death (8–39°C), and reproduction (10–35°C) (Grewal *et al.*, 1994, 2004, 2006). *Heterorhabditis floridensis* was found to have an extremely broad temperature niche breadth for infectivity with activity from 17°C to 39°C (Shapiro-Ilan *et al.*, 2014b).

Grewal (2000) demonstrated that temperature has a strong influence on IJ survival in storage and this influence varies with EPN species. For example, 50% of *S. feltiae* IJs survived in tap water for 42–44 and 27–30 weeks at 5°C and 25°C, respectively. The survival of *S. riobrave* IJs was not much different between 25°C and 5°C, perhaps due to its lack of cold adaptation. Heat tolerance also varies substantially among species and strains of EPNs. The survival of IJs exposed to 40°C for 2 h varied between 13% and 90% among 15 *H. bacteriophora* strains (Grewal *et al.*, 2002) and between 37% and 82% in 15 *S. carpocapsae* strains (Somasekhar *et al.*, 2002).

Mukuka *et al.* (2010) screened 36 natural populations and 18 hybrid or inbred strains of heterorhabditids for their response to high temperature. Nematodes were tested with or without prior adaptation to heat at 35°C for 3 h. The mean tolerated temperature ranged from 33.3°C to 40.1°C for non-adapted and from 34.8°C to 39.2°C for adapted strain populations. *H. indica*

was the most tolerant, followed by *H. bacteriophora* and *H. megidis*. Correlation between heat tolerance and mean annual temperature at place of origin of the strains was weak.

Temperature tolerance in EPN IJs also appears to be facilitated by trehalose accumulation (see Grewal *et al.*, 2006). Jagdale and Grewal (2003) showed that *S. feltiae*, *S. carpocapsae*, and *S. riobrave* accumulated trehalose when acclimated at either 5°C or 35°C, but the amount of trehalose accumulation differed by species and temperature. Enhanced heat tolerance (at 40°C for 8 h) was positively correlated with the increased trehalose levels in warm- and cold-acclimated *S. carpocapsae* and *S. feltiae* but not in *S. riobrave*.

Production of *hsp70* 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) analyzed the molecular organization of *hsp70* genes in five species and isolates of heterorhabditids and one steinernematid species using PCR and restriction fragment length polymorphism (RFLP). They found homology of *hsp70* genes of *C. elegans*. In addition, RFLPs with *hsp70* probes revealed different banding patterns for *Heterorhabditis* species and isolates. The analysis of the *H. bacteriophora* genome revealed a large number of HSPs, including Hsp1, Hsp3, Hsp6, Hsp12.2, Hsp16.2, Hsp17, Hsp25, Hsp43, Hsp60, Hsp70, and other HSPs such as STI-1, STC-1, DAF-21, DNJ-12 and DNJ-13 (Grewal *et al.*, 2011).

In yeasts, deletion of either *tps1* or *tps2* genes, which encode the T6PS complex, causes an inability to accumulate trehalose during heat shock, which, in turn, significantly reduces thermo-tolerance in yeast (Bell *et al.*, 1992; De Virgilio *et al.*, 1993, 1994; Hottiger *et al.*, 1994). Since *H. bacteriophora* accumulates trehalose and increases T6PS activity after heat shock (Jagdale *et al.*, 2005), it is possible that this enzyme in nematodes, as in yeast, may also be acting as an HSP and performing a role in thermo-tolerance.

HSF1 is a heat shock transcription factor functioning as a transcriptional regulator of stress-induced gene expression and is involved in nematode larval development, innate immunity, and regulation of adult lifespan (Baugh and Sternberg, 2006; Singh and Aballay, 2006). The

skinhead transcription factor (SKN-1) functions in the p38 mitogen-activated protein kinase pathway to regulate the oxidative stress response and in parallel to DAF-16 in the DAF-2-mediated insulin/IGF-1-like signaling pathway to regulate adult lifespan (Tullet *et al.*, 2008). Other transcriptional factors/regulators identified in *H. bacteriophora* include GFL-1, CUL-4, LAG-1, DIN-1, and DJR-1.1.

Oxidative stress

Oxygen is an essential component for maintaining life of most organisms, including nematodes. There is a wide range of tolerance to oxygen concentrations among different species of Nematoda. Kitazume *et al.* (2018) examined the effects of low oxygen concentrations on four distinct species, including the model nematode *C. elegans*, as well as *Pristionchus pacificus*, *Panagrolaimus superbus* and *Bursaphelenchus xylophilus* (a plant parasite that can be maintained using fungi in the laboratory). They showed behaviour and survival of nematodes under anoxic conditions and found that *B. xylophilus* was more tolerant of anoxia than the other species.

Most intensive studies on nematode responses to hypoxia have been conducted with *C. elegans* (e.g., Van Voorhies and Ward, 2000; Padilla *et al.*, 2002; Gray *et al.*, 2004; Leiser *et al.*, 2013; LaMacchia *et al.*, 2015; Padmanabha *et al.*, 2015; Miller and Roth, 2009). The nematode *C. elegans*, like other animals, requires oxygen to develop and survive, but can maintain a normal metabolic rate at low oxygen concentrations of 3.6 kPa and has a near-normal metabolic rate at oxygen concentrations as low as 2 kPa (Van Voorhies and Ward, 2000; Padilla *et al.*, 2002). Interestingly, a correlation between oxidative stress tolerance and lifespan has been reported. Certain degrees of hypoxia (0.5% or 0.51 kPa oxygen) were reported to extend the lifespan of *C. elegans* (Leiser *et al.*, 2013). Johnson *et al.* (2001) found that the so-called *C. elegans* "Age" mutants were associated with oxidative stress along with heat- and UV-stress resistance. Other studies reported that long-lived dauer mutants had increased resistance to oxidative stress as well as upregulated antioxidant genes (Honda and Honda, 1999; Johnson *et al.*, 2002).

Under lower oxygen concentrations (anoxia), however, the majority of nematodes died within 96–144 h (Van Voorhies and Ward, 2000). Hypoxia-inducible factor 1 (HIF-1) is a key transcription factor in the detection of oxygen depletion and in mediating the response to hypoxia to maintain cellular oxygen homeostasis. Macnee and Hejmadi (2015) investigated hypoxia signaling *in vivo* using a *C. elegans* HIF-1 mutant model. This mutant *C. elegans* showed an impaired chemosensory response following a 48 h hypoxia exposure. One of the most deleterious effects of dehydration in the cell is oxidative damage (Hermes-Lima and Zenteno-Savin, 2002; Franca *et al.*, 2007). Besides protecting the cell structure from collapse due to gradual removal of water molecules, the desiccation process has been shown to make nematodes and other anhydrobiotes more sensitive to reactive oxygen species (ROS) (Burnell and Tunnacliffe, 2011). ROS cause substantial damage to membrane lipids because lipid peroxidation proceeds by a chain-reaction mechanism in which the lipid radicals interact with oxygen, leading to the formation of other radical species that further propagate the peroxidation process (Bokoch and Diebold, 2002; reviewed by Niki, 2009). A ten-fold increase in oxidation as a result of dehydration has been recorded in yeast cells (Pereira *et al.*, 2001). It is suspected that greater oxidation also plays a major role in death caused by dehydration in nematodes. Thus, protection against oxidation by enzymatic breakdown of oxygen radicals is crucial during water loss. A universal primary defense against oxygen toxicity is superoxide dismutase (SOD), which catalyzes the breakdown of the superoxide anion into hydrogen peroxide and oxygen. Hydrogen peroxide is then broken down to water and oxygen by several enzymes, the main one being catalase. Catalase is involved in the dehydration response of *Plectus murrayi* (Adhikari *et al.*, 2009) as well as in the Antarctic midge *Belgica antarctica* (Lopez-Martinez *et al.*, 2008). Several broad-spectrum detoxification gene families are also present in *P. murrayi*. These include UDP-glucuronosyltransferases (UGTs) and glutathione *S*-transferases (GSTs) (Adhikari *et al.*, 2009).

Many enzymatic antioxidant defense systems have been reported to be upregulated in response to desiccation or osmotic stress in microorganisms, plants, and animals, and in both

desiccation-sensitive and anhydrobiotic nematodes. Some examples in nematodes include: ALDH in *S. feltiae* (Gal *et al.*, 2003; Yaari *et al.*, 2015); glutaredoxin (GRx) and mitochondrial manganese superoxide dismutase (MnSOD) in *S. carpocapsae* (Tyson *et al.*, 2007); GRx (Browne *et al.*, 2004) and glutathione peroxidase (GPx) (Reardon *et al.*, 2010; Yaari *et al.*, 2015) in *A. avenae*; and ALDH, GST, and GPx in *P. murrayi* (Adhikari *et al.*, 2009).

Erkut *et al.* (2013) indicated that *C. elegans* uses cytoplasmic superoxide dismutases to eliminate superoxide, and glutathione peroxidases as well as catalases to convert peroxide into water. However, the molecular basis for oxidative stress is poorly understood in nematodes. A number of ESTs encoding proteins that detoxify ROS, like SOD, Ras-related protein, and GST, are expressed in *P. murrayi* in response to desiccation. GSTs are a diverse superfamily of multifunctional proteins that play prominent roles in detoxification metabolism in nematodes. Expression of ESTs similar to *C. elegans* *sod-1*, which encodes a copper/zinc superoxide dismutase, is also abundant (Lindblom and Dodd, 2006). More than a dozen different GSTs have been isolated from *C. elegans* (Van Rossum *et al.*, 2001) and these detoxifying enzymes are reported to be involved in several functions, including xenobiotic detoxification and oxidative stress tolerance (Leiers *et al.*, 2003; Lindblom and Dodd, 2006).

Oxygen stress and oxidative effect have been intensely studied with EPNs. This is due to the deleterious effect of lack of oxygen at fermentation, storage, and even under certain field conditions. There are large differences in the ability of EPN species and strains to withstand hypoxic conditions (summarized by Grewal *et al.*, 2011). Burman and Pye (1980) reported that *S. carpocapsae* can withstand oxygen tensions of as low as 0.5% saturation at 20°C. In sandy soil, survival of *S. carpocapsae* and *S. glaseri* decreased as oxygen concentration decreased from 20% to 1% (Kung *et al.*, 1990). In *H. bacteriophora*, Grewal *et al.* (2002) found a strong correlation of DJ longevity with heat, UV radiation, and hypoxia tolerance. While *H. bacteriophora* can withstand anoxic conditions in water at 25°C for only 4 days (Grewal *et al.*, 2002), *S. carpocapsae* can tolerate such conditions for up to 10 days (Somasekhar *et al.*, 2002). Also there appears to be more variation in

hypoxia tolerance among strains of *H. bacteriophora* (Grewal *et al.*, 2002) than those of *S. carpocapsae* (Somasekhar *et al.*, 2002). Qiu and Bedding (1999) reported that *S. carpocapsae* IJs incubated in M9 buffer at 23°C under absolute anaerobic conditions were fully inactivated in 16 h but could be revived when returned to aerobic conditions if exposure to anaerobic conditions was not more than 7 days. The survival time under anaerobic conditions was significantly affected by temperature, with 90% survival times being 20, 7, and 5 days at 5°C, 23°C, and 28°C, respectively.

In addition to this finding, we recently found a strong correlation between oxidative stress tolerance and DJ longevity in *H. bacteriophora* (Sumaya *et al.*, 2017). The same strains that expressed increased oxidative stress resistance also elicited a high longevity and soil persistence. Sumaya *et al.* (2018) used oxidative stress tolerance assays as a selection parameter to improve *H. bacteriophora* IJ longevity following hybridization and mutagenesis.

Conclusions and Future Directions

In this chapter the amazing capabilities of nematodes to respond to fluctuating environmental conditions have been summarized. Naturally, many studies concentrate on single stress factors. However, under natural conditions the animals may encounter a combination of threats. For example, soil temperature is determined by factors that control heat transfer. Moist soil has greater conductance and lower rise in temperature than dry soil when exposed to the same heat source. In another example, 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 (Glazer, 2002).

Since nematodes are the most numerous and diverse members of the meiofauna in marine, freshwater, and soil environments (Yeates *et al.*, 2009), understanding their environmental physiology will contribute to our knowledge of how these ecosystems function (see also Chapter 7).

Despite the fact that substantial progress had been made in exploring the behavioral, physiological, genetic, and molecular mechanisms of nematode response to stresses, a full understanding of these mechanisms requires further research. Advances in molecular research technique and approaches such as the use of CRISPR-Cas9, RNAi, deep sequencing, and bioinformatics analytical tool for different “omic” data (genomics, proteomics, lipidomics, etc.) will enable us to deepen and expand the understanding of the survival mechanisms.

Storey (2015) suggested that mechanisms previously associated mainly with epigenetic regulation can also contribute to reversible suppression of gene expression in hypometabolic state-associated animals’ response to environmental stresses such as extremes of heat or cold, drought, and oxygen limitation. Indeed, situations such as anoxia tolerance in turtles show coordinated changes in histone post-translational modifications (acetylation, phosphorylation), and activities of histone deacetylases, consistent with their use as mechanisms for suppressing gene expression during hypometabolism. Other potential mechanisms of gene silencing in hypometabolic states include altered expression of miRNAs that can provide post-transcriptional suppression of mRNA translation. Furthermore, mechanisms first identified in epigenetic regulation (e.g., protein acetylation) are now proving to apply to many central metabolic enzymes (e.g., lactate dehydrogenase), suggesting a new layer of regulatory control that can contribute to coordinating the depression of metabolic rate. These provide new dimensions for exploration in survival mechanisms of nematodes and other animals.

Notably, there are common physiological mechanisms and molecules involved in the different survival mechanisms. These include detection of and sensing changes in the environment, signal transduction pathways, membrane stabilizers, and oxidation protection mechanisms. Trehalose is a particular example of a compatible solute which is used by many organisms to counteract osmotic stress (Grewal *et al.*, 2006). The common features and molecules involved in the various survival mechanisms are summarized in Table 9.1.

In the light of reduction in use of chemical pesticides against animal- and plant-parasitic

Table 9.1. Summary of the various mechanisms involved in the response of nematodes to different environmental stresses.

Physiological and molecular mechanisms		Environmental stress							
		Desiccation		Osmotic pressure		Extreme temp.		Oxygen	
		Slow	Fast	Hypotonic	Hypertonic	High	Low	Low O ₂	Oxidation
Detection of conditions	Kinases	+	ND	?	?	+	+	+	-
	Phosphatases	+	ND	?	?	+	+	+	-
	Transcription factors	+	ND	?	?	+	+	+	-
Behavior response	Slowing or ceasing movement	+	+	+	+	+	+	+	+
	Coiling	+	+	-	-	-	-	-	-
	Aggregation	+	-	-	-	-	-	-	-
	Shrinking	+	+	+	+	-	-	-	-
Gene expression									
Effector proteins	LEA (late embryogenesis abundant) proteins	+	+	+	+	+	+	+	+
	HSPs (heat shock proteins)	+	+	+	+	+	+	+	+
	Other chaperones	+	?	?	+	+	+	?	+
	Antioxidants	+	?	?	?	+	+	+	+
	Aquaporins	+		+	+	-	-	ND	ND
	Proteosomal proteins								
Enzymes	Novel stress proteins								
	TPS (Trehalose phosphate synthase)	+	+	+	+	+	+	+	-
	Other compatible solutes	+	+	+	+	+	+	+	+
	Detoxification enzymes	+	ND	+	+	ND	ND	+	+
	Nonenzymatic antioxidative systems	+	+	+	+	+	+	+	+

ND, not done

nematodes, understanding the survival mechanisms of these pests may provide new environmentally acceptable approaches to combat these pests in agriculture and other settings. As indicated here, the developmental stages that are most tolerant to environmental stresses are those that are outside the host (IJs, cysts, eggs). Studying the behavioral, biochemical, and genetic aspects of survival under stress conditions will lead to development of control methods such as modification of the environment (i.e., increased humidity and moisture when hosts are not available).

Survival of EPNs is a key factor in the development and expansion of their use as biological control agents. In the past three decades much research has focused on physiological mechanisms of stress tolerance in the IJs. Studies on molecular mechanisms controlling IJ longevity and stress tolerance have only accelerated in the past decade. The availability of genomic sequence data and putative proteomes provides a large number of genes that could be useful to increase persistence of IJs and their survival in the soil. Several known stress-tolerance genes, such as heat shock proteins, trehalose-related molecules and pathways, as well as their orthologs and paralogs that have been expanded in EPN genomes, remain to be functionally tested. EPN research is burgeoning with possibility, but much remains intractable without the application of more molecular tools (Lu *et al.*, 2016). The field advanced significantly with the sequencing of these genomes, but whether this will lead to actual improvements in the field efficacy of EPN biocontrol remains to be seen. The survival of EPNs and their activity under wider ranges of ambient temperatures as well as relative humidities will extend the range of pests and agricultural crops where they can be used—in particular, their use on exposed plant surfaces against foliage and fruit pests.

The link between survival mechanisms, particularly the response to heat stress, and nematode longevity, as well as the fascinating ability to

survive long periods under extreme conditions (low relative humidity or sub-zero temperatures), provide the foundation to explore these mechanisms in other organisms. Kaczmarek *et al.* (2019) recently reviewed knowledge about the impact of anhydrobiosis on longevity and fitness of nematodes, rotifers, and tardigrades, indicating that group anhydrobiosis has been proposed to eliminate or slow down aging symptoms.

According to Kaczmarek *et al.* (2019), the impact of anhydrobiosis on animal aging is illustrated by two models based on experimental data, namely the “Sleeping Beauty” and “Picture of Dorian Grey” models. According to the “Sleeping Beauty” model, anhydrobiotic organisms do not age during anhydrobiosis, whereas the “Picture of Dorian Grey” model predicts that the anhydrobiotic organism ages, at least during the initial stage of anhydrobiosis. This notion has been characterized among rotifers and tardigrades but has yet to be determined among nematodes. Thus, further research should focus on molecular adaptations underlying cytoprotective mechanisms responsible for the lack of aging symptoms. Moreover, a better understanding of anhydrobiotic animals and their life strategies can provide fundamental answers in many branches of applied sciences, such as medicine, biotechnology, and astrobiology. This applies, for example, to dry vaccines, preservation of biological materials for transplantation or food production, enzymes working in a small amount of water, and mechanisms of DNA protection and repair.

This chapter concludes with a recent statement of John Crowe, one of the pioneers and a leading scientist in anhydrobiosis research. In reference to an earlier statement (Crowe, 1971) in which he stated that “desiccation tolerance has essentially been explained”, he later commented: “Now it is clear that we were optimistic in this conclusion, and that what we had thought to be a simple solution is not so simple at all” (Crowe, 2014). This can be applied to all other survival mechanisms described here.

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10 Molecular Toxicology in *Caenorhabditis elegans*

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Introduction

So far, a large amount of evidence has been raised that the nematode *Caenorhabditis elegans*, a classic model animal, has the potential to be used for toxicological study of various environmental toxicants or stresses (Leung *et al.*, 2008; Wang, 2018, 2019). Due to the short life cycle, small size, ease in cultivation, and especially the sensitivity to environmental exposures, *C. elegans* can be employed to detect the potential toxicity of environmental toxicants even at environmentally relevant concentrations (Wang, 2018, 2019). Additionally, *C. elegans* has been systematically used for the study of target organ toxicology and response to environmental toxicants or stresses (Wang, 2019). With the aid of a series of sensitive sublethal endpoints, the animal model of *C. elegans* is helpful for understanding the response to environmental toxicants or stresses, or toxicity induction by environmental toxicants, or stresses at a whole-animal level.

More importantly, during the past almost 20 years, a large amount of data on molecular toxicology has been obtained in *C. elegans*. In *C. elegans*, the molecular signaling pathways and related molecular events are conserved to a great degree with those in mammals and in human beings. Meanwhile, rich genetic resources and well-described genetic backgrounds are available

for *C. elegans*. These backgrounds contribute greatly to the establishment of knowledge systems on molecular toxicology in nematodes. This chapter focuses on the introduction of molecular signaling pathways involved in regulating the toxicity of environmental toxicants or stresses in nematodes. The introduced molecular signaling pathways are: (i) oxidative stress-related molecular signals; (ii) MAPK signaling pathways; (iii) insulin and the related signaling pathways; (iv) development-related signaling pathways; (v) cell death and DNA damage-related signaling pathways; (vi) metabolism-related signaling pathways; (vii) protective response-related signaling pathways; (viii) G-protein coupled receptors and ion channels and the downstream cytoplasmic signals; and (ix) epigenetic regulation signals. These molecular signaling pathways provide the basis for understanding the molecular toxicology at the whole-animal level in nematodes.

Oxidative Stress-related Molecular Signals

Activation of oxidative stress is normally considered as a crucial cellular contributor to toxicity induction of various environmental toxicants or stress (Leung *et al.*, 2008; Wang, 2018, 2019). Besides this, oxidative stress-related molecular

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signals can further act as important molecular regulators of toxicity induced by different environmental toxicants or stresses in nematodes.

Roles of mitochondrial complex signals

Complex I (NADH ubiquinone oxidoreductase)

GAS-1 *gas-1* encodes a 49 kDa subunit of complex I. With thiolated graphene oxide (GO-SH) as an example of environmental toxicants, it was found that prolonged exposure to GO-SH ($\geq 100 \mu\text{g/l}$) caused significant decrease in locomotion behavior, reduction in reproductive capacity, and induction of intestinal reactive oxygen species (ROS) production (Ding *et al.*, 2018). Meanwhile, among the examined genes required for the control of oxidative stress, exposure to GO-SH ($100 \mu\text{g/l}$) decreased the expression of *gas-1* (Ding *et al.*, 2018). In nematodes, a more severe decrease in locomotion behavior, reduction in brood size, and induction of intestinal ROS production could be detected in GO-SH ($100 \mu\text{g/l}$) exposed *gas-1* mutant nematodes than those in GO-SH ($100 \mu\text{g/l}$) exposed wild-type nematodes (Ding *et al.*, 2018). Therefore, mutation of *gas-1* may induce a susceptibility to the toxicity of environmental toxicants or stresses, such as GO-SH. For the cellular basis for the formation of susceptibility to toxicity of environmental toxicants or stresses in *gas-1* mutant nematodes, activation of oxidative damage, formation of more severe accumulation of environmental toxicants in the body or the targeted organs, and enhancement in the intestinal permeability acted as the important cellular contributors (Ding *et al.*, 2018). *daf-16* encodes a fork-head transcription factor in the insulin signaling pathway. DAF-16 normally resides in the cytoplasm in wild-type nematodes, and it resides constitutively in the nuclei of *gas-1* mutant nematodes under normal conditions (Kondo *et al.*, 2005). It was observed that supplementation with antioxidant (coenzyme Q10) could reverse this nuclear translocation of DAF-16 in *gas-1* mutant nematodes (Kondo *et al.*, 2005). Thus, for the molecular basis for the formation of susceptibility to toxicity of environmental toxicants or stresses in *gas-1* mutant nematodes, *gas-1* mutation-mediated mitochondrial perturbations may regulate

toxicity induction by at least affecting the insulin signaling pathways (Kondo *et al.*, 2005).

NUO-1 *nuo-1* encodes an NDUFV1 subunit of complex I. Transgenic strains carrying mutated *nuo-1* showed susceptibility to hyperoxia or paraquat, a generator of superoxide (Grad *et al.*, 2004), which implied that *nuo-1* mutants are hypersensitive to environmental toxicants or stresses.

Complex II (succinate ubiquinone oxidoreductase)

mev-1 encodes a cytochrome-b large subunit of complex II. In nematodes, simulated microgravity stress significantly decreased transcriptional expressions of *mev-1* and *gas-1*, and increased transcriptional expression of *isp-1* (Zhao *et al.*, 2017a). Meanwhile, mutation of *mev-1* could strengthen the induction of intestinal ROS production in simulated microgravity-treated nematodes (Zhao *et al.*, 2017a), which suggests that mutation of *mev-1* induces a susceptibility to toxicity of environmental toxicants or stresses. Similarly, *mev-1* mutation-mediated mitochondrial perturbations may further regulate toxicity induction of environmental toxicants or stresses by affecting the insulin signaling pathways (Kondo *et al.*, 2005).

Complex III

isp-1 encodes an iron sulfur component of complex III. In nematodes, exposure to both environmental toxicants (such as graphene oxide (GO)) and stresses (such as simulated microgravity) could increase the transcriptional expression of *isp-1* (Zhao *et al.*, 2017a; Wu *et al.*, 2013a). Meanwhile, mutation of *isp-1* induces a resistance to toxicity of environmental toxicants (such as GO) or stresses (Zhao *et al.*, 2017a; Wu *et al.*, 2014a). Moreover, ISP-1 may further positively regulate the formation of transgenerational toxicity in nematodes exposed to cadmium telluride (CdTe) quantum dots (QDs), since mutation or intestine-specific RNAi of *isp-1* caused resistance to the transgenerational toxicity of CdTe QDs, and prevented the translocation of CdTe QDs from the exposed nematodes to their progeny (Liu *et al.*, 2015).

For the molecular mechanism of the observed resistance to toxicity of environmental toxicants or stresses in *isp-1* mutant nematodes, it was found that genes regulated by DAF-16 could be upregulated by *isp-1* mutation, and both DAF-16 and its multiple interacting proteins (MATH-33, IMB-2, CST-1/2, and BAR-1) were required for the longevity of the long-lived phenotype of *isp-1* mutant nematodes (Senchuk *et al.*, 2018). Thus, ISP-1 may act upstream of the insulin pathway to regulate various biological processes, including the stress response. Moreover, elevated ROS levels could help the *isp-1* mutant nematodes to activate multiple stress-response pathways, including mitochondrial unfolded protein response (mt UPR), SKN-1-mediated stress response, and antioxidant enzymes (Dues *et al.*, 2017). Furthermore, in nematodes exposed to environmental toxicants (such as GO), mutation of *isp-1* could dysregulate the expressions of some long non-coding RNAs (lncRNAs) (Wu *et al.*, 2016), suggesting that the mutation of *isp-1* may reduce GO toxicity by affecting the functions of certain lncRNAs.

Complex IV

The long lifespan could be observed in nematodes with RNAi knockdown in any of the three complex IV subunits, including cytochrome *c* oxidase (COX) IV (W09C5.8 and Y37D8A.14) (Suthammarak *et al.*, 2009). In nematodes, COX deficiencies could decrease intrinsic complex I enzymatic activity, as well as complex I–III enzymatic activity (Suthammarak *et al.*, 2009), suggesting that complex IV may regulate the stress response by affecting the activity of complex I.

Coenzyme Q (ubiquinone, CoQ) synthesis

clk-1 encodes a ubiquinone biosynthesis protein COQ7. Exposure to toxicants (such as GO or multi-walled carbon nanotubes (MWCNTs)) could significantly increase the transcriptional expression of *clk-1* (Wu *et al.*, 2013a,b). Meanwhile, mutation of *clk-1* induced a resistance to toxicity of environmental toxicants (such as Hg, GO, or CdTe QDs) (Liu *et al.*, 2012; Wu *et al.*, 2014a; Liu *et al.*, 2015). During the control of stress response, CLK-1 may also act upstream of the insulin pathway (Senchuk *et al.*, 2018). In the nucleus, CLK-1 may further mediate a retrograde

signaling pathway in response to mitochondrial ROS, and thus act as a barometer of oxidative metabolism (Monaghan *et al.*, 2015). Additionally, some dysregulated lncRNAs induced by GO exposure could be reversed by *clk-1* mutation (Wu *et al.*, 2016), suggesting that CLK-1 potentially regulated GO toxicity by influencing the functions of certain lncRNAs.

Roles of superoxide dismutases (SODs)

Superoxide dismutases (SOD-1–SOD-5) are enzymes that catalytically remove the superoxide radical ($\cdot\text{O}_2^-$) so as to protect organisms from oxidative damage. *sod-1* encodes a Cu/Zn SOD. After Cd exposure, the 24 h LC_{50} of Cd observed was in the order of wild-type (N2) > *sod-1(or13)* > *daf-16(mu86)* (Roh *et al.*, 2009), which suggests the induction of susceptibility to environmental toxicants in *sod-1* mutant nematodes. *sod-2* and *sod-3* encode mitochondrial Mn-SODs. Exposure to environmental toxicants or stresses could dysregulate the expressions of SOD-2 and SOD-3. For example, exposure to titanium dioxide nanoparticles (TiO_2 -NPs, 4, 10, 60, and 90 nm) could increase the expressions of *sod-2* and *sod-3* (Li, Y.-X. *et al.*, 2012). Meanwhile, mutation of *sod-2* or *sod-3* induces a susceptibility to toxicity of environmental toxicants, such as TiO_2 -NPs (Rui *et al.*, 2013; Wu *et al.*, 2014b). During the control of stress response, SOD-3 acts downstream of intestinal DAF-16 in the insulin signaling pathway (Zhao *et al.*, 2016a), and some antimicrobial genes (such as *F55G11.4* and *spp-1*) were further identified as the downstream targets of SOD-3 (Ren *et al.*, 2017). *sod-4* encodes an extracellular Cu/Zn SOD, which could be activated by environmental toxicants or stresses (Zhao *et al.*, 2017a; Wu *et al.*, 2017). Mutation or RNAi knockdown of *sod-4* also induced a susceptibility to the toxicity of environmental toxicants, such as coal combustion-related $\text{PM}_{2.5}$ (Wu *et al.*, 2017). *sod-5* encodes a cytoplasmic Cu/Zn SOD. Exposure to environmental pollutants (such as the original surface water sample collected from the backwater area of Three Gorges Reservoir (TGR) region) or stress (such as simulated microgravity) could increase the expression of *sod-5* (Zhao *et al.*, 2017a; Xiao *et al.*, 2018). It was further found that mutation of *sod-5* also resulted in a

susceptibility of nematodes to the toxicity of environmental pollutants, such as coal combustion-related PM_{2.5} (Wu *et al.*, 2017).

Roles of catalase (CTL-1-3) proteins

Exposure to toxicants (such as copper and phoxim) could increase both the activity of CAT and the transcriptional expressions of *ctl-1* and/or *ctl-2* (Song *et al.*, 2014; Han *et al.*, 2017). Using different endpoints, the *ctl-1(ok1242)* and *ctl-3(ok2042)* mutants showed hypersensitivity to the toxicity of environmental toxicants, including phoxim, copper, or coal combustion-related PM_{2.5} (Song *et al.*, 2014; Han *et al.*, 2017; Wu *et al.*, 2017).

Roles of glutathione-requiring prostaglandin D synthases (GSTs)

Among the *gst* genes, at least the expressions of *gst-1* and *gst-4* could be increased by exposure to toxicants (such as NaF and methylmercury (MeHg)) or stresses (such as simulated microgravity) (Helmcke and Aschner, 2010; Li, Q. *et al.*, 2012; Zhao *et al.*, 2017a). Meanwhile, mutation of *gst* genes, such as *gst-4*, could significantly enhance the toxicity of simulated microgravity treatment (Zhao *et al.*, 2017a), suggesting that mutation of *gst* genes may induce a susceptibility to toxicity of environmental toxicants or stresses.

MAPK Signaling Pathways

In nematodes, there are three important mitogen-activated protein kinase (MAPK) signals: p38 MAPK, JNK MAPK, and ERK MAPK.

p38 MAPK signaling pathway

The core p38 MAPK signaling pathway contains components of *pmk-1* encoded MAPK, *sek-1* encoded MAPK kinase (MAPKK), and *nsy-1* encoded MAPK kinase kinase (MAPKKK). In nematodes, exposure to environmental

toxicants or stress could dysregulate the expression of genes encoding the p38 MAPK signaling pathway. For example, simulated microgravity treatment or prolonged exposure to GO (100 mg/l) all could significantly increase the transcriptional expression of *nsy-1*, *sek-1*, and *pmk-1*, and the expression of phosphorylated PMK-1 (Zhao *et al.*, 2016b; Li *et al.*, 2018). Different from this, exposure to multi-walled carbon nanotubes (MWCNTs) decreased the expression of *pmk-1*, *sek-1*, and *nsy-1*, and inhibited translational expression of PMK-1::GFP in the intestine and phosphorylation of PMK-1 (Shakoor *et al.*, 2016). Thus, the dysregulation pattern of the p38 MAPK signaling pathway may be different under different stress conditions.

The p38 MAPK signaling pathway is involved in the control of toxicity induced by various environmental toxicants or stresses. With GO as an example, exposure to GO (100 mg/l) induced the formation of a more severe reduction in lifespan, decrease in locomotion behavior, and induction of intestinal ROS production in *pmk-1(km25)*, *sek-1(ag1)*, or *nsy-1(ag3)* mutants than those in wild-type nematodes (Zhao *et al.*, 2016b), suggesting that mutations of genes encoding the core p38 MAPK signaling pathway induced a susceptibility to toxicity of environmental toxicants or stresses. In contrast, overexpression of *pmk-1*, *sek-1*, or *nsy-1* caused resistance to the toxicity of GO in inducing intestinal ROS production (Zhao *et al.*, 2016b).

In nematodes, although *pmk-1* is broadly expressed in multiple tissues, only the intestinal signaling cascade of the p38 MAPK signaling pathway regulates the toxicity of environmental toxicants (such as GO) or stresses (such as simulated microgravity) (Zhao *et al.*, 2016b; Li *et al.*, 2018). In the intestine, the transcriptional factor of SKN-1/Nrf was identified as one of the downstream targets for PMK-1 to regulate the toxicity of environmental toxicants or stresses. With the GO as an example, intestinal RNAi knockdown of *skn-1* could cause a susceptibility to GO toxicity (Zhao *et al.*, 2016b). More importantly, intestinal RNAi knockdown of *skn-1* could suppress the resistance of nematodes overexpressing intestinal *pmk-1* to GO toxicity (Zhao *et al.*, 2016b). Moreover, some proteins were further identified as the downstream targets of intestinal SKN-1 in regulating the toxicity of environmental toxicants. One of the downstream

targets is Phase II detoxification protein GST-4, since SKN-1 and GST-4 could act in the same genetic pathway to regulate GO toxicity (Zhao *et al.*, 2016b). Another identified important target for SKN-1 is GCS-1, an ortholog of γ -glutamine cysteine synthetase heavy chain, because both *gcs-1* promoter activation and GST-4 expression were significantly suppressed by feeding with *skn-1*(RNAi) in *Pseudomonas aeruginosa* PA14-infected nematodes (Papp *et al.*, 2012). In the intestine, the transcriptional factor of ATF-7 was identified as another downstream target for PMK-1 to regulate the toxicity of environmental toxicants or stresses. In simulated microgravity-treated nematodes, *atf-7* mutation could significantly reduce the toxicity in transgenic strains overexpressing intestinal *pmk-1* (Li *et al.*, 2018). In nematodes, some antimicrobial proteins could also act as the targets for intestinal PMK-1 to regulate the stress response, such as pathogen infection (Troemel *et al.*, 2006).

Some genes have been further identified as the upregulators of the p38 MAPK signaling pathway in response to environmental toxicants or stresses.

Duox1/BLI-3

During the control of pathogen infection, ROS produced by Duox1/BLI-3 was the source of SKN-1 activation via p38 MAPK signaling (van der Hoeven *et al.*, 2011), suggesting that ROS generation by Duox1/BLI-3 may activate a protective SKN-1 response via the p38 MAPK signaling pathway against the pathogen infection.

TIR-1 and VHP-1

During the control of pathogen infection, suppression of MAPK phosphatase VHP-1 resulted in an increased PMK-1 phosphorylation, and TIR-1, a conserved Toll/IL-1 resistance (TIR) domain protein, has the function to activate p38 MAPK signaling independently of the Toll-like receptor ortholog TOL-1 (Papp *et al.*, 2012).

MEK-1

mek-1 encodes a MAP kinase kinase (MAPKK), and RNAi knockdown of *pmk-1* could further reduce the lifespan of *mek-1* mutant nematodes after pathogen infection (Kim *et al.*, 2004).

Mitochondrial complex I

Downregulating complex I genes *nuo-2* or *nduf-6* could cause a significant increase in p38 MAPK phosphorylation, and downregulation of at least five of the six tested mitochondrial complex I genes could upregulate four of the seven ATF-7-dependent genes induced by rotenone (Chikka *et al.*, 2016).

JNK signaling pathway

The c-Jun N-terminal kinase (JNK) signaling pathway mainly contains members of MEK-1, JKK-1, and JNK-1. *mek-1* and *jkk-1* encode MAP kinase kinases, homolog of human MKK-7a, and act as an activator of JNK. *jnk-1* encodes a serine/threonine kinase, homolog of human JNK, and acts as the sole member of the JNK subgroup of MAP kinase. With the GO as an example, after prolonged exposure, the transcriptional expressions of *mek-1*, *jkk-1*, and *jnk-1* were significantly decreased by GO exposure (10 mg/l) (Zhao *et al.*, 2015a), suggesting that exposure to certain environmental toxicants or stress can dysregulate the expression of JNK MAPK signal. The JNK MAPK signaling pathway regulates the toxicity of environmental toxicants or stresses. Mutation of *mek-1*, *jkk-1*, or *jnk-1* could induce a susceptibility to toxicity of toxicants, such as GO and nanopolystyrene (Zhao *et al.*, 2015a; Qu *et al.*, 2020a). The genetic interaction analysis further indicated that the *jnk-1(gk7);mek-1(ks54)* double mutant exhibited similar GO toxicity to that in the *mek-1(ks54)* or the *jnk-1(gk7)* single mutant, and the *jnk-1(gk7);jkk-1(km2)* double mutant showed similar GO toxicity to that in the *jkk-1(km2)* or *jnk-1(gk7)* single mutant (Zhao *et al.*, 2015a), suggesting that MEK-1 and JKK-1 act genetically in the same pathway with JNK-1 to regulate the toxicity of environmental toxicants or stresses.

During the control of stress response, some proteins have been identified as downstream targets of neuronal JNK-1. One of those downstream targets is SNB-1, a synaptobrevin. In nanopolystyrene-exposed nematodes, RNAi knockdown of *jnk-1* could significantly decrease *snb-1* expression, and RNAi knockdown of *snb-1* could suppress the resistance of nematodes overexpressing neuronal JNK-1 to nanopolystyrene

toxicity (Qu *et al.*, 2020a). Moreover, the JNK MAPK signaling pathway is regulated by MLK-1 MAPK kinase kinase (MAPKKK), MEK-1 MAPK kinase (MAPKK), and KGB-1 JNK-like MAPK (Mizuno *et al.*, 2008).

ERK signaling pathway

The core ERK signaling pathway contains the *mek-2*-encoded MAPK kinase MEK and *mpk-1*-encoded ERK. Exposure to environmental toxicants or stress could dysregulate the expression of genes encoding the ERK MAPK signaling pathway. With GO as an example, prolonged exposure to GO (10 or 100 mg/l) could significantly increase transcriptional expressions of *mpk-1* and *mek-2* (Qu *et al.*, 2017). The ERK MAPK signaling pathway is involved in the regulation of toxicity of environmental toxicants or stresses. Mutation of *mpk-1* or *mek-2* may induce a susceptibility to the toxicity of environmental toxicants, such as GO or nanopolystyrene (Qu *et al.*, 2017, 2020b).

Genetic interaction analysis demonstrated that mutation of *mpk-1* could significantly suppress the resistance of nematodes overexpressing neuronal *mek-2* to GO toxicity (Qu *et al.*, 2017), suggesting that MPK-1 acts downstream of MEK-2 to regulate the toxicity of environmental toxicants or stresses. MPK-1 is expressed in both the neurons and the germline. MPK-1 and MEK-2 could act in the neurons to regulate the toxicity of GO or nanopolystyrene (Qu *et al.*, 2017, 2020b). Nevertheless, germline-specific RNAi knock down of *mpk-1* could not significantly induce a susceptibility to toxicity or nanopolystyrene (Qu *et al.*, 2017, 2020b). Thus, the neuronal signaling cascade of MEK-2-MPK-1 may play a crucial role in regulating the stress response.

During the control of stress response, some proteins have been identified as the downstream targets of neuronal MPK-1. One of the downstream targets is SKN-1. Mutation of *skn-1* could significantly suppress the resistance of nematodes overexpressing neuronal *mpk-1* to GO toxicity (Qu *et al.*, 2017). Moreover, it was observed that mutation of *aex-3* encoding a guanine nucleotide exchange factor could significantly suppress the resistance of nematodes overexpressing the neuronal *skn-1b* to GO toxicity (Qu *et al.*,

2017), suggesting the formation of the neuronal signaling cascade of MEK-1-MPK-1-SKN-1b-AEX-3 in the regulation of toxicity of environmental toxicants or stresses. Besides SKN-1, three insulin peptides (INS-4, INS-39, and DAF-28) also acted as downstream targets of neuronal MPK-1 in regulating the stress response. Neuronal RNAi knockdown of *ins-4*, *ins-39*, or *daf-28* could inhibit the susceptibility of *mpk-1* (RNAi) nematodes to the toxicity of nanopolystyrene (Qu *et al.*, 2020b).

Some upstream regulators for the ERK MAPK signaling pathway in regulating the toxicity of environmental toxicants or stresses have also been identified. One of the upstream regulators is LIN-45, a Raf protein. In the ERK signaling pathway, LIN-45 normally acts upstream of the signaling cascade of MEK-1-MPK-1, and mutation of *lin-45* induced a susceptibility to toxicity of toxicants such as GO (Chen *et al.*, 2017). *pkc-1* encodes a serine/threonine protein kinase C (PKC) protein. Mutation of *lin-45* could noticeably inhibit the resistance of transgenic nematodes overexpressing neuronal PKC-1 to GO toxicity (Chen *et al.*, 2017), suggesting that LIN-45 acts downstream of neuronal PKC-1 to regulate the stress response. *nlg-1* encodes a neuroligin, a postsynaptic cell adhesion protein. Moreover, mutation of *pkc-1* could obviously suppress the resistance of transgenic nematodes overexpressing neuronal NLG-1 to GO toxicity (Chen *et al.*, 2017), suggesting that PKC-1 could further act downstream of neuronal NLG-1 to regulate the stress response. Therefore, a signaling cascade of NLG-1-PKC-1-LIN-45 was identified to act upstream of the ERK MAPK signaling pathway to regulate the toxicity of environmental toxicants or stresses.

Insulin and the Related Signaling Pathways

In the core insulin signaling pathway, insulin ligands bind to DAF-2/IGF-1 receptor (InR) to activate tyrosine kinase activity, which will allow the initiation of the cascade of several kinases (AGE-1/phosphatidylinositol 3-kinase (PI3K), PDK-1/3-phosphoinositide-dependent kinase 1, AKT-1/2/serine/threonine kinase Akt/PKB, and SGK-1/serine or threonine-protein

kinase). The activated kinases further phosphorylate and inactivate transcription factor DAF-16/FOXO, which thereby blocks the transcription of its multiple target genes.

In nematodes, environmental toxicants or stresses could dysregulate the expression of genes encoding the insulin signaling pathway. With GO as an example, prolonged exposure to GO (100 mg/l) could result in a significant increase in the expression levels of *daf-2*, *age-1*, *akt-1*, and *akt-2*, and a decrease in the expression levels of *daf-18* and *daf-16* (Zhao *et al.*, 2016a). Different from this, prolonged exposure to nanopolystyrene (1 µg/l) significantly decreased the transcriptional expressions of *daf-2*, *age-1*, and *akt-1*, and increased the transcriptional expression of *daf-16* (Shao *et al.*, 2019). Therefore, exposure to environmental toxicants or stresses with different toxicity may differently dysregulate the expression of genes in the insulin signaling pathway. Meanwhile, mutation of *daf-16* or *daf-18* could induce a susceptibility to toxicity of toxicants (such as GO or nanopolystyrene), whereas mutation of *daf-2*, *age-1*, *akt-1*, or *akt-2* could induce a resistance to toxicity of toxicants (such as GO or nanopolystyrene) (Zhao *et al.*, 2016a; Shao *et al.*, 2019).

Genetic interaction analysis demonstrated that DAF-16 acted downstream of DAF-2, AGE-1, AKT-1, or AKT-2 to regulate the toxicity of toxicants, because mutation of *daf-16* could effectively suppress the resistance of *daf-2(e1370)*, *age-1(hx546)*, *akt-1(ok525)*, or *akt-2(ok393)* mutant nematodes to GO toxicity (Zhao *et al.*, 2016a). Thus, a signaling cascade of DAF-2-AGE-1-AKT-1/2-DAF-16 in the insulin signaling pathway has been identified to be involved in the control of stress response. Meanwhile, mutation of *daf-18* could effectively inhibit the resistance of *age(hx546)* mutants to GO toxicity (Zhao *et al.*, 2016a), which suggests the suppressor role of DAF-18 on the function of AGE-1 in the regulation of GO toxicity.

The tissue-specific activity of DAF-16 in regulating the toxicity of toxicants has also been determined. *daf-16* is expressed in almost all tissues. During the control of nanopolystyrene toxicity, intestine-specific RNAi knockdown of *daf-16* resulted in susceptibility to the toxicity of nanopolystyrene (Shao *et al.*, 2019). In contrast, epidermal-, neuron-, muscle-, or germline-specific

RNAi knockdown of *daf-16* did not significantly affect the toxicity induction of nanopolystyrene (Shao *et al.*, 2019). Meanwhile, intestine-specific RNAi knockdown of *daf-2*, *age-1*, or *akt-1* resulted in the resistance of nematodes to the toxicity of nanopolystyrene (Shao *et al.*, 2019). Therefore, DAF-16, DAF-2, AGE-1, and AKT-1 acted in the intestinal cells to regulate the toxicity of nanopolystyrene.

During the control of stress response, a series of downstream targets have been identified for DAF-16. Moreover, some upregulators of the insulin signaling pathway in regulating the toxicity of environmental toxicants or stresses have been further identified.

SOD-3

SOD-3 is normally considered as the downstream target for DAF-16 in regulating the stress response. Genetic interaction analysis suggested that DAF-16 acted upstream of SOD-3 to regulate GO toxicity, because the resistance of transgenic strain of *Ex(Pges-1-daf-16)* overexpressing intestinal DAF-16 to GO toxicity could be inhibited by *sod-3* mutation (Zhao *et al.*, 2016a).

Antimicrobial proteins

Some genes encoding potential antimicrobial proteins have also been identified to act as the targeted genes for *daf-16* in regulating the toxicity of GO toxicity (Ren *et al.*, 2017). RNAi knockdown of *lys-1*, *dod-6*, *F55G11.4*, *lys-8*, or *spp-1* could significantly suppress the resistance of nematodes overexpressing intestinal *daf-16* to GO toxicity (Ren *et al.*, 2017), suggesting that LYS-1, DOD-6, F55G11.4, LYS-8, and SPP-1 act as downstream targets for intestinal DAF-16 in regulating GO toxicity. Among these proteins, F55G11.4 and SPP-1 acted further downstream of SOD-3 in the regulation of GO toxicity (Ren *et al.*, 2017).

MTL-1 and MTL-2

mtl-1 and *mtl-2* encode metallothioneins. During the control of toxicity of outdoor PM_{2.5}, MTL-1 or MTL-2 acted in the same genetic pathway with DAF-16 in regulating the toxicity of outdoor PM_{2.5} (Yang *et al.*, 2016a).

NATC-1

natc-1 encodes an evolutionarily conserved subunit of the N-terminal acetyltransferase C (NAT) complex. The *daf-16(mu86);natc-1(am138)* double-mutant animals displayed heat stress resistance similar to *natc-1* single-mutant animals (Warnhoff *et al.*, 2014).

HSF-1

The enhanced resistance of *daf-2(e1370)* and nematodes carrying additional *daf-16* gene copies to *Pseudomonas aeruginosa* infection was reduced by the RNAi knockdown of *hsf-1* (Singh and Aballay, 2006).

GPD-2

gpd-2 encodes a glyceraldehyde-3-phosphate dehydrogenase. The *daf-16* mutation significantly decreased the transcriptional expression of *gpb-2* after exposure to nanopolystyrene (1 µg/l), and RNAi knockdown of *gpd-2* could significantly suppress the resistance of transgenic strain overexpressing intestinal DAF-16 to the toxicity of nanopolystyrene (Shao *et al.*, 2019).

SMK-1

smk-1 encodes a homolog to mammalian SMEK. SMK-1 acted in the intestine to regulate GO toxicity (Yang *et al.*, 2016b). RNAi knockdown of *daf-16* could significantly suppress the protective effects of *smk-1* overexpression on both the lifespan and the locomotion behavior in GO-exposed nematodes (Yang *et al.*, 2016b), suggesting that SMK-1 acts upstream of DAF-16 to regulate the toxicity of environmental toxicants or stresses.

AAK-2

aak-2 encodes a catalytic alpha subunit of AMP-activated protein kinases (AMPKs). The toxicity in multi-walled carbon nanotubes (MWCNTs) (1 mg/l) exposed double mutant of *daf-16(mu86);aak-2(om524)* was similar to that in MWCNTs (1 mg/l) exposed single mutant of *aak-2(om524)* or *daf-16(mu86)* nematodes (Zhuang *et al.*, 2016), implying that AAK-2 can further act together with DAF-16 in the same genetic pathway to form a signaling cascade of AAK-2-DAF-16 to regulate the toxicity of MWCNTs.

HCF-1

hcf-1 encodes a conserved homolog of host cell factor 1. The paraquat resistance of the *hcf-1(pk924)* mutants was dependent on *daf-16*, as the *daf-16(mgDf47);hcf-1(pk924)* double mutant was sensitive to paraquat, similar to that of the *daf-16(mgDf47)* single mutant (Li *et al.*, 2008).

SIR-2.1/SIRT1

Under the paraquat or t-BOOH exposure conditions, mutation of *hcf-1* could suppress the susceptibility of *sir-2.1(ok434)* mutant nematodes to the toxicity of paraquat or t-BOOH (Rizki *et al.*, 2011), suggesting that SIR-2.1 can act upstream of the insulin signaling pathway to regulate the toxicity of environmental toxicants or stresses by suppressing the function of HCF-1. DAF-16 and SIR-2.1 could interact even under the stress condition, and this interaction depended on the 14-3-3 proteins as the SIR-2.1 binding partners (Berdichevsky *et al.*, 2006). The 14-3-3 proteins were required for the SIR-2.1-induced transcriptional activation of DAF-16 and stress resistance (Berdichevsky *et al.*, 2006).

PRDX-2

PRDX-2 is a single cytosolic 2-Cys Prx. Loss-of-function mutation of *prdx-2* increased the arsenite resistance by increasing DAF-16 activity (Olahova and Veal, 2015). The expressions of several DAF-16-activated genes (*mtl-1*, *sod-3*, *gst-7*), as well as the expression of *sod-3p::gfp*, were also increased in *prdx-2* mutant nematodes (Olahova and Veal, 2015). More importantly, mutation of *daf-16* could suppress the resistance of *prdx-2(RNAi)* nematodes to the toxicity of arsenite (Olahova and Veal, 2015).

Development-related Signaling Pathways

Wnt signaling pathway

In the Wnt signaling pathway, the Wnt ligands contain LIN-44, EGL-20, MOM-2, CWN-1, and CWN-2. Certain Wnt ligands have been shown to be involved in regulating the toxicity of environmental toxicants or stresses. With GO as an

example, mutation of *mom-2* or *egl-20* could not affect the formation of GO toxicity, whereas mutation of *cwn-1* or *lin-44* induced a resistance to GO toxicity, and mutation of *cwn-2* induced a susceptibility to GO toxicity (Zhi *et al.*, 2016a). Thus, the Wnt ligands of CWN-1, CWN-2, and LIN-44 play an important role in regulating the toxicity of environmental toxicants or stresses. During the control of GO toxicity, mutation of *cwn-1* or *lin-44* could inhibit the susceptibility of *cwn-2(ok895)* mutant to GO toxicity (Zhi *et al.*, 2016a). Meanwhile, CWN-1 and LIN-44 functioned in the same genetic pathway to regulate the GO toxicity (Zhi *et al.*, 2016a).

The major effector in the canonical Wnt signaling pathway is the transcriptional factor β -catenin BAR-1. After the binding of Wnt ligand(s) to a Frizzled receptor (LIN-17, MOM-5, MIG-1, or CFZ-2), APC complex containing APR-1/Axin, PRY-1/CK1a, KIN-19, and GSK-3 can be inhibited by Dishevelled proteins (MIG-5, DSH-1, and/or DSH-2) so as to lead to stabilization of β -catenin BAR-1 and its translocation into the nucleus. This canonical Wnt/ β -catenin signaling pathway has been further shown to be involved in regulating the toxicity of environmental toxicants or stresses. Among the components in the canonical Wnt/ β -catenin signaling pathway, GO (1 mg/l) could significantly decrease the expressions of *bar-1*, *dsh-1*, *dsh-2*, *mom-5*, and *cfz-2*, and increase the expressions of *apr-1*, *gsk-3*, *apr-1*, and *gsk-3* (Zhi *et al.*, 2017a). Different from this, simulated microgravity treatment could significantly increase the expression of *bar-1*, *dsh-2*, *mig-5*, *mig-1*, and *lin-17*, and decrease the expression of *pry-1* and *gsk-3* (Rui *et al.*, 2019). That is, different toxicants or stresses with different toxicity may induce the different dysregulation patterns of genes in the canonical Wnt/ β -catenin signaling pathway. Among the dysregulated genes by environmental toxicants or stresses, mutation of *bar-1*, *dsh-1*, *dsh-2*, *mig-1*, *mom-5*, or *cfz-2* induced a susceptibility to toxicity of GO or simulated microgravity (Zhi *et al.*, 2017a; Rui *et al.*, 2019). Meanwhile, mutation of *apr-1* or *gsk-3* induced a resistance to toxicity of GO or simulated microgravity (Zhi *et al.*, 2017a; Rui *et al.*, 2019).

In the canonical Wnt/ β -catenin signaling pathway, genetic interaction analysis indicated that APR-1 and/or GSK-3 acted upstream of BAR-1 in regulating the toxicity of GO or simu-

lated microgravity (Zhi *et al.*, 2017a; Rui *et al.*, 2019). DSH-1 and/or DSH-2 acted upstream of APR-1 or GSK-3 in regulating the toxicity of GO or simulated microgravity (Zhi *et al.*, 2017a; Rui *et al.*, 2019). Moreover, Frizzled receptors of MIG-1, MOM-5, and/or CFZ-2 acted upstream of DSH-1 or DSH-2 to regulate the toxicity of GO or simulated microgravity (Zhi *et al.*, 2017a; Rui *et al.*, 2019).

HMP-2 is another β -catenin protein. After acute exposure to GO, among the examined genes required for the control of development of intestinal apical junctions, intestine-specific RNAi knockdown of *hmp-2* caused enhanced induction of intestinal ROS production (Ren *et al.*, 2018). Additionally, intestine-specific RNAi knockdown of *hmp-2* significantly enhanced the accumulation of GO/Rho B in nematode bodies (Ren *et al.*, 2018). These results further demonstrate the important role of HMP-2 in regulating the toxicity of environmental toxicants or stresses.

During the control of stress response, some proteins have been identified as downstream targets of β -catenin BAR-1.

POP-1

POP-1 is a TCF/LEF transcriptional factor. BAR-1 mediates canonical Wnt signaling by forming a BAR-1/POP-1 physical complex to activate expression of its downstream target genes (Korswagen *et al.*, 2000). RNAi knockdown of *bar-1* could decrease the expression of *pop-1* in simulated microgravity-treated nematodes, and intestinal RNAi knockdown of *pop-1* induced a susceptibility to the toxicity of simulated microgravity (Zhao *et al.*, 2020).

DAF-16

RNAi knockdown of *bar-1* could decrease the expression of *daf-16* in simulated microgravity-treated nematodes, and intestinal RNAi knockdown of *daf-16* induced a susceptibility to the toxicity of simulated microgravity (Zhao *et al.*, 2020).

UNC-62

UNC-62 is a transcriptional factor. RNAi knockdown of *bar-1* could increase the expression of *unc-62* in simulated microgravity-treated

nematodes, and intestinal RNAi knockdown of *unc-62* induced a resistance to the toxicity of simulated microgravity (Zhao *et al.*, 2020). Genetic interaction analysis further demonstrated that POP-1 acted upstream of DAF-16 and UNC-62, and activated the function of DAF-16 or inhibited the function of UNC-62 in the intestine to regulate the response to simulated microgravity (Zhao *et al.*, 2020). UNC-62 further acted upstream of DAF-16 and inhibited the DAF-16 function to regulate the response to simulated microgravity (Zhao *et al.*, 2020).

EGL-5

egl-5 encodes a homeodomain transcription factor, orthologous to *Drosophila* Abd-B and the vertebrate Hox9–13 proteins. Among the potential targets of BAR-1, prolonged exposure to GO (1 mg/l) could only significantly decrease the expression of *egl-5*, and mutation of *egl-5* induced a susceptibility to GO toxicity (Zhi *et al.*, 2017a).

PRX-5

prx-5 encodes an ortholog of human receptor PEX (peroxisomal biogenesis factor). Intestinal RNAi knockdown of *bar-1* could significantly decrease the expression of *prx-5* in nematodes exposed to nanopolystyrene (1 µg/l), and intestinal RNAi knockdown of *prx-5* induced a susceptibility to the nanopolystyrene toxicity (Shao *et al.*, 2020). In nematodes, peroxisomal PTS1 proteins of KAT-1 and ACOX-1.6 acted downstream of intestinal PRX-5 in the peroxisome to regulate the response to nanopolystyrene (Shao *et al.*, 2020).

TGF-β signaling pathway

DBL-1-mediated TGF-β signaling pathway

DBL-1 is a TGF-β ligand. The *dbl-1* mutants exhibited a dramatically reduced lifespan in the presence of *S. marcescens* Db11 or *S. marcescens* Db1140 relative to wild-type nematodes (Mallo *et al.*, 2002), suggesting the involvement of DBL-1 in the regulation of toxicity of environmental toxicants or stresses. SMA-6 is a TGF-β receptor of DBL-1, SMA-3 and SMA-2 are two R-Smads, and SMA-4 is a Co-Smad. SMA-6, SMA-2, SMA-3, and SMA-4 were found to be involved in the

control of innate immune response to pathogen infection (Partridge *et al.*, 2010; Savage-Dunn and Padgett, 2017), and the signaling cascade of SMA-6-SMA-2/SMA-3/SMA-4 has been suggested to be involved in the regulation of toxicity of environmental toxicants or stresses.

DAF-7-mediated TGF-β signaling pathway

DAF-7 is also a TGF-β ligand. An increased expression of *daf-7* was observed in nematodes exposed to UV + TiO₂NPs, and mutation of *daf-7* could strengthen the reproductive toxicity induced by UV + TiO₂NPs (Kim *et al.*, 2017). In the DAF-7-mediated TGF-β signaling pathway, *daf-8* encodes an R-Smad protein, and *daf-5* encodes a transcriptional factor. Intestine-specific RNAi knockdown or mutation of *daf-8* induced a susceptibility to GO toxicity, whereas intestine-specific RNAi knockdown or mutation of *daf-5* induced a resistance to GO toxicity (Ren *et al.*, 2017). Mutation of *daf-5* could suppress the susceptibility of *daf-8(e1393)* mutant to GO toxicity (Ren *et al.*, 2017), suggesting that a signaling cascade of DAF-8-DAF-5 can be formed downstream of DAF-7 during the regulation of toxicity of environmental toxicants or stresses. Moreover, the DAF-16-LYS-8 in the insulin signaling pathway could also act upstream of the signaling cascade of DAF-8-DAF-5 in the DAF-7-mediated TGF-β signaling pathway to regulate the GO toxicity (Ren *et al.*, 2017).

Notch signaling pathway

glp-1 encodes an N-glycosylated transmembrane receptor of LIN-12/Notch signal. Using a germline-deficient mutant of *glp-1(e2141)*, it was observed that the *glp-1(e2141)* mutant nematodes displayed reduced levels of neurodegeneration following *P. aeruginosa* infection (Wu *et al.*, 2015), confirming that the sterility induced by *glp-1* mutation may confer a protective response to pathogen infection. Genetic analysis demonstrated that RNAi knockdown of *daf-16* in the insulin signaling pathway could suppress the resistance of *glp-1(e2141)* mutant nematodes to pathogen-induced neurodegeneration (Wu *et al.*, 2015), suggesting that DAF-16 is required for resistance to pathogen-induced neurodegeneration of *glp-1(e2141)* mutant nematodes.

It was further found that *glp-1* mutation significantly increased DAF-16 nuclear location in nematodes after infection with *Cryptococcus neoformans* (TeKippe and Aballay, 2010).

Developmental timing control-related signals

let-7 functions in regulating the timing of larval and adult transition (Reinhart *et al.*, 2000; Sokol, 2012). Prolonged exposure to MWCNTs (10 µg/l) or *P. aeruginosa* PA14 infection could cause a significant decrease in the expression of *let-7::GFP* (Zhao *et al.*, 2017b; Zhi *et al.*, 2017c). Meanwhile, mutation of *let-7* induced a resistance to MWCNT toxicity or to *P. aeruginosa* PA14 infection (Zhao *et al.*, 2017b; Zhi *et al.*, 2017c), suggesting the important function of *let-7* in the regulation of environmental toxicants or stresses. Tissue-specific activity analysis indicated that *let-7* acted in the intestine or the neurons to regulate the innate immune response to *P. aeruginosa* PA14 infection (Zhi *et al.*, 2017c).

During the control of stress response, some proteins have been identified as the downstream targets of *let-7*.

HBL-1 and LIN-41

HBL-1 and LIN-41 are the normally considered direct targets for *let-7* during regulation of the timing of larval and adult transition (Reinhart *et al.*, 2000; Sokol, 2012). In MWCNT-exposed nematodes, *let-7* mutation could significantly increase the expressions of both *hbl-1* and *lin-41*, and mutation of *hbl-1* or *lin-41* induced a susceptibility to MWCNT toxicity (Zhao *et al.*, 2017b). Moreover, mutation of *hbl-1* or *lin-41* could suppress the resistance of *let-7(mg279)* mutants to MWCNT toxicity (Zhao *et al.*, 2017b). During the control of MWCNT toxicity, TIR-1 and SYM-1 were further identified as the downstream targets of HBL-1, and a signaling cascade of *let-7*-HBL-1-TIR-1/SYM-1 has been suggested in the regulation of MWCNT toxicity (Zhao *et al.*, 2017b). *tir-1* encodes a Toll-interleukin 1 receptor (TIR) domain adaptor protein, and *sym-1* encodes a protein containing 15 contiguous leucine-rich repeats (LRRs). During the control of MWCNT toxicity, ALG-1 and ALG-2 were further identified as the downstream targets of

LIN-41, and ALG-1 or ALG-2 acted upstream of LIN-41 in the regulation of MWCNT toxicity (Zhao *et al.*, 2017b). *alg-1* and *alg-2* encode two members of RDE-1 proteins.

SDZ-24

SDZ-24 is an SKN-1-dependent zygotic protein. Mutation of *sdz-24* significantly suppressed the resistance of *let-7(mg279)* mutants to *P. aeruginosa* PA14 infection (Zhi *et al.*, 2017c). Loss-of-function mutation of *let-7* significantly increased SDZ-24::GFP expression under both the *E. coli* OP50 exposure condition and the *P. aeruginosa* PA14 exposure condition (Zhi *et al.*, 2017c). The *in vivo* 3'-UTR binding assay of *sdz-24* further confirmed that *let-7* may suppress the function of SDZ-24 through binding to its 3'-UTR and inhibiting its translation in *P. aeruginosa* PA14-infected nematodes (Zhi *et al.*, 2017c).

Cell Death and DNA Damage-related Signaling Pathways

Apoptosis signaling pathway

Prolonged exposure to GO (10–100 mg/l) could cause the production of more germ cell corpses compared with control based on AO staining results (Zhao *et al.*, 2016c). The core signaling pathway of *C. elegans* apoptosis is constituted by CED-3, CED-4, and CED-9 (Lettre and Hengartner, 2006). Prolonged exposure to GO (10 mg/l) could further significantly increase the expression of *ced-3* and *ced-4*, and decrease the expression of *ced-9* (Zhao *et al.*, 2016c). Meanwhile, mutation of *ced-3* or *ced-4* inhibited the germ cell apoptosis induced by GO exposure, and mutation of *ced-9* also altered the germ cell apoptosis induced by GO exposure (Zhao *et al.*, 2016c), suggesting the potential involvement of this core signaling pathway of apoptosis in regulating the reproductive toxicity of environmental toxicants or stresses.

During the control of stress response, some upregulators have been identified for the core apoptosis signaling pathway in regulating the toxicity of environmental toxicants or stresses. For example, PMK-1 inhibition by RNAi in *ced-9*

mutant nematodes could not reduce the high level of spontaneous gonadal apoptosis observed when fed on *E. coli* OP50 (Aballay *et al.*, 2003), implying that PMK-1 in the p38 MAPK signaling pathway may act as an upstream regulator for the core apoptosis signaling pathway in regulating the toxicity of environmental toxicants or stresses.

During the control of stress response, at least SIR-2.1, a member of the sirtuin family related to *Saccharomyces cerevisiae* Sir2p, acted as the downstream target of the core apoptosis signaling pathway in regulating the toxicity of environmental toxicants or stresses (Greiss *et al.*, 2008). *sir-2.1* was required for induction of germ cell apoptosis caused by ionizing radiation-induced DNA damage, and SIR-2.1 co-localized with the CED-4 in germ cells in nematodes exposed to ionizing irradiation (Greiss *et al.*, 2008).

DNA damage signaling pathway

In the DNA damage-related signaling pathway, DNA damage checkpoint EGL-1 functions as an upstream activator in the core apoptosis signaling pathway, and CEP-1/p53 functions in promoting DNA damage-induced apoptosis by activating EGL-1 (Hofmann *et al.*, 2002; O'Neil and Rose, 2006). CLK-2, an ortholog of telomere length-regulating protein Tel2p, and HUS-1 are required for the function of CEP-1 in activating DNA damage-induced apoptosis (Hofmann *et al.*, 2002; O'Neil and Rose, 2006). Prolonged exposure to GO (10 mg/l) could induce DNA damage and reduce the number of mitotic cells in the gonad, and significantly increase the expressions of *hus-1*, *clk-2*, *cep-1*, and *egl-1* (Zhao *et al.*, 2016c), suggesting the induction of germ DNA damage and alteration in the core molecular basis for DNA damage in GO-exposed nematodes. Meanwhile, mutation of *hus-1*, *clk-2*, *cep-1*, or *egl-1* could noticeably suppress the germline apoptosis induced by GO exposure (Zhao *et al.*, 2016c).

During the control of stress response, some upregulators have been identified for the core DNA damage-related signaling pathway in regulating the toxicity of environmental toxicants or stresses.

mir-360

Mutation of *mir-360* significantly enhanced the reproductive toxicity of GO (Zhao *et al.*, 2016c). CEP-1 was identified as a molecular target for *mir-360*, and the double mutant of *cep-1(RNAi);mir-360(n4635)* exposed to GO (10 mg/l) showed the similar phenotype of germline apoptosis to that in *cep-1(RNAi)* nematodes exposed to GO (10 mg/l) (Zhao *et al.*, 2016c).

ABL-1

abl-1 encodes c-Abl, a conserved nonreceptor tyrosine kinase that integrates the genotoxic stress responses. Mutation of *abl-1* caused hypersensitivity to radiation-induced apoptosis in the germline, and mutation of DNA damage checkpoint and p53 could prevent germ cell apoptosis in *abl-1(ok171)* mutant nematodes (Deng *et al.*, 2004).

NOL-6

nol-6 encodes a nucleolar RNA-associated protein (NRAP) that is involved in the early stages of ribosome biogenesis. During the control of innate immunity, mutation of *cep-1* could suppress enhanced resistance to *S. enterica*-mediated killing of *nol-6* RNAi nematodes, and the CEP-1-regulated genes were upregulated in *nol-6* RNAi nematodes (Fuhrman *et al.*, 2009).

Metabolism-related Signaling Pathways

Functions of fat metabolic sensors

SBP-1

shp-1 encodes a sterol response element binding protein (SREBP), a transcriptional regulator of fat and sterol synthesis. *shp-1* RNAi knockdown substantially reduced lifespan, and dramatically accelerated age-dependent declines in movement and feeding capacities under glucose-fed conditions (Lee *et al.*, 2015). Under glucose-fed conditions, the long lifespan in *sams-1* mutant nematodes could be suppressed by RNAi knockdown of *shp-1* (Lee *et al.*, 2015), suggesting that SAMS-1 acts upstream of SBP-1 to regulate the toxicity of environmental toxicants or stresses.

NHR-49

nhr-49 encodes a nuclear hormone receptor, and is the homology to mammalian peroxisome proliferator-activated receptor (PPAR α). Mutation or RNAi knockdown of *nhr-49* could induce a susceptibility to pathogen infection or toxicity of tBOOH (6 mM) (Sim and Hibberd, 2016; Goh *et al.*, 2018). After short-term food withdrawal (fasting), the induction of three mitochondrial fatty acid β -oxidation genes, *acs-2*, *acs-11*, and *hacd-1*, was virtually eliminated by *nhr-49* mutation (Gilst *et al.*, 2005), suggesting that at least some of the genes controlling the mitochondrial fatty acid β -oxidation process can act as the targeted genes for *nhr-49* in the regulation of toxicity of environmental toxicants or stresses.

MDT-15

mdt-15 encodes a homolog of mammalian PGC-1, and acts as co-activator for both NHR-49/PPAR α and SBP-1/SREBP. Exposure to nanopolystyrene (0.1–100 μ g/l) increased *mdt-15* expression, and loss-of-function mutation of *mdt-15* caused susceptibility to nanopolystyrene toxicity (Yang *et al.*, 2020).

During the control of stress response, some proteins have been identified as targets of MDT-15 in the regulation of toxicity of environmental toxicants or stresses. For example, RNAi knockdown of *sbp-1* effectively suppressed this resistance in nematodes overexpressing intestinal MDT-15 to nanopolystyrene toxicity (Yang *et al.*, 2020), which suggested the role of SBP-1 as the target of MDT-15 in regulating the response to nanopolystyrene.

Moreover, the p38 MAPK signal has been identified as the upregulator of MDT-15 in the regulation of toxicity of environmental toxicants or stresses. RNAi knockdown of *mdt-15* or *sbp-1* could suppress the resistance of a transgenic strain overexpressing intestinal PMK-1 to nanopolystyrene toxicity (Yang *et al.*, 2020). RNAi knockdown of *mdt-15* or *sbp-1* also inhibited the resistance of a transgenic strain overexpressing intestinal SKN-1 to nanopolystyrene toxicity (Yang *et al.*, 2020).

NHR-80

nhr-80 encodes an intestinal nuclear hormone receptor to regulate the expression of delta-9

desaturases. *nhr-80* was required for the increase in lifespan upon the removal of germline precursor cells, because the germline ablations in *nhr-80* mutant nematodes resulted in a significant decrease in survival when fed *Xenorhabdus nematophila* as compared with germline-ablated wild-type nematodes (Sinha and Rae, 2014).

AMPK signaling pathway

AMP-activated kinase (AMPK) is a major cellular fuel gauge. *aak-2* encodes the homolog of the catalytic subunit of mammalian AMPK. The *aak-2* mutant nematodes showed a hypersensitivity to heat or oxidative stress (Lee *et al.*, 2008). AAK-2 can be activated by the LKB1 homolog PAR-4 (Lee *et al.*, 2008). The *par-4* mutant nematodes also exhibited hypersensitivity to heat or oxidative stress (Matsuyama *et al.*, 2014), suggesting that a signaling cascade of PAR-4-AAK-2 was required for the regulation of toxicity of environmental toxicants or stresses.

ACC and FAS

ACC (POD-2) controls the synthesis of malonyl CoA from acetyl CoA, and FAS (FASN-1) controls the synthesis of saturated fatty acyl CoAs from malonyl CoA. Under glucose-fed conditions, RNAi knockdown of ACC (*pod-2* RNAi) or FAS (*fasn-1* RNAi) caused reduced lifespan (Lee *et al.*, 2015), suggesting their potential involvement in the regulation of toxicity of environmental toxicants or stresses.

FAT-6 and FAT-7

FAT-5, FAT-6, and FAT-7 control the change from saturated fatty acyl CoAs to monounsaturated fatty acyl CoAs. RNAi knockdown of *fat-6* or *fat-7* could enhance the toxicity of paraquat (10 mM) in decreasing viability, whereas RNAi knockdown of *fat-5* did not affect the toxicity of paraquat (10 mM) (Horikawa and Sakamoto, 2009). Moreover, the *fat-6(tm331);fat-7(wa36)* double mutation could suppress the resistance of *age-1(hx546)* mutant nematodes to cold stress in reducing lifespan (Savory *et al.*, 2011).

implying that FAT-6 and FAT-7 act downstream of the insulin signaling pathway to regulate the toxicity of environmental toxicants or stresses.

ELO proteins

ELO proteins (such as ELO-2, ELO-5, and ELO-6) control the synthesis of polyunsaturated fatty acyl CoAs from monounsaturated fatty acyl CoAs. At least, the RNAi knockdown of *elo-2* could enhance the toxicity of paraquat (10 mM) in decreasing viability (Horikawa and Sakamoto, 2009).

Fatty acid transport protein ACS-22

Using the blue food dye erioglaucine disodium, it was observed that mutation of *acs-22* caused obvious dye leakage from the intestinal lumen into the intestinal cells, and even the body cavity under normal conditions (Qu *et al.*, 2018), suggesting that ACS-22 regulates the functional state of the intestinal barrier. Meanwhile, the more severe toxicity of nanopolystyrene particles or multi-walled carbon nanotubes (MWCNTs) could be observed in *acs-22* mutant nematodes compared with that in wild-type nematodes (Zhi *et al.*, 2016b; Qu *et al.*, 2018). Mutation of *acs-22* could even disrupt the beneficial function of lactic acid bacteria (LAB) in preventing the toxicity of GO (Zhao *et al.*, 2015b).

Protective Response-related Signaling Pathways

Antimicrobial proteins

Bacterial or fungal pathogens can induce innate immune responses in nematodes by activating the expression of antimicrobial proteins (Sun *et al.*, 2016; Zhi *et al.*, 2017c). Exposure to environmental toxicants or stresses can also activate the expression of antimicrobial proteins. For example, acute exposure to GO (10 mg/l) could induce significant increase in expression of *lys-1*, *dod-6*, *F55G11.4*, *lys-8*, and *spp-1* (Ren *et al.*, 2017). *lys-1* and *lys-8* encode lysozymes, *dod-6*

encodes a protein downstream of DAF-16, *F55G11.4* encodes a protein containing a CUB-like domain, and *spp-1* encodes a caenopore. Meanwhile, mutation of *lys-1*, *lys-8*, or *spp-1* induced a susceptibility to GO toxicity, and RNAi knockdown of *dod-6* or *F55G11.4* also induced a susceptibility to GO toxicity (Ren *et al.*, 2017). The intestine-specific RNAi knockdown of the antimicrobial genes *lys-1*, *dod-6*, *F55G11.4*, *lys-8*, or *spp-1* caused a susceptibility to GO toxicity (Ren *et al.*, 2017), which implies that these antimicrobial proteins at least acted in the intestine in response to environmental toxicants or stresses.

Mitochondrial unfolded protein response (mt UPR)

HSP-6::GFP and HSP-60::GFP are markers of mt UPR. Infection with *P. aeruginosa* PA14 could induce mt UPR as indicated by increases in both HSP-60 and HSP-6 (Pellegrino *et al.*, 2014). Infection with *P. aeruginosa* strains lacking individual siderophore, pyocyanin, or cyanide toxin genes caused less mt UPR activation than wild-type *P. aeruginosa* (Pellegrino *et al.*, 2014), suggesting that multiple pathogen toxins will target mitochondrial function and result in mt UPR activation. Simulated microgravity treatment for 24 h could also activate significant mt UPR (Liu *et al.*, 2019).

For the molecular basis for mt UPR induced by environmental toxicants or stresses, it was first found that mt UPR is regulated by ATFS-1, a leucine zipper (bZIP) transcription factor containing both a mitochondrial targeting sequence (MTS) and a nuclear localization sequence (NLS). ATFS-1 mediates a mitochondrial-to-nuclear communication, and ATFS-1 is efficiently imported into the mitochondrial matrix under homeostatic conditions (Melber and Haynes, 2018). ATFS-1 not only induced mitochondrial protective genes, but also regulated innate immunity. The *atfs-1* gain-of-function mutant *atfs-1(et18)*, which constitutively activates mitochondrial UPR, accumulated less *P. aeruginosa*-GFP in the intestine, and survived longer than wild-type nematodes (Pellegrino *et al.*, 2014). The increase in mitochondrial chaperone reporter (*hsp-6* and *hsp-60opr::gfp*) activation in the intestine by *P. aeruginosa* exposure required

atfs-1, and was also correlated with increased nuclear accumulation of ATFS-1::GFP and NLS in ATFS-1 (Pellegrino *et al.*, 2014). During mitochondrial stress, more ATFS-1 accumulated within mitochondria was observed in *haf-1(ok705)* mutant nematodes (Nargund *et al.*, 2012), suggesting that HAF-1 affects mitochondrial UPR signaling by modulating mitochondrial import of ATFS-1.

Homeodomain-containing transcription factor DVE-1 and small ubiquitin-like protein UBL-5 were also required for the activation of mt UPR (Haynes *et al.*, 2007). Chromatin was specifically remodeled during mitochondrial dysfunction to promote mt UPR activation; and histone methyltransferase MET-2 in concert with LIN-65, along with two jumonji domain histone demethylases, JMJD-3.1 and JMJD-1.2, were required for the control of this process (Melber and Haynes, 2018). The chromatin state could be further stabilized by DVE-1 and UBL-5 (Melber and Haynes, 2018).

In nematodes exposed to environmental toxicants or stresses, some downstream targets have been identified for transcriptional factor ATFS-1 in the mt UPR signaling pathway. HSP-6, HSP-60, and DNJ-10 (mitochondrial chaperones), MPPA-1 (a subunit of the mitochondrial presequence processing protease), PPGN-1 (a matrix-localized protease), GLRX-5 (a glutaredoxin that functions in mitochondrial iron-sulfur cluster biogenesis), MRPS-14 (a subunit of the mitochondrial ribosome), and TSFM-1 (a mitochondrial translational elongation factor) could be induced during mitochondrial dysfunction by ATFS-1 (Nargund *et al.*, 2012; Melber and Haynes, 2018). Antimicrobial peptide *abf-2*, secreted lysozyme *lys-2*, and two C-type lectins could be induced during mitochondrial stress, and the induction of innate immune genes by *spg-7*(RNAi) also required ATFS-1 (Pellegrino *et al.*, 2014). ATFS-1 could further bind the promoters of oxidative phosphorylation machinery (OXPHOS) complex assembly factors and iron-sulfur biogenesis components. For example, ATFS-1 bound promoters of *nuaf-1* and *Y17G9B.5 (ECSIT)*, two NADH ubiquinone oxidoreductase assembly factors, and *lpd-8* (NFU1), an Fe-S cluster biogenesis component, and these genes could be induced during mitochondrial stress in an *atfs-1*-dependent manner (Nargund *et al.*, 2015).

Endoplasmic reticulum (ER) UPR

HSP-4 is the marker of ER UPR. Nanopolystyrene (100 nm) was selected as the example of environmental toxicants, and exposure to nanopolystyrene (1 µg/l) induced a significant increase in expression of intestinal HSP-4::GFP (Qu *et al.*, 2019a), suggesting the potential of ER UPR induction by environmental toxicants or stresses in nematodes. Meanwhile, intestine-specific RNAi knockdown of *hsp-4* could induce a susceptibility to nanopolystyrene toxicity (Qu *et al.*, 2019a).

For the molecular basis of ER UPR induced by environmental toxicants or stresses, IRE-1, PEK-1, and ATF-6 are three important ER UPR transducers. *ire-1* encodes a transmembrane serine/threonine protein kinase, *pek-1* encodes a transmembrane protein kinase, and *atf-6* encodes an ortholog of mammalian transmembrane ATF6α. Among these genes, both ATF-6 and IRE-1 are involved in the regulation of toxicity of environmental toxicants or stresses (Bischof *et al.*, 2008). *xbp-1* encodes a bZIP transcriptional factor. The *xbp-1* mRNA is an IRE-1 substrate or a direct target required for IRE-1 signaling (Calfon *et al.*, 2001). Mutations in either *ire-1* or *xbp-1* could abolish ER UPR induction (Calfon *et al.*, 2001), and mutation of *xbp-1* induced a susceptibility to environmental toxicants, such as nanopolystyrene (Qu *et al.*, 2019a). *hpl-2* encodes the homolog of heterochromatin protein 1 (HP-1), and inactivation of HPL-2 caused an XBP-1-dependent enhancement in resistance to ER stress (Kozłowski *et al.*, 2014). It was suggested that the retinoblastoma protein homolog LIN-35 and the LIN-13 zinc finger protein acted in the same pathway with HPL-2 to limit the ER stress response (Kozłowski *et al.*, 2014). Moreover, some genes (*exos-3* and *F48E8.6*) encoding subunits of the exosome complex that functions in mRNA surveillance were identified for the induction of ER UPR response (Sakaki *et al.*, 2012). ER UPR could be activated in nematodes with mutations of *smg-1* or *smg-6* encoding the products required for nonsense-mediated mRNA decay (NMD) (Sakaki *et al.*, 2012), suggesting the involvement of NMD-mediated mRNA quality in controlling the ER UPR response.

In nematodes exposed to environmental toxicants or stresses, some downstream targets have been identified for the transcriptional

factor XBP-1 in the ER UPR signaling pathway. Moreover, some proteins have been further identified as upregulators of ER UPR signaling in nematodes exposed to environmental toxicants or stresses.

HSP-4

RNAi knockdown of *hsp-4* could significantly suppress the resistance of nematodes overexpressing intestinal XBP-1 to nanopolystyrene toxicity (Qu *et al.*, 2019a).

ABU (activated in blocked UPR) proteins

RNAi knockdown of *abu-1* activated the ER stress marker *hsp-4::gfp* in otherwise normal animals, and killed 50% of ER-stressed *ire-1* and *xbp-1* mutant nematodes (Urano *et al.*, 2002).

p38 MAPK signaling cascade (PMK-1-SKN-1/ATF-7)

RNAi knockdown of *pmk-1*, *atf-7*, or *skn-1* significantly suppressed this induction of ER UPR in nematodes exposed to nanopolystyrene (1 µg/l), and XBP-1 acted as the downstream target of SKN-1 or ATF-7 during control of nanopolystyrene toxicity (Qu *et al.*, 2019a).

MDT-15 and SBP-1

The activation of HSP-4::GFP induced by nanopolystyrene exposure was significantly inhibited by RNAi knockdown of *mdt-15* or *sbp-1* (Yang *et al.*, 2020).

CDC-48-RUVB-2 signaling cascade

The degradation of AAA⁺ ATPase RUVB-2 by AAA⁺ ATPase p97/CDC-48 enhanced the expression of ER UPR response genes through an XBP1-dependent mechanism (Marza *et al.*, 2015).

CED-1

CED-1 is a phagocytic receptor recognizing cell corpses and initiating their engulfment. PQN (prion-like glutamine[Q]/asparagine[N]-rich domain-bearing protein)/ABU (activated in blocked unfolded protein response) proteins represent a non-canonical UPR response (Haskins *et al.*, 2008). CED-1 was identified to have the

function in activating the expression of *pqn/abu* genes during the control of innate immunity (Haskins *et al.*, 2008), suggesting that CED-1 regulated the non-canonical UPR response genes during the control of toxicity of environmental toxicants or stresses.

Autophagy

Using GFP::LGG-1 as a marker for autophagy induction, the autophagy can be dynamically altered by environmental toxicants or stresses. Infection with *P. aeruginosa* PA14 could markedly increase GFP::LGG-1 puncta in both seam cells and intestine (Zou *et al.*, 2014). A significant increase in the number of LGG-1::GFP positive puncta was observed in nematodes exposed to nanopolystyrene particles (10 µg/l) (Qu *et al.*, 2019b). Nevertheless, the observed increase in the number of LGG-1::GFP positive puncta in nanopolystyrene (10 µg/l) exposed nematodes was significantly inhibited by exposure to higher levels of nanopolystyrene (1000 µg/l) (Qu *et al.*, 2019b).

In nematodes, the autophagy genes *bec-1*, *lgg-1*, *lgg-2*, and *unc-51* were involved in the control of toxicity of environmental toxicants or stresses (Samokhvalov *et al.*, 2008; Qu *et al.*, 2019b). For example, RNAi knockdown of *lgg-1* induced a susceptibility to nanopolystyrene neurotoxicity on the development of D-type motor neurons (Qu *et al.*, 2019b). BEC-1, an ortholog of yeast and mammalian autophagy protein ATG6/VPS30/beclin1, acted upstream of both CED-3 and CTR-1 to regulate autophagy induction in nematodes exposed to environmental toxicants or stresses (Samokhvalov *et al.*, 2008).

For molecular control of autophagy in nematodes exposed to environmental toxicants or stresses, MiT transcription factor HLH-30/TFEB governs the autophagy induction. The induction of autophagy genes *lgg-1* and *lgg-2*, *unc-51*, and *atg-2*, *atg-13*, and *atg-16.2* (homologous to human ATG2, ATG13, and ATG16L1, respectively) was *hlh-30*-dependent in nematodes after pathogen infection (Visvikis *et al.*, 2014). HLH-30 overexpression could further suppress the neurodegeneration of D-type motor neurons and decrease locomotion behavior induced by exposure to nanopolystyrene (Qu *et al.*, 2019b). HPK-1, a Hipk homolog, was

necessary for induction of autophagosome formation and autophagy gene expression in response to dietary restriction (DR) or inactivation of target of rapamycin complex 1 (TORC1) (Das *et al.*, 2017). The autophagy-stimulating transcription factors *pha-4*/FoxA and *mxl-2*/Mlx, but not *hlh-30*/TFEB and nuclear hormone receptor *nhr-62*, were necessary for the observed extended longevity induced by HPK-1 overexpression (Das *et al.*, 2017).

Mitophagy

Mitophagy shares biological events with generalized autophagy, including development of an isolation membrane and maturation of autophagosome. Mitophagy induction required at least two specific regulators (PINK-1/PINK1 and PDR-1/Parkin) to target mitochondria for recycling, and exposure to phenanthroline or *P. aeruginosa* inhibited the mitochondrial import and degradation of PINK-1/PINK1::GFP (Kirienko *et al.*, 2015). Disruption of mitophagic regulators (*pink-1*/PINK1 and *pdr-1*/PARK2) could increase the lethality of nematodes treated with *P. aeruginosa* or phenanthroline (Kirienko *et al.*, 2015), suggesting the important role of mitophagy in regulating the stress response.

G-Protein Coupled Receptors and Ion Channels and Downstream Cytoplasmic Signals

It can be assumed that certain G-protein coupled receptors (GPCRs) or ion channels would be activated or suppressed after exposure to environmental toxicants/stresses. After the activation of GPCRs or ion channels, a certain downstream cytoplasmic signaling cascade will be affected, and functions of certain transcriptional factors and their targets will be further altered in nematodes exposed to environmental toxicants/stresses.

GPCRs

The important functions of insulin receptors and Wnt receptors in regulating the toxicity of

environmental toxicants or stresses have been discussed above.

Epidermal DCAR-1

During the control of innate immunity, three clones, targeting *dcar-1* (*dcar-1*(RNAi)), *frpr-11*(*frpr-11*(RNAi)) and *srv-21* (*srv-21*(RNAi)), were found to be able to decrease the expression of the antimicrobial peptide (AMP) reporter gene (*nlp-29p::gfp*) (Zugasti *et al.*, 2014). Unlike *frpr-11*(RNAi) or *srv-21*(RNAi), *dcar-1*(RNAi) did not affect the expression of *nlp-29p::gfp* in nematodes expressing a constitutively active form of the Gα protein GPA-12 (Zugasti *et al.*, 2014), suggesting that DCAR-1 may act upstream or in parallel with GPA-12. DCAR-1 acted in the epidermis to regulate the expression of AMP-encoding genes and the defense against fungal infection (Zugasti *et al.*, 2014). Activation of DCAR-1 was due to the endogenous ligands of NLP-28 and NLP-29, and loss-of-function mutation of *dcar-1* reduced the elevated expression of *nlp-29* (Zugasti *et al.*, 2014). In the epidermis, dihydrocaffeic acid (DHCA) was further identified as an exogenous ligand that activates the innate immune response via DCAR-1 (Zugasti *et al.*, 2014).

Intestinal FSHR-1

Of the 14 candidate LRR receptors, GPCR FSHR-1 was identified as a putative innate immune receptor in response to bacterial infection (Powell *et al.*, 2009). FSHR-1 acted in the intestine to regulate the transcription of a set of putative antimicrobial genes (Powell *et al.*, 2009). During the control of innate immunity, FSHR-1 acted in parallel to both the insulin (*daf-2*) and the p38 MAPK (*tir-1*, *nsy-1*, and *pmk-1*) pathways (Powell *et al.*, 2009).

Neuropeptide receptors

At least, NPR-1, NPR-4, NPR-9, and NPR-12 have been shown to be involved in the control of toxicity induced by environmental toxicants or stresses (Styer *et al.*, 2008; Yu *et al.*, 2016, 2018). Mutation of *npr-1* induced a susceptibility to *P. aeruginosa* infection, which was largely associated with decreased pathogen avoidance and decreased innate immune responses (Styer

et al., 2008). Expression of *npr-1* in the sensory neurons (AQR, PQR, and URX neurons) was required for control of innate immunity (Styer *et al.*, 2008). NPR-1 could regulate both PMK-1-dependent and independent innate immune responses (Styer *et al.*, 2008). NPR-4 is the receptor for neuropeptide FLP-4 released from ADL sensory neurons, and loss-of-function mutation of *npr-4* significantly decreased the index of choice between bacterial OP50 and pathogen PA14 (Yu *et al.*, 2016). Expression of NPR-4 in AIB interneurons was required for the control of this preference choice (Yu *et al.*, 2016). In AIB interneurons, SET-4, a histone H3 at lysine 4 (H3K4) methyltransferase, was identified as the downstream target of NPR-4 to regulate preference choice (Yu *et al.*, 2016). NPR-9 is a homolog of the gastrin-releasing peptide receptor (GRPR) expressed in AIB interneurons, and the *npr-9(tm1652)* mutant nematodes showed a resistance to PA14 infection (Yu *et al.*, 2018). NPR-9 could regulate the innate immune response to pathogen infection by antagonizing the activity of AIB interneurons (Yu *et al.*, 2018). NPR-12 was identified as the receptor of epidermal neuropeptide NLP-29, and mutation of *npr-12* completely blocked *Drechmeria conio-spora* infection-induced dendrite degeneration (E *et al.*, 2018). Autophagic machinery may be involved downstream of NPR-12 to transduce the degeneration signals in pathogen-infected nematodes (E *et al.*, 2018).

Neuronal SRH-220

In ADL sensory neurons, loss-of-function mutation of *srh-220* resulted in an enhanced index of choice between bacterial OP50 and pathogen PA14 (Yu *et al.*, 2016). ADL sensory neurons regulated preference choice through peptidergic signals, such as FLP-4 and NLP-10, which was controlled by SRH-220 since *flp-4* or *nlp-10* mutation suppressed the preference choice phenotype in *srh-220* mutant (Yu *et al.*, 2016).

Ion channels

Cyclic nucleotide-gated ion channels

The susceptibility of *npr-1(ad609)* mutant nematodes to *P. aeruginosa* could be rescued by

mutations in *tax-2* or *tax-4*, encoding a cyclic GMP-gated ion channel (Styer *et al.*, 2008), suggesting the involvement of these two ion channels in regulating the function of NPR-1 in controlling innate immunity. CNG-3 shows high homology with CNG channels, and CNG-3 acted upstream of TAX-4 to regulate thermal stress (Cho *et al.*, 2004).

Voltage-gated calcium ion channel UNC-2

UNC-2 was required for expression of the thermal stress-induced tryptophan hydroxylase (TPH-1) in serotonergic chemosensory ADF neurons by antagonizing the TGF- β signaling pathway (Estevez *et al.*, 2004). This thermal stress-induced *tpH-1::GFP* expression could be restored to a normal level in *unc-2* mutant nematodes by mutations in *daf-4* encoding a TGF- β receptor or by *unc-43(gf)* mutation (Estevez *et al.*, 2004).

Potassium ion channel KVS-1

Oxidation of KVS-1 during aging could cause sensory function loss, and protection of this KVS-1 channel from oxidation could preserve neuronal function (Cai and Sesti, 2009). The native KVS-1 currents could be modified by endogenous ROS or by oxidizing agents (Cai and Sesti, 2009).

Chloride intracellular channel EXL-1

Under thermal stress conditions, EXL-1, not EXC-4, responded specifically to heat stress and translocated from the cytoplasm to the nucleus in intestinal cells and body wall muscle cells (Liang *et al.*, 2017). The *exl-1* loss-of-function mutant nematodes were susceptible to heat stress (Liang *et al.*, 2017).

ARR-1/arrestin

ARR-1 is the sole GPCR adaptor protein arrestin-1, which can block G protein-mediated signaling (Singh and Aballay, 2012). ARR-1 acted in the neurons to regulate innate immunity against the pathogen infection by targeting different pathways, such as ER UPR signaling (Singh and Aballay, 2012). ARR-1 was required

for GPCR signaling in ADF, AFD, ASH, ASI, AQR, PQR, and URX neurons to regulate the innate immune response (Singh and Aballay, 2012).

G proteins

Gq α signaling

Gq α signaling antagonizes Go α signaling by affecting the levels of diacylglycerol (DAG). Gq α signaling is mediated by heterotrimeric G protein α q subunit (EGL-30), and EGL-30 further stimulates phospholipase C β (EGL-8) to produce DAG (Lackner *et al.*, 1999). Both the *egl-30* and the *egl-8* mutant nematodes have decreased DAG, decreased neuronal secretion, and susceptibility to pathogen infection (Lackner *et al.*, 1999; Kawli *et al.*, 2010).

Go α signaling

GOA-1 is a neuronal G protein Go α subunit, and EAT-16 is a regulator of G-protein signaling (RGS) protein acting downstream of GOA-1. It was found that the inhibition of feeding by pore-forming toxins (PFTs) required both the GOA-1 and the EAT-16 (Los *et al.*, 2013). G α RGS EGL-10 in the rectal epithelium could further act downstream of TPH-1-mediated neuronal serotonin signaling released from chemosensory neurons to regulate the innate immune response and to affect pathogen clearance (Anderson *et al.*, 2013).

PLC-DAG-PKD signaling

PLC-PKD-TFEB signaling cascade

The activation of transcription factor EB (TFEB) HLH-30 required DKF-1, a homolog of protein kinase D (PKD), in *Staphylococcus aureus*-infected nematodes (Najibi *et al.*, 2016). The activation of HLH-30 also required PLC-1, a phospholipase C (PLC), acting downstream of Gq α homolog EGL-30 and upstream of DKF-1 in *S. aureus*-infected nematodes (Najibi *et al.*, 2016). Thus, a conserved PLC-PKD-TFEB signaling cascade has been identified as being required for the regulation of innate immune response (Najibi *et al.*, 2016).

DKF-2

DKF-2 is a protein kinase D (PKD), which mediates the signal transduction acting downstream from phospholipase C and DAG. Pathogen infection could potentially activate DKF-2 expression, and nematodes lacking DKF-2 were hypersensitive to killing by bacteria (Ren *et al.*, 2009). TPA-1, a PKC δ homolog, was further identified to regulate activation and functions of DKF-2 in regulating innate immunity (Ren *et al.*, 2009), suggesting that a signaling cascade of DAG-TPA-1-DKF-2 was required for the control of innate immunity. Moreover, DKF-2 could promote the activation of PMK-1, and a-loop phosphorylation was required for DKF-2-mediated induction of antimicrobial genes (Ren *et al.*, 2009).

Ca²⁺ signaling

UNC-31

UNC-31 is a calcium activator. Inhibition of feeding by PFT required the neuronal-expressed UNC-31 (Los *et al.*, 2013). Inhibition of feeding by PFT in *unc-31(e928)* mutant nematodes could be restored when *unc-31* was selectively expressed in the neurons (Los *et al.*, 2013).

CRT-1

crt-1 encodes a calreticulin (CRT) to regulate Ca²⁺ homeostasis and chaperone activity (Park *et al.*, 2001). CRT-1 expression was obviously elevated under stress conditions (Park *et al.*, 2001), implying the possible involvement of CRT-1 in the regulation of toxicity of environmental toxicants or stresses.

Epigenetic Regulation of Toxicity of Environmental Toxicants or Stresses

Epigenetic regulation can be formed and reflected by several forms, such as methylation, acetylation, and control of small RNAs, which provides a possibility that a small set of molecules can act upstream and govern many important molecular signaling pathways to regulate the toxicity of environmental toxicants or stresses.

Methylation regulation

Methylation of histone H3K4

ASH, WDR-5, and SET-2 constitute an ASH-2 trithorax complex, which trimethylates the histone H3K4. ASH-2 is a trithorax group protein, WDR-5 is a WD40 repeat-containing protein, SET-2 is a histone H3 at lysine 4 (H3K4) methyltransferase, and RBR-2 is an H3K4 demethylase. During the control of preference choice behavior, mutation of *set-2*, *ash-2*, or *wdr-5* caused significant decreases in index of choice between bacterial OP50 and pathogen PA14; however, mutation of *rbr-2* did not affect preference choice behavior (Yu *et al.*, 2016). In this ASH-2 trithorax complex acting downstream of GPCR NPR-4, SET-2 functioned in the same pathway with ASH-2 and WDR-5 in the AIB interneurons to regulate preference choice (Yu *et al.*, 2016).

sams-1 encodes the synthase of *S*-adenosylmethionine (SAM), the sole methyl donor. RNAi knockdown of *sams-1* resulted in the activation of innate immune genes (Ding *et al.*, 2015), suggesting that low SAM can activate or attenuate innate immune response. Infection response genes could not cause the increase in histone methylation marks in *sams-1* (*lof*) mutant nematodes after *Pseudomonas* infection, and low SAM restricted H3K4me3, thereby limiting their expression in *sams-1* mutant nematodes (Ding *et al.*, 2015). The H3K4 modification occurs through the action of methyltransferases with functions in COMPASS methyltransferase complex (such as SET-2 and SET-16), and the genes requiring *set-16*/MLL-dependent methylation were preferentially affected by SAM depletion during the transcriptional response to pathogen infection (Ding *et al.*, 2015).

Methylation of histone H3K9

As discussed above, nuclear co-factor LIN-65 is a regulator for the activation of mt UPR response. The histone H3K9 is methylated sequentially by methyltransferases MET-2/SETDB1 in the cytoplasm and SET-25 in the nucleus (Towbin *et al.*, 2012). MET-2 was required for the LIN-65 nuclear accumulation induced by mt UPR response (Tian *et al.*, 2016). The full activation of mt UPR required di-methylation of histone H3K9, and the global chromatin

reorganization induced by mitochondrial stress was dependent on both MET-2 and LIN-65 (Tian *et al.*, 2016). Additionally, H3K9me2 was also required for mitochondrial stress-induced DVE-1 nuclear puncta formation during the mt UPR response (Tian *et al.*, 2016).

JMJD-1.2, a member of the KDM7 family, is a demethylase toward lysine residues on histone 3 (H3), and thus potentially controlled the level of H3 lysine 9, lysine 23, and lysine 27 di-methylation (H3K9/K23/K27me2) (Myers *et al.*, 2018). Mutation of *jmjd-1.2* induced a susceptibility to replication stress (Myers *et al.*, 2018). Moreover, both JMJD-1.2 and JMJD-3.1, two histone lysine demethylases, were also identified to be required for the induction of mt UPR response, since the reduction of function of JMJD-1.2 or JMJD-3.1 suppressed mt UPR induction (Merkwirth *et al.*, 2016).

Methylation of HIS-24K14

HIS-24 is a linker histone (H1) variant. Pathogen infection could increase the cellular levels of HIS-24K14me1, and HIS-24K14me1 localization was changed from being mostly nuclear to both nuclear and cytoplasmic in intestinal cells by pathogen infection (Studencka *et al.*, 2012). HPL-1 and HPL-2 are heterochromatin protein 1 (HP1)-like proteins. Moreover, HPL-1 could interact with HIS-24 monomethylated at lysine 14 (HIS-24K14me1), and HIS-24 recruited HPL proteins to the promoters of genes involved in the defense response against pathogen infection (Studencka *et al.*, 2012).

Methylated glycans

Tectonin 2 of the mushroom *Laccaria bicolor* (Lb-Tec2) was toxic for nematodes, and this toxicity formation depended on the binding to N-glycans (Wohlschlager *et al.*, 2014). Both Gram-negative bacterial agglutination and Lb-Tec2 depended on the recognition of methylated glycans (O-methylated mannose and fucose residues) as part of bacterial lipopolysaccharide (LPS) and cell-surface glycans in nematodes (Wohlschlager *et al.*, 2014). SAMT-1, a membrane transport protein for presumptive donor substrate of glycan methylation from cytoplasm to Golgi, was required for the regulation of Lb-Tec2 toxicity (Wohlschlager *et al.*, 2014).

Histone acetylation regulation

MYST family histone acetyltransferase complex

The MYST family histone acetyltransferase complex contains MYS-1 and TRR-1, and both of them could promote stress resistance and longevity (Ikeda *et al.*, 2017). Mutation of *mys-1* or *trr-1* suppressed the resistance of *daf-2* mutant nematodes to environmental stress (Ikeda *et al.*, 2017), suggesting that MYS-1 and TRR-1 act downstream of DAF-2 to regulate the toxicity of environmental toxicants or stresses. MYS-1 and TRR-1 could be further recruited to the promoter regions of *daf-16*, where they functioned in histone acetylation, including H4K16 acetylation (Ikeda *et al.*, 2017).

N-terminal acetyltransferase C (NAT) complex

natac-1 encodes an N-acetyltransferase, an auxiliary subunit of the N-terminal acetyltransferase C (NAT) complex. Loss-of-function mutations in *natac-1* caused resistance to a broad spectrum of stressors, including heat stress and oxidation stress (Warnhoff *et al.*, 2014). The mRNA levels of *natac-1* were suppressed by DAF-16 activity (Warnhoff *et al.*, 2014), implying that NATC-1 is a physiological target of DAF-16 and functions downstream of the insulin/IGF-1 signaling pathway to regulate the toxicity of environmental toxicants or stresses.

CBP-1

The sirtuin SIR-2.4 regulated DAF-16 acetylation indirectly by preventing acetyltransferase CBP-1-mediated acetylation under stress conditions (Chiang *et al.*, 2012). Thus, DAF-16 acetylation was modulated by the antagonistic activities of CBP-1 and SIR-2.4 (Chiang *et al.*, 2012).

MicroRNAs (miRNAs) regulation

In nematodes, dysregulated miRNAs could be detected in nematodes exposed to environmental toxicants or stresses. For example, using SOLiD sequencing technique, 31 dysregulated expressed miRNAs, including 23 upregulated

miRNAs and eight downregulated miRNAs, were identified in GO-exposed nematodes (Wu *et al.*, 2014c). Using the available mutants for the identified dysregulated miRNAs, it has been observed that mutation of *mir-244* or *mir-235* induced a susceptibility to GO toxicity in reducing lifespan and in enhancing aging-related phenotypes, and mutation of *mir-247/797*, *mir-73/74*, or *mir-231* induced a resistance to GO toxicity in reducing lifespan and in enhancing aging-related phenotypes (Wu *et al.*, 2014c). Using the SOLiD sequencing technique, seven dysregulated miRNAs (three upregulated miRNAs and four downregulated miRNAs) were identified in 100 nm nanopolystyrene (1 µg/l, a predicted environmental concentration) exposed nematodes (Qu *et al.*, 2019c). The more severe toxicity was observed in nanopolystyrene-exposed nematodes overexpressing *mir-76* or *mir-794* compared with nanopolystyrene-exposed wild-type nematodes, and overexpression of *mir-35*, *mir-38*, or *mir-354* suppressed the toxicity of nanopolystyrene (Qu *et al.*, 2019c), suggesting the involvement of *mir-35*, *mir-38*, *mir-76*, *mir-354*, and *mir-794* in regulating the response to nanopolystyrene.

In nematodes, the molecular basis for some miRNAs in regulating the toxicity of environmental toxicants or stresses has been determined. The molecular basis for *let-7* in the regulation of MWCNTs toxicity has been discussed above. Besides this, it was further found that *mir-35* acted in the intestine to regulate the response to nanopolystyrene by affecting the expression and functions of its target NDK-1, a homolog of NM23-H1 (Li *et al.*, 2020). An intestinal signaling cascade of *mir-35*-NDK-1-DAF-16/KSR-1/2 has been suggested to be required for the control of response to nanopolystyrene (Li *et al.*, 2020). Mutation of *sek-1* encoding a mitogen-activated protein kinase kinase (MAPKK) suppressed GO toxicity in *mir-73* mutant nematodes, which suggests the possible role of SEK-1 as the molecular target for *mir-73* in regulating GO toxicity (Zhao *et al.*, 2015a). *mir-84* and *mir-241* are members of the *let-7* family. SKN-1 was identified as a direct functional target of *let-7* miRNAs (*mir-84* and *mir-241*), and mutation of *skn-1* suppressed the resistance of *mir-84* or *mir-241* mutant nematodes to pathogen infection (Liu *et al.*, 2013). *mir-231* acted in the intestine to regulate GO toxicity by affecting the expression

and function of its downstream target of SMK-1, a protein that is orthologous to mammalian SMEK (suppressor of MEK null) proteins, and SMK-1 further acted upstream of DAF-16 to regulate GO toxicity (Yang *et al.*, 2016c). During *P. aeruginosa* PA14 infection, *mir-233* repressed the protein levels of endoplasmic reticulum Ca^{2+} -ATPase SCA-1 and activated XBP-1-mediated ER UPR response, and RNAi knockdown of *sca-1* suppressed the susceptibility of *mir-233* mutant nematodes to pathogen infection (Dai *et al.*, 2015). *mir-259* acted in the pharynx/intestinal valve to regulate MWCNT toxicity by affecting the expression and function of its target of RSKS-1, a putative ribosomal protein S6 kinase, and RSKS-1 further acted upstream of AAK-2 to regulate MWCNT toxicity (Zhuang *et al.*, 2016). *mir-355* acted downstream of PMK-1 to regulate the innate immune response to *P. aeruginosa* PA14 infection (Zhi *et al.*, 2017b). Meanwhile, *mir-355* acted upstream of DAF-16 and SKN-1 to regulate the innate immune response to *P. aeruginosa* PA14 infection (Zhi *et al.*, 2017b), suggesting that *mir-355* functions as an important link between the p38 MAPK signaling pathway and insulin signaling pathway in the regulation of innate immunity. The GO-exposed *cep-1(RNAi);mir-360(n4635)* showed the similar phenotype of germline apoptosis to that in GO-exposed *cep-1(RNAi)* nematodes (Zhao *et al.*, 2016c), implying the role of CEP-1 as the potential target for *mir-360* in regulating the reproductive toxicity of GO in inducing germline apoptosis. *mir-794* acted in the intestine to regulate nanopolystyrene toxicity by affecting the expression and functions of its multiple targets of DAF-16, SKN-1, and MDT-15 (Qiu *et al.*, 2020).

The mRNAs-miRNAs network can be further identified during the regulation of toxicity of environmental toxicants or stresses in nematodes. With GO as an example, an mRNAs-miRNAs network involved in the control of GO toxicity was suggested based on both the dysregulated mRNAs and the dysregulated miRNAs in GO-exposed nematodes (Zhao *et al.*, 2015a). Thus, in GO-exposed nematodes, certain miRNAs-mRNAs networks existed to regulate toxicity induction by influencing some important biological processes, such as oxidative stress, intestinal development and function, and defecation behavior (Zhao *et al.*, 2015a).

LncRNAs regulation

Long non-coding RNAs are normally defined as non-coding RNAs having at least 200 nucleotides. Exposure to certain environmental toxicants or stresses could dysregulate the expression of lncRNAs. Using Illumina HiSeq 2000 sequencing technique and following qRT-PCR confirmation, a total of 34 dysregulated expressed lncRNAs (30 downregulated lncRNAs and four upregulated lncRNAs) were identified in GO-exposed nematodes, and ten known lncRNAs, including nine downregulated lncRNAs (*linc-14*, *anr-24*, *linc-68*, *linc-103*, *linc-83*, *linc-24*, *anr-36*, *linc-5*, and *linc-37*) and one upregulated lncRNA (*linc-3*) (Wu *et al.*, 2016). In nematodes, RNAi knockdown of *linc-37* or *linc-14* could induce a resistance to GO toxicity (Wu *et al.*, 2016). Moreover, 37 lncRNAs were dysregulated by exposure to nanopolystyrene (1 $\mu\text{g/l}$) (Qu *et al.*, 2019d). Among these 37 dysregulated lncRNAs, 22 lncRNAs, including three known lncRNAs (*linc-7*, *linc-50*, and *linc-169*), were downregulated, and 15 lncRNAs, including five known lncRNAs (*linc-2*, *linc-9*, *linc-18*, *linc-32*, and *linc-61*), were upregulated (Qu *et al.*, 2019d). The qRT-PCR assay confirmed the decrease in expression of *linc-7*, *linc-50*, and *linc-169*, and the increase in expression of *linc-2*, *linc-9*, *linc-18*, *linc-32*, and *linc-61* in nanopolystyrene-exposed nematodes (Qu *et al.*, 2019d). Meanwhile, susceptibility was observed in nanopolystyrene-exposed *linc-2(RNAi)*, *linc-9(RNAi)*, or *linc-61(RNAi)* nematodes, and resistance was detected in nanopolystyrene-exposed *linc-18(RNAi)* or *linc-50(RNAi)* nematodes (Qu *et al.*, 2019d). These observations suggested two different responses to nanopolystyrene mediated by these five lncRNAs (*linc-2*, *linc-9*, *linc-61*, *linc-18*, and *linc-50*). On the one hand, alteration in *linc-18* mediated the toxicity induction of nanopolystyrene. On the other hand, alteration in *linc-2*, *linc-9*, *linc-50*, and *linc-61* expressions mediated a protective response for nematodes against nanopolystyrene toxicity.

During the control of GO toxicity, *linc-37* regulated GO toxicity by affecting the function of DAF-16 in the insulin signaling pathway (Wu *et al.*, 2016). Besides this, RNAi knockdown of *linc-37* could significantly affect the expression of *sod-1*, *sod-2*, *sod-3*, *sod-4*, *isp-1*, *clk-1*, *nhx-2*, *pkc-3*, *jkk-1*, *jnk-1*, *ced-3*, *ced-4*, *cep-1*, and *daf-16*

(Wu *et al.*, 2016), implying that *linc-37* may possibly regulate GO toxicity by targeting proteins required for the control of oxidative stress or intestinal development and function, or encoding JNK, insulin, or apoptosis and DNA damage-signaling pathways. Similarly, *linc-14* may also possibly regulate GO toxicity by targeting proteins required for the control of oxidative stress or intestinal development and function, or encoding JNK, insulin, or apoptosis and DNA damage-signaling pathways (Wu *et al.*, 2016).

Circular RNAs (circRNAs)

Using the Illumina HiSeq2500 sequencing technique, 43 dysregulated circRNAs were identified in GO (1 mg/l) exposed nematodes, and the 33 known dysregulated circRNAs are exon-shuffling-derived circRNAs (Shi *et al.*, 2019). Moreover, among the dysregulated circRNAs, RNAi knockdown of *circ_0000115*, *circ_0000247*, *circ_0000308*, or *circ_0000665* caused resistance of nematodes to GO toxicity, and RNAi knockdown of *circ_0000201* resulted in susceptibility of nematodes to GO toxicity (Shi *et al.*, 2019).

The most downregulated circRNA in nematodes exposed to GO is *circ_0000115* (Shi *et al.*, 2019). It was found that only RNAi knockdown of *circ_0000115* in the intestine or neurons could induce a resistance to GO toxicity (Shi *et al.*, 2019), suggesting that *circ_0000115* acted in the intestine and the neurons to regulate the response to GO. To identify the potential targets of *circ_0000115* during control of GO toxicity, an RNA pull-down experiment was performed to pull down proteins with or without the biotinylated probe of *circ_0000115* (Shi *et al.*, 2019). IFC-2, a protein present in the cytoplasm of intestinal cells, was identified as the downstream target of intestinal *circ_0000115* in regulating GO toxicity (Shi *et al.*, 2019).

Perspectives

This chapter has systematically introduced several important molecular signaling pathways involved in the control of toxicity of environmental toxicants or stresses (Table 10.1). These molecular signaling pathways are: (i) oxidative

stress-related molecular signals; (ii) MAPK signaling pathways; (iii) insulin and related signaling pathways; (iv) development-related signaling pathways; (v) cell death and DNA damage-related signaling pathways; and (vi) metabolism-related signaling pathways. These molecular signaling pathways have a crucial function in regulating the toxicity induction, as well as the response, in nematodes exposed to environmental toxicants or stresses. Nevertheless, more efforts are still needed to further clarify the detailed underlying mechanisms for these molecular signaling pathways in regulating the toxicity of environmental toxicants or stresses. With the insulin signaling pathway as an example, the detailed insulin signaling pathway involved in the control of toxicity from different environmental toxicants or stresses may be different. Among the large number of targeted genes (predicted) for *daf-16*, only a few genes have been proven to act as the downstream targeted genes for *daf-16* in regulating the toxicity of environmental toxicants or stresses. It is still unclear whether the rest of the predicted genes can also act as targeted genes for *daf-16* in the regulation of toxicity of environmental toxicants or stresses. The data obtained so far may further imply the formation of a large physical interaction surrounding DAF-16 in regulating the toxicity of environmental toxicants or stresses. The identification of exact scaffold molecules in this large complex may provide an important basis for further screening of related components and the thorough elucidation of the underlying mechanism for the insulin signaling pathway in regulating the toxicity of environmental toxicants or stresses.

In this chapter, we have also focused on several protective response-related signaling pathways to discuss their involvement in the control of toxicity of environmental toxicants or stresses (Table 10.1). Undoubtedly, more protective response-related signaling pathways will be identified as being involved in the control of toxicity of environmental toxicants or stresses, or to replace the importance of already identified protective response-related signaling pathways. Some new protective responses may be further formed during the evolution. Actually, after exposure to low-dose environmental toxicants or stresses, the alteration in at least the MAPK signaling pathway, insulin and related signaling

Table 10.1. Molecular signals involved in the regulation of response to environmental toxicants or stresses in *C. elegans*.

Molecule groups	Proteins	References
Mitochondrial complex I	GAS-1 and NUO-1	Ding <i>et al.</i> , 2018; Grad and Lemire, 2004
Mitochondrial complex II	MEV-1	Zhao <i>et al.</i> , 2017a
Mitochondrial complex III	ISP-1	Zhao <i>et al.</i> , 2017a; Wu <i>et al.</i> , 2013a; Wu <i>et al.</i> , 2014a; Liu <i>et al.</i> , 2015
Mitochondrial complex IV	W09C5.8 and Y37D8A.14	Suthammarak <i>et al.</i> , 2009
Superoxide dismutase (SOD)	SOD-1, SOD-2, SOD-3, SOD-4, and SOD-5	Roh <i>et al.</i> , 2009; Li, Y.-X. <i>et al.</i> , 2012; Rui <i>et al.</i> , 2013; Wu <i>et al.</i> , 2014b; Zhao <i>et al.</i> , 2017a; Wu <i>et al.</i> , 2017; Xiao <i>et al.</i> , 2018
Catalase proteins	CTL-1, CTL-2, and CTL-3	Song <i>et al.</i> , 2014; Han <i>et al.</i> , 2017; Wu <i>et al.</i> , 2017
Glutathione-requiring prostaglandin D synthases (GSTs)	GST-1, GST-4,	Helmcke and Aschner, 2010; Li, Q. <i>et al.</i> , 2012; Zhao <i>et al.</i> , 2017a
p38 MAPK signaling pathway	PMK-1, SEK-1, NSY-1, SKN-1, ATF-7, GST-4, and GCS-1	Zhao <i>et al.</i> , 2016b; Li <i>et al.</i> , 2018; Shakoor <i>et al.</i> , 2016; Papp <i>et al.</i> , 2012
JNK signaling pathway	MEK-1, JKK-1, JNK-1, and SNB-1	Zhao <i>et al.</i> , 2015a; Qu <i>et al.</i> , 2020a
ERK signaling pathway	MEK-2, MPK-1, LIN-45, PKC-1, NLG-1, SKN-1b, AEX-3, INS-4, INS-39, and DAF-28	Qu <i>et al.</i> , 2017; Qu <i>et al.</i> , 2020b; Chen <i>et al.</i> , 2017
Insulin and the related signaling pathway	DAF-2, AGE-1, AKT-1, AKT-2, DAF-16, SOD-3, MTL-1, MTL-2, NATC-1, HSF-1, and GPD-2	Zhao <i>et al.</i> , 2016a; Shao <i>et al.</i> , 2019; Ren <i>et al.</i> , 2017; Yang <i>et al.</i> , 2016a; Warnhoff <i>et al.</i> , 2014; Singh and Aballay, 2006
Wnt signaling pathway	CWN-1, LIN-44, CWN-2, BAR-1, DSH-1, DSH-2, MIG-1, MOM-5, CFZ-2, APR-1, GSK-3, HMP-2, POP-1, DAF-16, UNC-62, EGL-5, and PRX-5	Zhi <i>et al.</i> , 2016a; Zhi <i>et al.</i> , 2017a; Rui <i>et al.</i> , 2019; Ren <i>et al.</i> , 2018; Zhao <i>et al.</i> , 2020; Shao <i>et al.</i> , 2020
DBL-1-mediated TGF- β signaling pathway	DBL-1, SMA-6, SMA-2, SMA-3, and SMA-4	Mallo <i>et al.</i> , 2002; Partridge <i>et al.</i> , 2010; Savage-Dunn and Padgett, 2017
DAF-7-mediated TGF- β signaling pathway	DAF-7, DAF-8, and DAF-5	Kim <i>et al.</i> , 2017; Ren <i>et al.</i> , 2017
Notch signaling pathway	GLP-1	Wu <i>et al.</i> , 2015; TeKippe and Aballay, 2010
Developmental timing control-related signals	<i>let-7</i> , HBL-1, LIN-41, TIR-1, SYM-1, ALG-1, ALG-2, and SDZ-24	Zhao <i>et al.</i> , 2017b; Zhi <i>et al.</i> , 2017c
Apoptosis signaling pathway	CED-3, CED-4, CED-9, and SIR-2.1	Zhao <i>et al.</i> , 2016c; Aballay <i>et al.</i> , 2003; Greiss <i>et al.</i> , 2008
DNA damage signaling pathway	EGL-1, CEP-1, CLK-2, and HUS-1	Zhao <i>et al.</i> , 2016c
Fat metabolic sensors	SBP-1, NHR-49, MDT-15, and NHR-80	Lee <i>et al.</i> , 2015; Sim and Hibberd, 2016; Goh <i>et al.</i> , 2018; Yang <i>et al.</i> , 2020; Sinha and Rae, 2014
AMPK signaling pathway	AAK-2, LKB-1, and PAR-4	Lee <i>et al.</i> , 2008; Matsuyama <i>et al.</i> , 2014
ACC and FAS	POD-2 and FASN-1	Lee <i>et al.</i> , 2015
Saturated fatty acyl CoAs	FAT-6 and FAT-7	Horikawa and Sakamoto, 2009; Savory <i>et al.</i> , 2011

Continued

Table 10.1. Continued.

Molecule groups	Proteins	References
Fatty acid transport protein	ACS-22	Zhi <i>et al.</i> , 2016a; Qu <i>et al.</i> , 2018
Antimicrobial proteins	LYS-1, DOD-6, F55G11.4, LYS-8, and SPP-1	Ren <i>et al.</i> , 2017
Mitochondrial unfolded protein response (mt UPR)	HSP-6, HSP-60, ATFS-1, DVE-1, UBL-5, MET-2, LIN-65, JMJD-3.1, and JMJD-1.2	Pellegrino <i>et al.</i> , 2014; Liu <i>et al.</i> , 2019; Melber and Haynes, 2018; Nargund <i>et al.</i> , 2012; Haynes <i>et al.</i> , 2007
Endoplasmic reticulum (ER) UPR	HSP-4, IRE-1, PEK-1, ATF-6, XBP-1, and ABU-1	Qu <i>et al.</i> , 2019a; Bischof <i>et al.</i> , 2008; Calfon <i>et al.</i> , 2001; Urano <i>et al.</i> , 2002
Autophagy	LGG-1, LGG-2, BEC-1, UNC-51, HLH-30, and HPK-1	Samokhvalov <i>et al.</i> , 2008; Qu <i>et al.</i> , 2019b; Visvikis <i>et al.</i> , 2014; Das <i>et al.</i> , 2017
Mitophagy	PINK-1/PINK1 and PDR-1	Kirienko <i>et al.</i> , 2015
G-protein coupled receptors	DCAR-1, FSHR-1, NPR-1, NPR-4, NPR-9, NPR-12, and SRH-220	Zugasti <i>et al.</i> , 2014; Powell <i>et al.</i> , 2009; Styer <i>et al.</i> , 2008; Yu <i>et al.</i> , 2016; Yu <i>et al.</i> , 2018
Ion channels	TAX-2, TAX-4, CNG-3, UNC-2, KVS-1, EXL-1	Styer <i>et al.</i> , 2008; Cho <i>et al.</i> , 2004; Estevez <i>et al.</i> , 2004; Cai and Sesti, 2009; Liang <i>et al.</i> , 2017
Arrestin	ARR-1	Singh and Aballay, 2012
G proteins	EGL-30 and GOA-1	Lackner <i>et al.</i> , 1999; Kawli <i>et al.</i> , 2010; Los <i>et al.</i> , 2013
PLC-DAG-PKD signaling	DKF-1, PLC-1, and DKF-2	Najibi <i>et al.</i> , 2016; Ren <i>et al.</i> , 2009
Ca ²⁺ signaling	UNC-31 and CRT-1	Los <i>et al.</i> , 2013; Park <i>et al.</i> , 2001
Methylation of histone H3K4	ASH, WDR-5, SET-2, and SAMS-1	Yu <i>et al.</i> , 2016; Ding <i>et al.</i> , 2015
Methylation of histone H3K9	MET-2, JMJD-1.2, and JMJD-3.1	Tian <i>et al.</i> , 2016; Myers <i>et al.</i> , 2018; Merkwirth <i>et al.</i> , 2016
Methylation of HIS-24K14	HIS-24	Studencka <i>et al.</i> , 2012
Methylated glycans	SAMT-1	Wohlschlager <i>et al.</i> , 2014
MYST family histone acetyltransferase complex	MYS-1 and TRR-1	Ikeda <i>et al.</i> , 2017
N-terminal acetyltransferase C (NAT) complex	NATC-1	Warnhoff <i>et al.</i> , 2014
microRNAs	<i>mir-244</i> , <i>mir-23</i> , <i>mir-247</i> , <i>mir-73</i> , <i>mir-231</i> , <i>mir-35</i> , <i>mir-38</i> , <i>mir-76</i> , <i>mir-354</i> , <i>mir-794</i> , <i>let-7</i> , <i>mir-84</i> , <i>mir-241</i> , <i>mir-233</i> , <i>mir-259</i> , <i>mir-355</i> , and <i>mir-360</i>	Wu <i>et al.</i> , 2014c; Qu <i>et al.</i> , 2019c; Li <i>et al.</i> , 2020; Zhao <i>et al.</i> , 2015a; Liu <i>et al.</i> , 2013; Yang <i>et al.</i> , 2016c; Dai <i>et al.</i> , 2015; Zhuang <i>et al.</i> , 2016; Zhi <i>et al.</i> , 2017b; Zhao <i>et al.</i> , 2016c; Qiu <i>et al.</i> , 2020
Long non-coding RNAs	<i>linc-37</i> , <i>linc-14</i> , <i>linc-2</i> , <i>linc-9</i> , <i>linc-61</i> , <i>linc-18</i> , and <i>linc-50</i>	Wu <i>et al.</i> , 2016; Qu <i>et al.</i> , 2019d
Circular RNAs	<i>circ_0000115</i> , <i>circ_0000247</i> , <i>circ_0000308</i> , <i>circ_0000665</i> , and <i>circ_0000201</i>	Shi <i>et al.</i> , 2019

pathways, development-related signaling pathways, and metabolism-related signaling pathways may also mediate the protective response.

Additionally, some commonly shared molecular signals may exist for these protective responses for nematodes against environmental toxicants

or stresses. The detailed cross-talk events in sub-cellular compartments or certain tissues, as well as their possible communication between different cells or tissues, also require further elucidation.

Moreover, we have discussed the crucial roles of GPCRs and ion channels in regulating the toxicity induction of environmental toxicants or stresses (Table 10.1). Nevertheless, only very limited GPCRs and ion channels have been identified to be involved in the regulation of toxicity of environmental toxicants or stresses. Among the huge number of GPCRs that exist in the cells of nematodes, the exact functions for most of the GPCRs are still unknown even under normal conditions. For different environmental toxicants and stresses, we do not deny that they may activate some shared and conserved molecular responses mediated by GPCRs and ion channels. Nevertheless, a certain difference may exist for the molecular responses (mediated by

GPCRs and ion channels) to environmental toxicants compared with those to environmental stresses.

Finally, we have also focused on methylation, acetylation, miRNAs, lncRNAs, and circRNAs to discuss the involvement of epigenetic mechanisms in the regulation of toxicity of environmental toxicants or stresses (Table 10.1). The study of epigenetic regulation may help us identify more important signaling pathways involved in the regulation of toxicity of environmental toxicants or stresses. In fact, more convincing data are still needed to prove the possible or potential pivotal roles of different forms of epigenetic signals in regulating different biological processes. More importantly, the roles of epigenetic mechanisms in regulating the trans-generational toxicity of environmental toxicants or stresses and the underlying mechanisms are still largely unclear.

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11 Nematode Use for Testing Theoretical Models of Behavioral Ecology

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Introduction: Models of Behavioral Ecology

Behavioral ecology is the study of behavior in the context of fitness; such studies seek to understand why animals do what they do from an evolutionary standpoint. We have structured this chapter to address models in two different contexts. First, we have the use of nematodes as model organisms, which is the common thread of this volume. But second is the use of theoretical models in behavioral ecological research as vehicles to generate testable hypotheses. Thus, we will discuss how nematodes can be used as systems to test hypotheses generated by some of the central model constructs used in this line of research. There is a rich literature of examples of nematodes being used for this purpose, but we also see the striking diversity of life history parameters within the phylum as a significant strength when developing nematodes as useful tools that guide scientific inquiry.

We have divided the chapter into six sections, based on categories of animal activities: (i) resource acquisition; (ii) competition; (iii) communication; (iv) dispersal; (v) aging; and (vi) learning. Behavioral ecology can be divided into categories in numerous ways; these particular categories serve to fit nematode life histories. Other hierarchical systems describe behavioral

ecological thought structures. For example, the widely used text by Davies *et al.* (2012) includes categories that are relevant to birds and mammals, but not nematodes, such as “parental care” or “social behaviors”. Other categories used by Davies *et al.* (2012) may not seem relevant to nematodes at first glance but have been shown recently to have surprising relevance to nematodes, such as “cooperation” or “associative learning”. We address these categories here.

Caenorhabditis elegans is one of the “big six” models used in biology (others include bacteria, two yeast species, *Drosophila melanogaster*, and *Mus musculus*), and there are thousands of research groups worldwide working with this one species of worm (Matthews and Vosshall, 2020). Thus, it is not surprising that when a literature search is conducted using the terms “nematode” and “behavior”, almost 60% of the papers listed for 2020 focus on either *C. elegans*, other *Caenorhabditis* species, or another commonly used model group, *Pristionchus* spp. But there are practical reasons for studying the behavior of nematodes that are not models. If nematode species are categorized into five groups that include known models, herbivores, parasites of invertebrates, parasites of vertebrates, and free-living but not models, the distribution of 2020 papers found on the Web of Science database is shown in Fig. 11.1. Admittedly, this survey was not all-inclusive and

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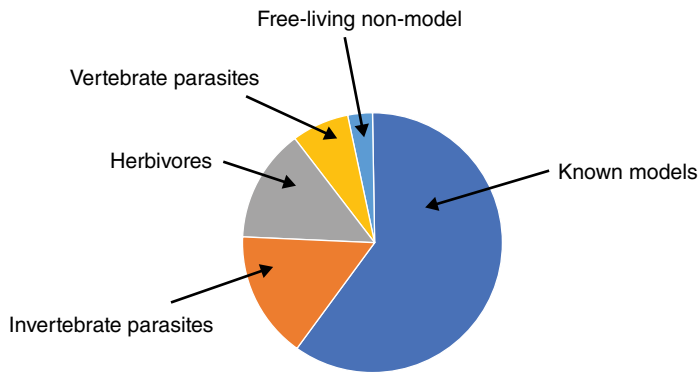


Fig. 11.1. Distribution of nematode groups represented in a keyword search conducted using the Web of Science database for 2020 (available at: <https://clarivate.com/products/web-of-science> accessed January 2021).

consisted of searching using the Web of Science. Most of the non-model nematode species are of interest due to their economic impact on public health or agriculture. For example, herbivorous nematodes cause an estimated US\$80 billion to US\$180 billion annual loss (management costs plus yield reduction) to crops (Bernard *et al.*, 2017). Obviously, understanding their behavior is essential to management of these pests in agricultural settings. Similar claims can be made for parasites, though some of the parasite species are beneficial (e.g., entomopathogenic nematodes). The overwhelming majority of citations for herbivores and parasites were for those associated with hosts that are economically important, but ignored parasites and herbivores associated with host species that were not. The slimmest portion of papers focus on free-living nematodes that are not known as “model systems”, but there are studies on these species as well.

The diversity of life history traits within the Nematoda suggests that there exist systems that would be ideal for testing any hypothesis generated using theoretical models of behavioral ecology. Free-living nematodes like *C. elegans* are powerful models for many biological systems. But this is not the only species to test hypotheses about behavioral ecology. There are well-known species with parasitic life histories, for example entomopathogenic nematodes, that can be used to learn about parasite behavior (Campbell *et al.*, 2003; Rengarajan and Hallem, 2016); there are predators that can illuminate predator/prey interactions, where the nematodes can function as predators (Sidhu and Kanwar, 2020) or prey

(Yang *et al.*, 2020); and herbivorous nematode species are ideal for studying competition (Mateille *et al.*, 2020). In this chapter, our goal is to provide the background for researchers to choose nematode species for testing hypotheses in behavioral ecology based on optimal life history traits.

Resource Acquisition

While free-living and parasitic nematodes that have been studied appear to respond strongly to sensory cues (Gang and Hallem, 2016), the specifics of responses vary across life history strategies. The similarities among these nematodes with respect to searching for food or hosts renders them useful model systems for understanding how nematode parasites of humans or livestock infect their hosts. For example, plant-parasitic, insect-parasitic, and mammal-parasitic nematodes all respond to carbon dioxide (CO_2). Despite this commonality, within at least one group (entomopathogenic nematodes) CO_2 occupies a different place in the hierarchical response framework between species that appear to differ in terms of foraging strategy, suggesting ecological complexity that has not been fully explored. Specifically, the ambush-foraging species *Steinernema carpocapsae* responds to CO_2 significantly more strongly after having contacted host cuticle, whereas the cruise-foraging congener *S. glaseri* utilizes CO_2 as a long-distance host-finding cue (Lewis *et al.*, 1992, 1993). This diversity of

responses among nematode species and life histories to a common and oft-encountered cue, such as CO₂, offers opportunities to compare information acquisition and decision-making capabilities between parasitic and free-living species.

Resource acquisition, as we consider it here, can generally be divided into several different components. For our purposes, we include foraging, recognition, and exploitation; resources we consider in this chapter include food, hosts (in the case of parasitic nematodes), or mates.

Foraging

Foraging is most simply defined as searching for resources. Behavioral ecology has focused a great deal on foraging, and many interrelated foraging theories have been (or could be) adapted to ask questions of different types of nematodes. These include optimal foraging theory (Pyke, 2019) along with related concepts such as the marginal value theorem (Charnov, 1976), and optimal diet theory models (Sih and Christensen, 2001). Optimal foraging theory predicts that in a patchy resource environment, and as a result of evolution by natural selection, animals should make decisions that maximize their net energy gain. In any particular situation, then, a researcher needs to identify the appropriate currency (generally energy captured through food) and constraints (energy and time used to capture, subdue, and handle food) to model the optimal foraging strategy. These currencies and constraints can be expected to vary significantly between free-living and parasitic nematodes. The goals of parasites and free-living nematodes can also differ. Adult free-living nematodes search for food as they develop, find mates (for some) and reproduce, whereas the infective stage of many endoparasitic nematodes search exclusively for a host. There are no competing goals for the latter parasites, thus they make an unusually simple model for those studying foraging.

Free-living nematodes that feed on bacteria, fungi, or soil fauna (including other nematodes) live in an environment that can be considered resource-rich, given common estimates of density for bacteria (10¹⁰ cells per gram of soil), fungi (10⁶), protozoa (10⁴), and nematodes (10²) (Raynaud

and Nunan, 2014; Weil and Brady, 2016). Regardless, consumption will likely deplete resources, or shift communities to less palatable species, resulting in an optimality cost. Many researchers have investigated foraging theory using the model nematode *C. elegans*, even identifying some of the underlying neuronal bases of foraging (Milward *et al.*, 2011) and the genetic basis of patch-leaving behavior (Bendesky *et al.*, 2011). While those are beyond the scope of this chapter, other researchers have used this model system to investigate multi-stage search behaviors such as the switch from local to long-distance (“global”) search patterns (Calhoun *et al.*, 2014), the existence of alternative foraging strategies (Greene *et al.*, 2016), and the effect of starvation on long-term foraging strategies (Pradhan *et al.*, 2019). Many of these approaches could be adapted easily to other free-living nematodes, provided that the culture of both the nematode and its food source can be easily maintained. Indeed, nematodes have also been included as a prey source in foraging studies: by using two different size classes of the nematode *Acrobeloides nanus*, Hohberg and Traunspurger (2009) showed that the predatory eutardigrade *Macrobiotus richtersi* optimized energy acquisition by more quickly abandoning partially consumed prey under high-density conditions, in accordance with predictions from the marginal value theorem.

In crowded environments, the marginal value theorem predicts that animals should leave a patch at a time that optimizes net energy gain per unit time. These and other optimal-foraging approaches would work well for most free-living nematodes, regardless of their prey source. A nematophagous nematode, for example, should leave a nematode-rich patch at a point when it has exploited the local nematode population to a point where the energy lost by traveling to a new patch will be offset by the increased energy gain in the new patch. While these approaches work well for nematodes that feed more or less continuously, they are not likely to work for endoparasitic nematodes, including parasites of plants, insects, and mammals. Many of these species have non-developmental and non-feeding infective stages that forage for a host and resume development once they invade a host. Subsequent generations emerge to forage again for a different host (or infect a single host at each stage, in the case of complex life histories

exhibited by vector-borne nematodes such as *Wuchereria bancrofti*). As a result, energy “gain” from multiple feeding events is not relevant, even though the nematode still experiences constraints resulting from travel and host invasion. Optimization of a behavior is based upon repeated actions, but infective-stage parasites invade a single host, so are not amenable to testing these theoretical underpinnings.

The trade-offs involved in optimal foraging have also been expanded to consider the constraint of risk, whether it be risk of reduced energy gain or predation. Risk-sensitive foraging theory predicts that most animals will avoid risk if they can do so and maintain optimal energy levels, and that animals will only become risk-prone if that is the only way to meet their net energy requirements (McNamara and Houston, 1992). Typically, assessments of risk-sensitive foraging contrast constant food availability with some sort of unpredictable food availability and examine how foraging decisions change. In the case of nematodes, the availability of cultures of various nematode predators or parasites may allow for an effective incorporation of risk of predation into these investigations. For example, using a combination of nematode-trapping fungi and/or predatory nematodes (e.g., the toothed variant of *Pristionchus pacificus*, see below) could make for interesting arenas within which to investigate risk-sensitive foraging in a bacterial-feeding species such as *C. elegans*.

Risk sensitivity has been employed with nematodes to describe the decision-making process of insect-parasitic nematodes when presented with a host. Nematodes in the genus *Steinernema* use a mass-attack strategy to overcome host defenses (Lewis *et al.*, 2006) because it takes more than an individual nematode to initiate an infection. There are two risks for invading nematodes: (i) if they are the first nematodes to invade a host, it is likely that the host immune system will impact their success negatively; and (ii) if they invade too late, the nutritional quality within the host will have declined to the point where the success of their progeny would be in doubt (Hsieh *et al.*, 2008). Thus, hosts that are recently infected would be the least risky, since their immune systems are compromised, there are potential mates present, and there is enough resource remaining to assure success of progeny. Hsieh *et al.* (2008) tested this hypothesis by

developing a model of invasion behavior based on empirical data, and it was supported.

Recognition

Building on the framework of optimal foraging theory, the recognition portion of resource acquisition suggests that organisms should be able to recognize signals or cues that indicate, and allow the organism to discriminate among, a variety of potential food sources, mates, or hosts.

C. elegans shows significant food choice preferences when those traits are examined. In laboratory trials, *C. elegans* prefers *Bacillus mycoides* and *Bacillus soli* to the OP50 strain of *Escherichia coli* (*E. coli* OP50 is the bacterial strain upon which most laboratory cultures of *C. elegans* are maintained); when these preferred bacteria were fed to wild-type laboratory strains of *C. elegans*, the result was extended lifespan, suggesting a fitness benefit associated with this recognition (Abada *et al.*, 2009). This adds a fitness dimension to research on dietary choice (Shtonda and Avery, 2006; Frey *et al.*, 2010). Similar research on the soil-dwelling bacterial-feeding nematodes *Mesorhabditis* sp. and *Acrobeloides* sp. showed that, when exposed to five different bacteria, the nematodes ingested bacteria with preferred characteristics, and when fed solely on that species they demonstrated higher rates of reproduction (Liu *et al.*, 2017).

Plant-parasitic nematodes respond strongly to root volatiles (reviewed in Sikder and Vestergård, 2019). Metabolites such as methyl salicylate (Murungi *et al.*, 2018), salicylic acid (Wuyts *et al.*, 2006), and dopamine (Wuyts *et al.*, 2006) are attractive, while those containing compounds such as dimethyl phthalate (Yang *et al.*, 2016) or thymol (Kihika *et al.*, 2017) were avoided. While studies such as these demonstrate that plant-feeding nematodes can discriminate among potential host species, the research to date has not indicated whether nematodes are choosing to infect species upon which the nematodes perform best. The fitness consequences of these foraging decisions may be a useful component to add to research on plant-parasitic nematode responses, particularly given the broad host range of generalist nematode parasites such as the southern root-knot nematode *Meloidogyne incognita*, contrasted with more specialized

nematodes such as *Globodera pallida*. In addition, the attraction to root-produced volatiles appears to transcend feeding groups, with both root-feeding and insect-parasitic nematodes attracted to some key components that are upregulated after insect attack (Ali *et al.*, 2011).

Omnivorous and predatory nematodes have been less well studied than many of these groups, but there are some examples of effective recognition. Lightfoot *et al.* (2019) identified peptides that promote self-recognition in *P. pacificus*; this sort of mechanism can distinguish between heterospecifics and even distinguish self-progeny from closely related strains. *P. pacificus* exhibits polyphenism, with one form specialized for feeding on bacteria and the other “toothed” form capable of feeding on nematodes. Given this polyphenism, *P. pacificus* may be an excellent model system for studying optimality models under differing environmental conditions, or even differing conditions of food availability and competition.

Entomopathogenic nematodes respond to a range of insect-associated and plant-produced compounds; models have assumed that the particular response for a species of entomopathogenic nematode is: (i) optimal for that species; and (ii) somehow tied to foraging strategy. Controlled laboratory trials with entomopathogenic nematodes have shown that even a ubiquitous cue such as CO₂ is filtered through the hierarchical response framework (or fixed action pattern) differently for different species (see the introduction to the Resource Acquisition section above). For example, species such as *S. carpocapsae* that have been described as “ambush” foragers due to their tail-standing habit respond significantly more strongly to CO₂ after having made contact with host cuticle, while broader-ranging “cruise” foragers respond to CO₂ prior to contacting a potential host. Entomopathogenic nematodes appear to respond most strongly to cues associated with “optimal” hosts that support maximum production of infective juveniles in future generations (Lewis *et al.*, 1996) (see below). This optimal decision making is to be expected, as a foraging infective-stage juvenile can only infect a single host and the fitness consequences for a poor decision would be significant. Similarly, entomopathogenic nematodes also respond to plant-associated compounds, including both induced compounds such as volatiles and root-cap exudates;

combining CO₂ with root volatiles appears to have synergistic effects, promoting more attraction than either of the two alone (Turlings *et al.*, 2012). To the extent these compounds are positively and consistently associated with increased probability of encountering a potential insect host, the assumption of optimality holds. That said, results seen in the laboratory may not always translate to soil systems, where there is an inherently more complex set of cues and conditions (Spence *et al.*, 2008).

There are many species of nematode parasites that infect vertebrates, but these are often limited to mouse models of human-parasitic nematodes. Studies of these species seldom address aspects of parasite biology and are limited to testing antihelminth treatments (Keiser *et al.*, 2016) or models of the human immune response to parasite invasion (Tao and Reese, 2017).

Exploitation

In the exploitation phase of resource acquisition, theory suggests that nematodes should optimize their exploitation of a resource once it has been found and recognized. What this looks like and how it could be tested will likely vary substantially among different nematode feeding strategies. This is essentially an application of the marginal value theorem, in which the currency (calories per unit time) and constraints (energy and travel time associated with the ability to leave the exploitation site) would need to be modeled mathematically to determine whether the organism in question is demonstrating what would be considered “optimal” exploitation behavior.

In general, endoparasitic nematodes (plant or animal) will not be useful for investigations of exploitation, as they resume development after penetrating the host and cannot leave after they have assessed and made the decision to infect. While this limits the value of studying exploitation in these species, the significance of the infection decision in the lifelong fitness of the nematode (a poor decision may result in death or profound fitness consequences) allows us to ask questions using endoparasites that may not be suitable for repeating feeders.

Ectoparasitic nematodes, such as the widespread plant-parasitic *Pratylenchus penetrans*, move between the host plant and the soil repeatedly

over the course of feeding and development. Given the necrotic lesions that result from *P. penetrans* infection, and the possibility of secondary infection that results, one might hypothesize that increased levels of feeding would result in decreased host root quality. This might be an excellent application of the marginal value theorem, as given the broad host range of *P. penetrans* (> 160 host species) (Hooker, 1981) and the extensive nature of most plant root systems, it may be advantageous for the nematode to recognize decreasing quality and move to either a different location in the root system or a different plant root system entirely. This suggests that an adaptation of the marginal value theorem may be appropriate in situations where the predator feeds on an organism that is orders of magnitude larger than itself—one might hypothesize that local degradation rather than overexploitation of the resource would trigger leaving a local area for a more optimal one.

Optimal strategies above and beyond resource acquisition: bet hedging and the preference–performance hypothesis

Additional models that fall under the broad umbrella of optimality sometimes focus on specific aspects of the life cycle of parasites. For example, Fenton and Hudson (2002) discussed at length the potential for “bet hedging” to heavily influence the approach of free-living stages of parasites. In these situations, from an evolutionary perspective, a species should employ a strategy that increases the probability of encountering a host, even under highly variable spatial or temporal conditions. The ultimate cost to a parasite that takes too long to infect a host is that it may run out of energy and die or become weakened with age to the extent that infection is not possible. High degrees of infectivity often result in a greater degree of energy expenditure, while the reduced energy expenditure associated with intermediate infectivity may be more capable of enduring periods of unpredictable host availability.

When the progeny of a parasite are likely to be faced with unpredictable host availability, the optimum strategy identified by Fenton and Hudson (2002) was to bet hedge by generating offspring with varying infection strategies.

Interestingly, the authors reported on the example of trematode cercariae that alternate between an intermediate host (snails) and humans. Even though all progeny produced from a single cercaria-infected snail are identical, due to asexual reproduction, there was still significant variance in the emergence from the intermediate host. This strongly suggests that bet hedging is a genotypic response to predictable variability in host presence. The logical extension from this framework is that the genotype codes for a temporal distribution of exploitation rates, much as what has been seen in some instances in entomopathogenic nematodes (Bohan and Hominick, 1996; Fairbairn *et al.*, 2000).

Another hypothesis that is relevant to nematodes is the preference–performance hypothesis (Jaenike, 1978; Valladares and Lawton, 1991). This theoretical framework has been applied to foliar insect parasitoids; it suggests that when the potential for relocation is limited due to low larval mobility, there is strong pressure on ovipositing females to be highly selective in their oviposition decisions. This can also be applied to individual foraging decisions, rather than just oviposition decisions, as organisms should make decisions with optimum consequences for individual fitness. This hypothesis has most often been applied to insects such as leaf miners, but a paper by Johnson *et al.* (2006) suggested that extending this paradigm to insects with a soil-dwelling larval stage was appropriate. While Johnson *et al.* (2006) suggested that insect larvae may be less capable of moving through soils than nematodes, the distance a plant-parasitic nematode can move throughout its larval stage(s) is certainly limited. In these cases, both maternal oviposition decisions and individual foraging decisions might be expected to have significant consequences for lifelong fitness.

Preference–performance optimization has also been used in investigations of entomopathogenic nematodes. For example, Lewis *et al.* (1996) examined the recognition response to contact with cuticle of putative hosts, as well as subsequent mortality in the hosts and nematode reproduction within the host. *Steinernema carpocapsae* infective juveniles were exposed to ten different species of arthropods from seven different orders; the degree of attraction of infective juveniles to these hosts was positively correlated with both host mortality and the level of reproduction

that the host supported. This sort of study design is useful for investigating preference–performance, as it assesses both attraction and multiple aspects of host preference (host mortality and entomopathogenic nematode reproduction).

Competition

Competition occurs when the use or defense of a resource by one individual reduces the availability of that resource to other individuals. There are two conditions that need to be met: (i) competitors must have the same needs regarding the resource; and (ii) that resource must be limiting. Nematodes as a group present many opportunities to test hypotheses of competition among individuals; there are myriad tractable systems where resource availability can limit individual success, and nematodes offer relatively simple ways to “keep score” of which individuals win. Competition is often divided in two ways: (i) interspecific versus interspecific competition; and (ii) exploitation (scramble) versus resource defense (interference) competition. Nematode systems provide ideal opportunities for testing hypotheses in all these relationships.

Using models to address hypotheses about competition is not new. Since Gause *et al.* (1934) described the principle of competitive exclusion using two species of *Paramecium*, there has been a long history of setting up competition experiments in artificial conditions to make predictions about the underpinnings and outcomes of competitive relationships. These models have trade-offs. Extremely artificial systems (such as *Paramecium* in a Petri dish) have the advantage of being amenable to direct observation of interactions and of outcomes, but one wonders how close predictions made based on these systems can be to nature. Other systems that are more realistic are more complicated, difficult to assess, and often are more expensive to develop. For example, birds in the genus *Periparus* have been used to test relationships between interspecific competition and species coexistence; there are several species that live together and their environment can be manipulated, but they cannot easily be subjects in laboratory tests. An advantage that nematodes may offer is that the same species, or group of species, can often be tested

using multiple scales that vary from Petri dish assays to small microcosms in the laboratory to large and complex field conditions.

Parasitic nematodes are particularly amenable to these sorts of scale-up testing. Plant-parasitic nematodes can provide examples of interspecific resource defense competition. Such studies have been conducted with the intent to measure the suitability of infected plants to other herbivores. These herbivore assemblages are not necessarily limited to nematodes; Kaplan *et al.* (2011) focused on the interaction between infection with *M. incognita* and the aphid *Mysus persicae* and found that, on tobacco plants, nematode infection reduced aphid performance, but the presence of aphids did not impact nematode performance. There are also examples of studies with the aim of understanding the impact of multiple species of nematodes on crop damage (Umesh *et al.*, 1994; Diez *et al.*, 2003; Garcia-Mendivil *et al.*, 2018), which, in addition to learning about herbivore competition, have applications to agriculture. What these studies have in common is the claim that herbivore interactions are mediated by the plant. Changes in plant chemistry induced by nematode infection can alter the acceptability and suitability of that plant as a host, which can be classified as resource defense by the first invaders. The ease of quantifying success is an excellent argument for using nematodes as models. Root-knot nematodes and cyst nematodes are both sedentary endoparasitic nematodes where mature females are relatively easy to find on plant roots and their eggs are obvious and easy to count. These are critical characteristics to determine “winners and losers” of a competitive interaction. There are thousands of examples of nematode community interactions in a single host. Most well-known examples have direct and important relevance to food production.

Entomopathogenic nematodes also offer systems amenable to studying competition in terms of intraspecific scramble competition. Here, adult parasitic male nematodes compete for access to females within the host. Nematodes in the genus *Steinernema* are obligate insect parasites and all but one species are amphimictic. The infective juvenile enters the host hemocoel and resumes development into an adult. Adult males engage in lethal fights over access to females (Zenner *et al.*, 2014). Males will attack males of different species and kill them; early-entering males reach adulthood

sooner than those entering later and enjoy the advantage of size. This situation lends itself to scenarios where competition occurs between species in terms of males for dominance within an infected host. Further, males will attack and kill conspecific males at a rate inversely proportional to their degree of familial relatedness (Kapranas *et al.*, 2015). This system has been developed as a powerful tool to study kin selection, sexual selection, and competition. The advantage from an experimental aspect is the ease with which hosts are infected. A challenge is the need to observe these interactions using a technique where hemolymph from a host is suspended beneath a microscope slide and nematodes are released into the hanging drop (see Gilarte *et al.*, 2015 for an explanation of the technique).

Communication and Dispersal

Communication among animals has been defined as “the action of or cue given by one organism [the sender] that is perceived by and thus alters the probability pattern of behavior in another organism [the receiver] in a fashion adaptive to either one or both of the participants” (Wilson, 1975). Pheromones (*pherein*, to transfer; *hormon*, to excite) are semiochemicals involved in intraspecific communication. The first pheromones were identified from insects, but communication systems based on pheromones have been described in mammals (including humans), fish, and birds. While insect pheromone systems are the most studied, pheromones impacting the behavior of nematodes have recently gained attention. Compared with insects, nematodes remain relative newcomers (8023 papers found on the Scopus database with the keywords “insect” AND “pheromone” versus 339 papers with the keywords “nematode” AND “pheromone”). Yet as the details of their communication systems become better understood, they will become useful tools because of their functional and ecological diversity, relatively simple biology and physiology, short generation time, and extensive knowledge of their genetics.

It can be argued that the study of nematode pheromones began when Butcher *et al.* (2007) identified ascarosides (small molecules derived

from dideoxysugar ascarylose) as key pheromones in the development of *C. elegans* dauer larvae. The structural and partially functional elucidation of these semiochemicals indeed facilitated research on specific receptors and physiological pathways as well as on the behavioral and developmental responses of this nematode species (Muirhead and Srinivasan, 2020). Ascarosides are evolutionarily conserved (Choe *et al.*, 2012b) and influence the behavior of nematodes across at least three functional groups (i.e., free-living, entomopathogenic, and plant-parasitic) (Kaplan *et al.*, 2012) yet not always to the same extent (Noguez *et al.*, 2012).

In addition to the development of dauer larvae in *C. elegans*, ascarosides profoundly influence nematode behavior. Social behavior of this species is also at least partially governed by ascarosides (comprehensively reviewed in: Butcher, 2017; Muirhead and Srinivasan, 2020). A highly diverse library of ascarosides is involved in several key steps in the life cycle of the free-living nematode *C. elegans*. To cite only two of them, *C. elegans* aggregates in response to ascarosides and indole ascarosides (Srinivasan *et al.*, 2012), and a blend of three ascarosides constitutes the mating signal in this same nematode species (Srinivasan *et al.*, 2008). Other free-living species employ complex sex pheromone signaling. For example, *Panagrellus redivivus* produces sex-specific attractants and repellents (Choe *et al.*, 2012a). *P. redivivus* females release the ascaroside ascr#1 to attract males and repel potentially competing females, whereas males of this species release the dihydroxy derivative dhas#18, which attracts females (Duggal, 1978; Choe *et al.*, 2012a).

Entomopathogenic nematode infective juveniles also respond to ascarosides. Noguez *et al.* (2012) demonstrated that infective juvenile formation in the host cadaver at high nematode density is due not only to lack of food (Johnigk and Ehlers, 1999) but also to the release of ascarosides preventing infective juvenile recovery to the next developmental stage and therefore leading to the accumulation of infective juveniles in the insect cadaver (Noguez *et al.*, 2012). At emergence from the insect cadavers, ascarosides also act as dispersal signals in various species (Kaplan *et al.*, 2012; Hartley *et al.*, 2019). Entomopathogenic nematode species produce pheromone blends comprising different ascarosides, but differing in their concentrations (Hartley

et al., 2019). Based on experimental data, Kaplan *et al.* (2020) developed a conceptual model of the role of ascarosides in three critical steps in entomopathogenic nematode infective juveniles: Exodus (emergence from host cadaver), Dispersal, and Forage. The pheromone blends involved in the Exodus vary among species but are not known to be species-specific; there is some cross-species activity of particular pheromones (Kaplan *et al.*, 2020). During the Exodus stage, infective juveniles respond to pheromones diffusing from the insect cadaver itself (Kaplan *et al.*, 2020). As they disperse (without finding a suitable host), this signal fades with the distance from the cadaver (Kaplan *et al.*, 2020). It remains unclear what pheromones are involved in the transition from dispersing to actually foraging (Kaplan *et al.*, 2020). The authors hypothesized that, similar to the juvenile hormone in insects, the presence of cadaver-derived pheromones prevents infective juveniles from organized foraging (Kaplan *et al.*, 2020). Yet, during the Forage step, it is hypothesized that infective juveniles aggregate, and that ascarosides are somehow involved (Shapiro-Ilan *et al.*, 2014; Kaplan *et al.*, 2020). The release of these pheromones might be triggered by environmental cues (e.g., host cues, plant cues) and such blends are expected to be rather species-specific to increase reproductive success (Kaplan *et al.*, 2020). The presence of leaders and followers in infective juvenile populations (Hsieh *et al.*, 2008; Bal *et al.*, 2014; Shapiro-Ilan *et al.*, 2014) could support this hypothesis, as pheromones have been used to boost entomopathogenic nematode efficacy (Oliveira-Hofman *et al.*, 2019).

Aging and Behavior

We include a section on aging because essentially all behavioral phenomena change to some degree as time passes. Specific examples of using nematodes as models in aging research are limited to instances where behavioral characteristics are central.

According to Arking (1999), aging is the time-independent series of cumulative, progressive, intrinsic, and deleterious functional and structural changes that begin to manifest at reproductive maturity and eventually culminate in death. A key aspect of the definition is the term “time-independent”. Aging is commonly

measured with a calendar (as do some of the studies discussed hereafter), yet various biological markers are more amenable to generalizations than chronological time. Lipid content or muscle tissue degradation are such markers for nematodes, and so are behavioral characters, such as movement, or decision making. Arking (1999) defined aging as beginning “at reproductive maturity,” but this presents special cases for some nematode life cycles, some of which include a non-developmental stage called the dauer stage, which forms due to various sorts of environmental cues. This juvenile stage is not reproductive, yet changes occurring in dauer juveniles are included here because of the pivotal importance of this developmental stage to nematode biology, and because the changes are cumulative, progressive, intrinsic, and deleterious.

Several biological markers change with age and were reviewed by Henderson *et al.* (2006). The morphology of nematodes changes as they age; older worms often display less defined body shapes and dark regions, and a closer look often reveals tissue degradation (e.g., Klass, 1977; Bolanowski *et al.*, 1981). There are also several physiological changes happening in aging nematodes (Henderson *et al.*, 2006). Here, we focus on the impact of aging on the mobility of free-living and parasitic nematodes as well as on the infectivity of parasitic nematodes.

Aging of free-living nematodes

The overwhelming majority of the published literature on aging in free-living nematodes focuses on *C. elegans* as a model, but this species was not even listed in the index of the first edition of the well-known reference book *Handbook of the Biology of Aging* in 1977. Most of the available literature focuses on physiological and genetic aspects of *C. elegans* aging (e.g., Henderson *et al.*, 2006) which are beyond the scope of this chapter and are detailed in Chapter 2.

Impact of aging on mobility and locomotion

In most models of behavioral ecology addressed here, the ability to move plays a role. Age-dependent

behavioral changes are intuitively apparent and manifest as a decrease in mobility and locomotion. For example, adult *C. elegans* gradually slow their movement and eventually cease moving (and defecating) (Bolanowski *et al.*, 1981). Hosono *et al.* (1980) showed in early work that the locomotory rate of *C. elegans* decreased quickly after egg-laying peaked, but there is significant variation in timing among individuals. Using the behavioral classification established by Hosono *et al.* (1980), Herndon *et al.* (2002) showed similar results, attributing this behavioral shift to deterioration of muscle tissues. They also found that predicting age-related behavior was more accurate when using behavioral classes than chronological age, supporting the utility of time independence, as stated in the Arking (1999) definition of aging. In addition to muscle deterioration, *C. elegans* mitochondrial activity is affected by aging whereas neurons remain fully functional (Wang *et al.*, 2019).

Aging of parasitic nematodes

Parasitic nematodes must adapt to at least two extremely different environments during their life cycle. Most species spend a portion of their lives as free-living nematodes, with very similar biology and behavior to fully free-living worms; additionally, they must interact parasitically with a host (plant or animal) to achieve development and reproduction. We would hypothesize that aging has very different effects on these different developmental stages (free-living versus parasitic). The free-living stages of parasitic nematodes are exposed to similar extrinsic factors causing mortality as free-living nematodes, so we would expect similar extrinsic control in the free-living stage.

An important difference between entirely free-living nematodes and parasitic nematodes is the potential maximum lifespan. The average lifespan of free-living nematodes at 20°C is about 62 days (± 15) whereas parasitic nematodes that successfully infect a host can live up to 947 days (± 297) (Gems, 2000). Several factors contribute to the evolution of increased longevity in parasitic nematodes, especially after infection of their host: (i) assured nutrient supply from the host; (ii) decreased interspecific competition; (iii) relatively protected nature of the host environment

(for example, protected from environmental disturbance, pathogens, predators, variability, and conspecifics); and (iv) longevity of the host itself (Gems, 2000). Conditions inside hosts do relieve some sources of extrinsic mortality in parasitic-stage nematodes, but not all (see section on Competition).

In entomopathogenic nematodes, the infective stage is the only free-living stage (Gaugler *et al.*, 1997). These so-called infective juveniles are analogous to the dauer juveniles of *C. elegans*. The main difference between these two groups is that, in entomopathogenic nematodes, infective juveniles must retain their ability to infect a host. An additional consideration is that this infective juvenile stage is non-developmental, non-feeding, and not reproductively mature; hence, to model effects, the phrase “aging” must be applied with careful consideration of alternate (non-calendar-based) assessments.

This ability to find and infect a host is to some extent related to their foraging strategies; research to date has indicated that aging affects species differently, and that some of these effects may vary based on foraging behavior. Foraging behavior in entomopathogenic nematode infective juveniles is considered to distribute along a continuum spanning from “ambushers,” rather sessile in soil, to “cruisers,” which seek hosts by moving through the soil (Campbell and Gaugler, 1993). Most species are distributed along this behavioral continuum, with most displaying both ambusher and cruiser behaviors, according to abiotic and biotic conditions.

Different species demonstrate different patterns in activity as the time period they have spent outside of their natal host increases (i.e., as they “age” as infective juveniles). Lewis *et al.* (1997) demonstrated that soon after emergence from their hosts, *S. carpocapsae* spent most of their time standing on their tail, a typical behavior of ambusher nematodes. Two to four weeks after emergence, a behavioral shift began, as infective juveniles decreased their standing rate to adopt a more motile behavior (Lewis *et al.*, 1997). The success rate of ambushing *S. carpocapsae* decreased with age, and their ability to stand on their tails also diminished (Lewis *et al.*, 1997). The typically cruising entomopathogenic nematode *Heterorhabditis megidis* foraged with a different temporal pattern; when released on an agar plate, they moved vigorously when

1.5 weeks old, remained mostly stationary from 2.5 to 3.5 weeks old, and again moved vigorously at 4.5 weeks old (Boff and Smits, 2001). Within the same genus, the cruiser *Heterorhabditis bacteriophora* decreased forward crawling by almost 50% after 21 days, shifting to backward crawling and curling thereafter (Brown *et al.*, 2002). Pausing and head waving, theoretical indicators of searching intensity, did not change with age (Brown *et al.*, 2002). Lipid reserves, the only source of energy for the non-feeding infective juvenile stage of entomopathogenic nematodes, declined with age, and were correlated with mobility decline (Brown *et al.*, 2002). Thus, comparing free-living with parasitic life histories with respect to behavioral changes with age can be accomplished using closely related nematode species (*C. elegans* and *Heterorhabditis* spp. are in the same order).

Impact of aging on infectivity in parasitic nematodes

Most parasitic nematodes with active infective stages experience a gradual reduction of infectivity, regardless of whether their hosts are plants or animals. Because of their commercial nature, several studies on the impact of aging (i.e., shelf life) on entomopathogenic nematode infectivity have been conducted. Incorporating these studies into models of aging may (or may not) be complicated by the applied nature of these assessments, as results in the laboratory often fail to translate into more natural conditions (Spence *et al.*, 2008).

The impact of aging on infectivity (e.g., Selvan *et al.*, 1993) as well as the time required to kill their insect host (Katti *et al.*, 2006) varies across entomopathogenic nematode species. The time it takes to kill a host can be pivotal when looking at models of interspecific competition, which is altered by aging (Aalders *et al.*, 2017). Several approaches to reduce the impact of aging on the infectivity of entomopathogenic nematodes have been tested over time. Breeding (e.g., Sumaya *et al.*, 2018) or specific storage formulations (e.g., Grewal, 2000a,b; Ruiz-Vega *et al.*, 2018) have shown the most promising results. One interesting, but complicating, characteristic of entomopathogenic nematodes is their symbiotic

relationship with entomopathogenic bacteria, because the bacteria cells play a role in the infection process and also age.

Strategies to delay the effects of aging

Some species of nematodes, in response to harsh conditions, have the ability to enter a dormant state that improves their chances of survival and therefore extends their lifespan (Evans and Womersley, 1980). Nematodes can either enter diapause, a state that implies cessation of ontogenic development of the individuals, or quiescence, where ontogenic development continues but is slowed down because of reduced metabolic rate.

In entomopathogenic nematodes, quiescence of infective juveniles has been shown to enhance their shelf life (Hiltpold *et al.*, 2015; Jaffuel *et al.*, 2015) as well as to boost their infectivity after resuming activity (Hiltpold *et al.*, 2015). Diapause in the eggs of plant-parasitic nematodes can be triggered by host-plant factors (Evans, 1987). Conversely, root exudates from host plants also trigger egg hatching of plant-parasitic nematodes (e.g., Mizukubo and Oka, 2009). This intricate interaction with roots and root signals allows plant-pathogenic nematodes to synchronize perfectly with host availability and phenology.

Learning

We address learning in this chapter because the degree to which learning takes place in nematodes is usually measured by behavioral responses to stimuli. Learning and memory are traits that were first attributed to nematodes in the 1980s. *C. elegans* has been used to link learning to physiological states in ways that would not be possible in more complicated animals. Habituation, the simplest example of learning, is a progressive decrease of behavioral response probability with repetition of a stimulus. This is followed by “dishabituation”, which is generally measured as the amount of time it takes to recover that original response. Habituation was described for *C. elegans* based on the nematode’s response to mechanosensory stimuli such as touch or striking a Petri dish that housed the worm (e.g., Rankin *et al.*, 1990). Often the response measured is a

reversal in a worm's direction of movement. Habituation itself is often used to measure other states of nematodes, such as how habituation changes with age. For example, Beck and Rankin (1993) did not see a change in the rates or magnitudes of habituation or dishabituation with age in *C. elegans* using the tap test. With more sophisticated tests, the responses of *C. elegans* to simple stimuli, like taps on the dish, have been shown to be flexible and more variable than originally thought, and are similar to optogenetic stimuli (Liu *et al.*, 2018). More recently, a long-term habituation response has been described in plant-parasitic nematodes in response to phytochemicals (Fleming *et al.*, 2017). Habituation to plant chemicals, in this case, is an adaptive response that allows the nematode to ignore stimuli that do not improve its chances of survival and reproduction. These simple assays have been used for decades to dissect the neurophysiological aspects of learning.

Features of associative learning have been described for *C. elegans*. Associative learning is generally defined by an organism's ability to perceive contingency relations between events and their environment. An example of an instantaneous behavioral response to previous conditioning is that of Pavlov's dogs, where the dogs recognized that the sound of a bell preceded the appearance of food, so they began to salivate (Pavlov, 1927). Aversive behaviors have been recorded in *C. elegans* (Zhang *et al.*, 2005), specifically in the ability to associate pathogenic odors with malaise, or a worm's version of "illness". The advantage conferred by such ability is that *C. elegans* can protect itself from pathogens by leaving a lawn of pathogenic bacteria before infection of intoxication. Because *C. elegans* feeds on bacteria, and the soil has bacteria suitable for food as well as pathogens, the benefit to *C. elegans* is obvious.

Associative learning may also provide an early warning system that, through memory, could allow nematodes to initiate physiological

or behavioral responses to existential threats based on experience (Eliezer *et al.*, 2019). These "memories" enable rapid and systemic protective responses that confer a fitness advantage when the worms are faced with various environmental hardships. The fact that these worms exhibit such behaviors enables detailed dissection of the physiological bases of learning that can be scaled to more complicated animals where this would be much more challenging. Eliezer *et al.* (2019) exposed *C. elegans* to isoamyl alcohol during periods of starvation, and found that after the worms had been reintroduced to food, the odor of isoamyl alcohol induced a withdrawal response whereas this same chemical caused untrained worms to "linger near the odor". This response was evoked 48 h after the initial training. The physiological bases of this response are beyond the scope of this chapter but using mutant worms that are defective for particular pathways enables understanding of these responses, heretofore impossible.

Conclusions

Nematodes offer some unusual advantages for behavioral studies. In general, they are small and easy to accommodate in laboratory conditions. Nematodes are also amenable to study in a range of conditions from extremely artificial, such as a Petri plate with agar, to field studies and everything in between. Perhaps one of the strongest arguments for studying nematode behavior to test general hypotheses is the ability to test species with wildly different life history traits in similar conditions. For example, free-living bacteriovores, insect parasites, and herbivores might be tested for their responses to various resources in very similar arenas. Thus, comparative studies of the evolution of parasitism, or host range expansion, could be studied in ways where data friction would be minimized, since similar data would be produced by all species involved.

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12 Entomopathogenic Nematodes as Models for Inundative Biological Control

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Introduction

Biological control (or biocontrol) is defined as the introduction or manipulation of natural enemies (parasites, parasitoids, or pathogens) to suppress pest populations (Debach, 1974; Grewal *et al.*, 2005). Approaches to biocontrol include classical, inoculative, inundative, and environmental manipulation (Dara *et al.*, 2018). **Classical biocontrol** (also called introduction establishment) consists of releasing exotic natural enemies into a new locality; the expectation is that the natural enemies will establish in the new ecosystem and reintroduction is not likely to be necessary. **Inoculative control** (or inoculative augmentation) is similar to classical biocontrol except one expects the introduced natural enemy population to recycle for several generations or seasons, at which time a reintroduction is necessary in order to continue suppressing the target pest. **Inundative biocontrol** is the addition of natural enemies to a system without the expectation of significant recycling; the expectation is that one will need to reapply the organism each year or each season. This short-term approach is often seen as akin to how chemical insecticides are used. **Environmental manipulation** (or conservation biocontrol) does not involve directly introducing the natural enemy population

to the system, but rather entails altering the environment to facilitate the natural enemies' preservation and growth. This chapter focuses on inundative biocontrol approaches.

A subcategory of biological control is microbial control. Microbial control is the management of pests with beneficial microorganisms, which may include bacteria, fungi, nematodes, protists, and viruses (Dara *et al.*, 2018). Depending on the host–pathogen system, microbial control can be accomplished using any of the biocontrol approaches (introduction, inoculative or inundative, or conservation), but many of the applications are implemented inundatively in a “biopesticide” approach. This chapter focuses on entomopathogenic nematodes as a model to study and improve inundative approaches to biocontrol. The chapter is reflective as a model for microbial control in particular but also can extend to other (non-microbial) biocontrol agents.

Basic biology and life cycle

Entomopathogenic nematodes (EPNs) are defined as insect-parasitic nematodes that are mutualistically associated with symbiotic bacteria; and all nematode life stages, except for the dauer stage (infective juvenile (IJ) stage), exist exclusively inside

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the host (Grewal *et al.*, 2005). Historically, EPNs have been considered to encompass only the families Steinernematidae and Heterorhabditidae. There have, however, been suggestions to expand the definition of EPN beyond these two families; Dillman *et al.* (2012) included some other insect-killing nematodes that are associated with bacteria, such as certain species in the genus *Oschelius*. Nonetheless, for the purposes of this chapter we will restrict our focus to the families Steinernematidae and Heterorhabditidae and their respective genera *Heterorhabditis* Poinar and *Steinernema* Travassos. We limit the chapter to these two genera because they are the only EPNs that have been substantially developed for commercial applications in inundative biocontrol approaches.

More than 115 species of heterorhabditids and steinernematids have been described to date (> 20 heterorhabditids and > 100 steinernematids), yet only about one dozen species have been incorporated into commercial applications (Shapiro-Ilan *et al.*, 2017; Bhat *et al.*, 2020). The nematodes kill insect hosts with the assistance of their symbiotic partners; *Heterorhabditis* spp. nematodes are associated with *Photorhabdus* spp. bacterial symbionts, and *Steinernema* spp. are associated with *Xenorhabdus* spp. bacteria (Bird and Akhurst, 1983; Poinar, 1990; Lewis and Clarke, 2012). Both nematode and bacterium contribute to the mutualistic partnership (Akhurst and Boemare, 1990; Forst and Clarke, 2002; Hazir *et al.*, 2003; Stock and Goodrich-Blair, 2008).

In nature, the EPN life cycle is carried out in a soil environment (Shapiro-Ilan *et al.*, 2018). The only free-living stage, the IJ, enters the insect hosts through natural openings (anus, mouth, or spiracles) or occasionally through the cuticle. The IJs release their symbiotic bacteria after entering the host's hemocoel. The invading IJs and their symbiotic bacteria release various virulence factors to overcome the host's immune system and cause death (Forst *et al.*, 1997; Simões *et al.*, 2000; Waterfield *et al.*, 2009; Jing *et al.*, 2010; Kenney *et al.*, 2021). Both nematodes and bacteria proliferate within the host, which dies due to septicemia or toxemia. Generally, the nematodes carry out two or more generations within the insect cadaver, but smaller hosts may only allow one generation. As nutrition in the host cadaver becomes depleted, the specialized IJ stage forms, and the IJs exit the cadaver to search for new hosts to infect. One difference between heterorhabditid and steinernematid life

cycles is that, in the former, all IJs molt into hermaphrodites in the first generation, and subsequent generations consist of males, females, and hermaphrodites (Koltai *et al.*, 1995); in contrast, steinernematids consist solely of amphimictic forms, i.e., males and females (with the exception of *Steinernema hermaphroditum*) (Griffin *et al.*, 2001). A generalized life cycle for EPNs is depicted in Fig. 12.1 (Shapiro-Ilan *et al.*, 2017).

Foraging behavior

A significant advantage in using EPNs for inundative biocontrol relative to other biopesticides is that the nematodes can actively disperse and find hosts. Within a common paradigm of EPN foraging strategies, host-seeking behavior exists as a continuum between ambushers and cruisers (Lewis *et al.*, 1992; Campbell and Lewis, 2002; Lewis, 2002; Shapiro-Ilan *et al.*, 2018). Ambushers (such as *Steinernema carpocapsae* and *S. scapterisci*) are typically characterized by a relatively low dispersal ability and a tendency to stay near the soil surface, where they may stand on their tails waiting for a host to pass. Cruisers (such as *Steinernema glaseri* and various heterorhabditids) are highly mobile and actively search for suitable hosts. However, many EPN species exhibit behavior that falls between the two extremes (ambusher and cruiser) and are considered intermediate foragers (examples include *Steinernema riobrave* and *S. feltiae*) (Campbell and Gaugler, 1997). Importantly, there are many examples in which cruiser or ambusher foragers have effectively controlled insect pests regardless of host mobility or location in the soil profile (Shapiro-Ilan *et al.*, 2014a; Wilson *et al.*, 2012). Additionally, research by Kruitbos *et al.* (2010) indicated that the environment can impact nematode foraging behavior. Nonetheless, a vast number of studies support the ambusher/cruiser model (Schroeder and Beavers, 1987; Lewis *et al.*, 1992; Grewal *et al.*, 1994a; Lewis, 2002; Lewis and Clarke, 2012).

Biocontrol production and application technology

For commercial biocontrol applications, EPNs are mass produced using *in vivo* or *in vitro*

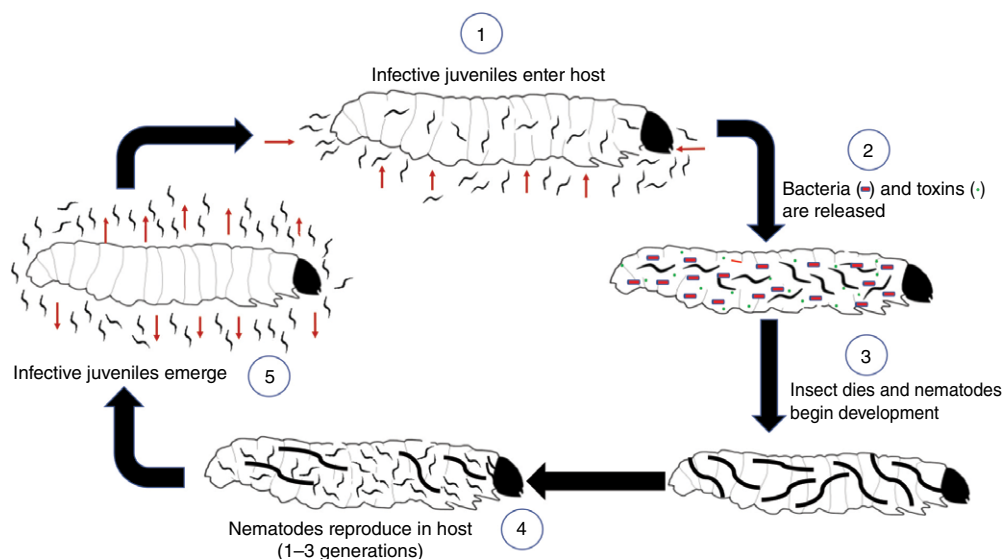


Fig. 12.1. A generalized life cycle of entomopathogenic nematodes. (Illustration by Selcuk Hazir, Aydin Adnan Menderes University, Turkey.)

approaches (Shapiro-Ilan *et al.*, 2012, 2014a). The bulk of current commercial production utilizes *in vitro* production, yet *in vivo* production continues to be employed by some relatively small-scale production companies. *In vitro* production, which consists of either solid or liquid fermentation, is generally more cost effective than *in vivo* approaches, and thus nematode products stemming from *in vitro* production are less expensive to end-users (Shapiro-Ilan *et al.*, 2012, 2014a).

To facilitate commercial biocontrol applications, EPNs are formulated to increase stability and ease of handling. EPN formulations for application are diverse and have included activated charcoal, alginate and polyacrylamide gels, clay, diatomaceous earth, paste, peat, sponge, vermiculite, and water-dispersible granules (WDGs) (Grewal, 2002; Shapiro-Ilan *et al.*, 2012; Guo *et al.*, 2017). Application is generally achieved in aqueous suspension, as EPNs are amenable to distribution using most types of agricultural equipment such as aerial sprayers, pressurized sprayers, mist blowers, and electrostatic sprayers, as well as various irrigation systems (Wright *et al.*, 2005; Shapiro-Ilan *et al.*, 2006, 2012, 2017). Other approaches for EPN application can include irrigation systems (e.g., microjet), trunk-sprayers, subsurface injection, or baits (Wright *et al.*, 2005; Shapiro-Ilan *et al.*, 2015a).

Justification of EPNs as a model system for inundative biocontrol

Several factors make EPNs excellent models for inundative biocontrol, including the various biological and commercial qualities that have facilitated their success, as well as the vast amount of research that has gone into optimizing EPN efficacy. Qualities that render EPNs attractive as biocontrol agents and contribute to their success include their host-finding capability, amenability to mass production and formulation, and fast-killing ability relative to other inundative microbial agents (e.g., entomopathogenic viruses and fungi) (Shapiro-Ilan *et al.*, 2017).

Another attractive quality is the EPN's versatility. As mentioned above, EPNs are applied using a wide variety of agricultural equipment and formulations. Moreover, unlike some microbial biopesticides, EPNs (with only a couple of exceptions) have a wide host range, with some species capable of infecting dozens of insect species across five or more orders (Poinar, 1979; Klein, 1990; Hajek and Shapiro-Ilan, 2018) (Table 12.1). Due to these attractive qualities, EPNs have been applied to control a broad array of economically important insect pests encompassing diverse crops (both annual and perennial) as well as urban and forest systems (Grewal *et al.*, 2005) (Table 12.1).

Table 12.1. Pests targeted commercially with entomopathogenic nematodes (EPNs).

Pest common name ^a	Pest scientific name	Key crop(s) targeted	Primary EPNs used
Artichoke plume moth	<i>Platyptilia carduidactyla</i> (Riley)	Artichoke	Sc
Armyworms	Lepidoptera: Noctuidae	Vegetables	Sc, Sf, Sr
Banana moth	<i>Opogona sachari</i> Bojer	Ornamentals	Hb, Sc
Banana root borer	<i>Cosmopolites sordidus</i> (Gemar)	Banana	Sc, Sf, Sg
Billbug	<i>Sphenophorus</i> spp. (Coleoptera: Curculionidae)	Turf	Hb, Sc
Black cutworm	<i>Agrotis ipsilon</i> (Hufnagel)	Turf, vegetables	Sc
Black vine weevil	<i>Otiorhynchus sulcatus</i> (F.)	Berries, ornamentals	Hb, Hd, Hm, Hmeg, Sc, Sg
Borers (e.g., peachtree borer)	<i>Synanthedon</i> spp. and other sesiids	Fruit trees and ornamentals	Hb, Sc, Sf
Citrus root weevil	<i>Pachnaeus</i> spp. (Coleoptera: Curculionidae)	Citrus, ornamentals	Sr, Hb
Codling moth	<i>Cydia pomonella</i> (L.)	Pome fruit	Sc, Sf
Corn earworm	<i>Helicoverpa zea</i> Boddie	Vegetables	Sc, Sf, Sr
Corn rootworm	<i>Diabrotica</i> spp.	Vegetables	Hb, Sc
Cranberry girdler	<i>Chrysoteuchia topiaria</i> (Zeller)	Cranberries	Sc
Crane fly	Diptera: Tipulidae	Turf	Sc
Diaprepes root weevil	<i>Diaprepes abbreviatus</i> (L.)	Citrus, ornamentals	Hb, Sr
False codling moth	<i>Thaumatotibia leucotreta</i> (Meyrick)	Citrus	Hb, Hz
Fungus gnats	Diptera: Sciaridae	Mushrooms, greenhouse	Sf, Hb
Iris borer	<i>Macronoctua onusta</i> Grote	Iris	Hb, Sc
Large pine weevil	<i>Hylobius albitis</i> (L.)	Forest plantings	Hd, Sc
Leaf miners	<i>Liriomyza</i> spp. (Diptera: Agromyzidae)	Vegetables, ornamentals	Sc, Sf
Mole crickets	<i>Scapteriscus</i> spp.	Turf	Sc, Sr, Sscap
Navel orangeworm	<i>Amyelois transitella</i> (Walker)	Nut and fruit trees	Sc
Plum curculio	<i>Conotrachelus nenuphar</i> (Herbst)	Fruit trees	Sr, Sf
Scarab grubs	Coleoptera: Scarabaeidae	Turf, ornamentals	Hb, Sc, Sg, Ss, Sk, Hz
Shore flies	<i>Scatella</i> spp.	Ornamentals	Sc, Sf
Strawberry root weevil	<i>Otiorhynchus ovatus</i> (L.)	Berries	Hm, Sc
Sweetpotato weevil	<i>Cylas formicarius</i> (Summers)	Sweet potato	Hb, Sc, Sf
Western flower thrips	<i>Frankliniella occidentalis</i> (Pergande)	Greenhouse, flowers	Sc, Sf

^aAt least one scientific paper reported $\geq 75\%$ suppression of these pests in the field; not meant to be an exhaustive list. Hb, *Heterorhabditis bacteriophora*; Hd, *H. downesi*; Hm, *H. marelata*; Hmeg, *H. megidis*; Hz, *H. zealandica*; Sc, *Steinernema carpocapsae*; Sf, *S. feltiae*; Sg, *S. glaseri*; Sk, *S. kushidai*; Sr, *S. riobrave*; Sscap, *S. scapterisci*; Ss, *S. scarabaei*.

Despite the level of success achieved with EPNs in inundative biocontrol, a number of biotic and abiotic factors have limited wider use (Shapiro-Ilan *et al.*, 2006, 2018). To overcome

these challenges, various approaches have been researched and implemented to improve biocontrol efficacy. The approaches used to improve EPN biocontrol provide a solid foundation for

developing solutions to increase efficacy in other biocontrol agents, and thus EPNs serve as a strong model in this fashion. In the next couple of sections, we explore the challenges that EPNs face in biocontrol and the methodologies developed to overcome these challenges.

Factors Affecting Efficacy

Efficacy of EPNs in inundative biocontrol has varied considerably (Shapiro-Ilan *et al.*, 2006, 2018). The various abiotic and biotic factors that affect EPN survival, dispersal, and infection underlie this variation. Abiotic factors consist of moisture levels, exposure to ultraviolet (UV) radiation, and temperature, as well as various physical parameters in the soil (see also Chapter 9, Stress and Survival Mechanisms). Biotic factors include EPN virulence and rate of application as well as interactions with other organisms.

Abiotic factors

Appropriate moisture/relative humidity levels are critical to achieving biocontrol efficacy when applying EPNs. Adequate moisture is required for EPN survival, dispersal, and infectivity (Kaya, 1990; Koppenhöfer *et al.*, 1995; Grant and Villani, 2003; Shapiro-Ilan *et al.*, 2006, 2012; Guyer *et al.*, 2018). Nematode infectivity is generally highest at intermediate soil moistures (−10 to −100 kPa), and lower in wet (−1 kPa) or moderately dry (−1000 kPa) soil (Koppenhöfer *et al.*, 1995). However, optimum soil moisture ranges for infectivity and survival vary among EPN species.

In certain cases, when EPN activity has been reduced due to low moisture levels, activity can be restored when moisture levels return to more suitable ranges (Glazer, 2002; Grant and Villani, 2003).

EPN survival, virulence, and reproduction can be negatively affected by UV radiation (Gaugler *et al.*, 1992; Fujiie and Yokoyama, 1998). Therefore, to avoid direct UV exposure, EPN applications to soil should ideally be made early in the morning or evening (Shapiro-Ilan *et al.*, 2012). Using ample irrigation to wash the IJs into the soil can also minimize effects of UV

radiation and reduces potential desiccation and exposure to high temperatures. UV tolerance varies considerably among EPN species and strains; for those species that have been characterized, *S. carpocapsae* appears to possess the highest level of UV tolerance (Shapiro-Ilan *et al.*, 2015b).

Temperature has a critical influence on EPN survival, infectivity, reproductive capacity, and efficacy (Kaya, 1990; Shapiro-Ilan *et al.*, 2012, 2014b). Extended exposure to high or low temperatures (> 40°C or < 0°C) is lethal to most EPN species (Brown and Gaugler, 1996). EPN species and strains vary in their optimum temperatures for survival, infection, and reproduction (Grewal *et al.*, 1994b; Hazir *et al.*, 2001; Karagoz *et al.*, 2009; Erbas *et al.*, 2014) and some may be considered cold- or heat-tolerant relative to other species. For example, some species that are considered relatively cold-tolerant (for example, efficacy is maintained at < 15°C), include *Heterorhabditis megidis*, *S. feltiae*, and *Heterorhabditis marelata* (Kung *et al.*, 1991; Grewal *et al.*, 1994b; Berry *et al.*, 1997; Hazir *et al.*, 2001). Nematodes that are relatively heat-tolerant (with efficacy still being achieved at > 30°C) include, for example, *Heterorhabditis indica*, *H. floridensis*, *S. glaseri*, *S. riobrave*, and *Steinernema siamkayai* (Ramalingam *et al.*, 2011; Shapiro-Ilan *et al.*, 2012, 2014b).

A number of other soil characteristics, beyond temperature and moisture, can impact EPN survival or efficacy. For example, soil texture can influence EPN efficacy (Kaya, 1990; Barbercheck, 1992). In general, soils with high clay content correlate with lower EPN efficacy, whereas sandy soils tend to be more conducive; nonetheless, exceptions to this general rule exist and EPNs have been efficacious in a wide variety of soil types (Georgis and Gaugler, 1991; Shapiro *et al.*, 2000; Blatt *et al.*, 2021). Generally, soil pH in the range of 4–8 does not have a substantial effect on EPN survival, but a pH ≥ 10 could have an adverse impact on survival and efficacy (Kung *et al.*, 1990).

Biotic factors

Arguably, the most important component of designing an effective inundative biocontrol

program is matching the appropriate EPN species or strain to the target pest. The selection should consider the biology and ecology of both the nematode and the target pest. Factors of importance include EPN virulence to the target pest, host-finding ability, and environmental tolerance. A high level of virulence is critical; if the nematode lacks a high level of virulence toward the target pest, successful biocontrol is unlikely. In some cases, however, a shortcoming in virulence may be overcome with persistence in the environment (Shapiro-Ilan *et al.*, 2002, 2006). Host-finding is also important; as indicated above, strategies of EPN host-finding are classified as a continuum between ambushers and cruiser type, and these behaviors may influence the choice of nematode to be used. Additionally, the EPN must be able to survive and infect under the environmental conditions of the target site; for example, temperature and soil characteristics must be suitable.

EPN application rate is critical to achieving biocontrol efficacy. A sufficient density of IJs is needed to allow for successful encounters with the target host. As a rule of thumb, for soil applications, a minimal application rate of 2.5×10^9 IJs/ha (25 IJs/cm²) is considered standard (Georgis *et al.*, 1995; Shapiro-Ilan *et al.*, 2002, 2017). Lower rates may be feasible when the host is particularly susceptible, or possibly in controlled environments (such as in greenhouses) (Levine and Oloumi-Sadeghi, 1992; Shapiro-Ilan *et al.*, 2002). In contrast, higher rates are required for pests that are innately less susceptible to EPNs, or that occupy cryptic habitats (McCoy *et al.*, 2000; Shapiro-Ilan *et al.*, 2002; Demir *et al.*, 2015).

Biotic agents in the soil can have positive, negative or neutral interactions with EPNs (Kaya, 2002; Gulcu *et al.*, 2012; Ulug *et al.*, 2014; Shapiro-Ilan *et al.*, 2017; Raja *et al.*, 2021). Examples of organisms that have a negative effect on EPN survival or efficacy include nematophagous fungi (Jaffee and Strong, 2005; El-Borai *et al.*, 2009, 2011) and certain mite species known to be effective predators of EPNs (Epsky *et al.*, 1988; Karagoz *et al.*, 2007; Ekmen *et al.*, 2010a,b; Cakmak *et al.*, 2013). Additionally, the host plant can impact efficacy via tri-trophic interactions (Hazir *et al.*, 2016).

When EPNs interact with other microbial biocontrol agents or biocontrol agents, the out-

come in terms of efficacy can be antagonistic, additive, or synergistic. Thus, EPNs can be purposefully combined with other biocontrol agents to achieve higher levels (i.e., synergistic) of biocontrol efficacy. Examples of synergistic interactions include EPNs combined with *Paenibacillus popilliae* (Thurston *et al.*, 1994), *Bacillus thuringiensis* (Koppenhöfer and Kaya, 1997), and the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium brunneum* (Ansari *et al.*, 2006; Wakil *et al.*, 2017). Antagonists include *B. bassiana* (Barbercheck and Kaya, 1990; Brinkman and Gardner, 2000) and *Isaria fumosorosea* (Shapiro-Ilan *et al.*, 2004). The nature of the interaction (antagonism, synergism, or additivity) depends on several factors, such as the nematode and host species as well as application rates and timing; note for example that *B. bassiana* has been observed as a synergist or as an antagonist, depending on the target host and other circumstances (Barbercheck and Kaya, 1990; Wakil *et al.*, 2017). Beneficial, negative, or neutral interactions between EPNs and other biocontrol agents also occur, such as with parasitic Hymenoptera (Lacey *et al.*, 2003; Everard *et al.*, 2009; Mbata and Shapiro-Ilan, 2010; Mbata *et al.*, 2019).

Approaches to Improve Efficacy

As indicated in the previous section, there are a number of factors that can hinder biocontrol efficacy. As a result, pest control efficacy using EPNs can vary greatly (Shapiro-Ilan *et al.*, 2002, 2018). However, there are a number of methods by which EPN efficacy can be improved. One component that can be improved is the organism itself, i.e., via EPN strain improvement. EPN strain improvement can be accomplished by discovering new strains with superior biocontrol traits or via genetic improvement programs. Genetic improvement can entail directed selection targeting specific traits, hybridization, or transgenic methods. EPN strain improvement is covered in depth in Chapter 14 of this volume and therefore we will not go into further detail on this subject. Rather, we will discuss other important approaches to improving EPN efficacy, including approaches to improve production, formulation, and application technology.

Improving production technology

Improvements in EPN mass production technology can result in enhanced cost efficiency and improved nematode quality (e.g., enhanced biocontrol traits). For *in vivo* production, the most important limitations are the cost of labor and insect hosts. These issues have been addressed by mechanizing the process, including insect production, inoculation, harvest, and packaging (Morales-Ramos *et al.*, 2011, 2013; Shapiro-Ilan *et al.*, 2016a). *In vitro* production can also be improved through optimization of media or fermentation parameters (Shapiro-Ilan *et al.*, 2014a; Leite *et al.*, 2016a,b, 2017).

Improving formulation and application technology

Due to EPN sensitivity to UV and desiccation, above-ground applications have been a major limiting factor in expanding EPN utility in biocontrol (Shapiro-Ilan *et al.*, 2018). Thus, recent research on developing protective formulations has led to substantial improvements in efficacy and opened new avenues for EPN application. For example, a sprayable gel, Barricade®, has been used in above-ground applications to protect EPNs from environmental stress. Barricade® is a fire gel that is commonly used for protecting houses or other structures from fire (Shapiro-Ilan *et al.*, 2010a), but was deemed to have potential for EPN use due to its high water-holding capacity. The gel was first used to facilitate improved *S. carpocapsae* applications for control of the lesser peachtree borer, *Synanthedon pictipes* (Shapiro-Ilan *et al.*, 2010a). Initially, the gel was applied after EPN application as topical protection. However, it was subsequently discovered that Barricade® could be applied as a tank mix (in a single application) and still provide protection to the EPNs (Antwi and Reddy, 2016; Dito *et al.*, 2016; Shapiro-Ilan *et al.*, 2016b). Barricade® can also be applied to soil with EPN applications; in this case the gel is used to retain soil moisture in lieu of irrigation (Shapiro-Ilan *et al.*, 2015a). Several other effective formulations have been developed for above-ground EPN application, such as those based on a surfactant and polymer mixture (Schroer and Ehlers,

2005), chitosan (Llàcer *et al.*, 2009), a wood flour foam (Lacey *et al.*, 2010), or other adjuvants (Van Niekerk and Malan, 2015).

As indicated above, interactions between EPNs and other biocontrol agents can be synergistic. Thus, these synergistic interactions can be optimized as a mechanism to improve EPN efficacy. The level of control and synergy will depend on the EPN and host species used, as well as timing and application rates. Synergy has also been documented with some chemical insecticides (Koppenhöfer and Grewal, 2005; Koppenhöfer and Fuzy, 2008; Morales-Rodriguez and Peck, 2009; Mbata and Shapiro-Ilan, 2013; Ozdemir *et al.*, 2021) and therefore such combinations can also be leveraged for improved efficacy.

Another potential avenue to enhance EPN application is application in EPN-infected hosts, also called the “cadaver approach” (Jansson *et al.*, 1993; Shapiro and Glazer, 1996; Dolinski *et al.*, 2015). In this approach, the infected host cadavers are applied to the target site and pest suppression is subsequently achieved upon emergence of the progeny IJs. Compared with the standard method of aqueous application, the cadaver approach has been shown to be superior in terms of EPN dispersal (Shapiro and Glazer, 1996), infectivity (Shapiro and Lewis, 1999), survival (Perez *et al.*, 2003), efficacy (Shapiro-Ilan *et al.*, 2003), and environmental tolerance (Gulzar *et al.*, 2020); on the other hand, some studies did not report a benefit in the cadaver approach (Bruck *et al.*, 2005; Ramalingam *et al.*, 2015). Protective formulations have been developed to protect the cadavers from rupturing or sticking together (Shapiro-Ilan *et al.*, 2001, 2010b; Del Valle *et al.*, 2009), and development of mechanized equipment for field distribution has been accomplished (Zhu *et al.*, 2011). Using a similar approach, pre-infected hosts (still living and mobile) can be used to carry the next generation of IJs to cryptic target sites (such as in wood galleries), and thereby facilitate enhanced control in locations that are hard to reach (Gumus *et al.*, 2015).

A relatively new and promising mechanism to enhance application efficacy is to leverage compounds produced by the host plant or the nematodes to direct EPN behavior. EPNs respond to certain plant compounds such as call-for-help signals that recruit EPNs when plants are under attack by phytophagous insects (Hiltbold and

Turlings, 2012). Possibly, these plant compounds can be used to draw EPNs to target sites. EPNs can “learn” to respond better to particular chemicals upon exposure, which may also enhance the potential to use such compounds to improve insect control with EPNs (Willett *et al.*, 2018). In another example, volatile compounds 1-octen-3-ol and 3-octanone from the entomopathogenic fungi *M. brunneum* significantly attract EPNs and thus may be manipulated to impact efficacy (Humjadi *et al.*, 2021).

Another set of compounds that directly influence EPN behavior is ascaroside pheromones (Kaplan *et al.*, 2012). Ascarosides impact EPN dispersal and infectivity and thus can be used to enhance biocontrol efficacy (Oliveira-Hofman *et al.*, 2019; Shapiro-Ilan *et al.*, 2019). For example, in laboratory and greenhouse experiments, pheromone extracts have improved biocontrol of pecan weevil, *Curculio caryae*, and black soldier fly, *Hermetia illucens* (Oliveira-Hofman *et al.*, 2019). The concept has yet to be proven under field conditions.

Biocontrol in Practice: Successes and Failures

In terms of achieving high levels of efficacy and grower implementation, application of EPNs for inundative biocontrol has resulted in numerous success stories as well as many failures (Shapiro-Ilan *et al.*, 2002, 2018). Success versus failure depends largely on the abiotic and biotic factors discussed above, but other issues such as economics also play a strong role. If competing tactics (such as chemical insecticides) are significantly less expensive, or EPN application is simply cost-prohibitive, then the biocontrol strategy will not be implemented. Therefore, approaches that encompass mechanisms to reduce cost, such as a reduced target area or reduced rates, are more likely to succeed. Overall, high-value crops with conducive abiotic parameters, such as irrigated perennial systems with natural shading, are more attractive for biocontrol with EPNs than, for example, non-irrigated low-value row crops with full direct sun. A full treatment of all the successes and failures is well beyond the scope of this chapter; below we provide just a few examples to illustrate the issues involved.

Failures in EPN application are often due to poor economics or inability to achieve sufficient efficacy. In the USA, high levels of efficacy have been achieved against a major corn pest, western corn rootworm, *Diabrotica virgifera virgifera* (Jackson and Hesler, 1995), but the tactic has yet to be implemented, given the cost relative to the value of the crop and competing tactics. However, high efficacy and implementation to control against *D. virgifera virgifera* have succeeded in Europe (Toepfer *et al.*, 2010; E-nema, 2020), presumably due to stricter regulatory policies on the use of chemical insecticides (thus such regulatory factors can also be important). Control of wireworms (Coleoptera: Elateridae) with EPNs faces dual challenges of low virulence and economics (many applications would be in low-value crops) (Eidt and Thurston, 1995; Sandhi *et al.*, 2020a,b). However, despite the lack of success in implementing EPNs for control of wireworms or *D. virgifera virgifera* in the USA, research is underway to improve the system, such as by exploring more persistent EPN strains, or applying EPNs as a component of an integrated pest management (IPM) system rather than a stand-alone tactic. Such approaches and others as indicated above to improve efficacy could turn a failure into a success.

A major success story in EPN biocontrol is the targeting of citrus root weevils, particularly *Diaprepes abbreviatus* (Shapiro-Ilan *et al.*, 2005). The adult insects feed on young leaves, and eggs are oviposited on leaf surfaces. Neonates fall to the soil, where they develop to adults; the larvae feed on the roots of citrus trees, causing major damage. After screening a number of EPN species, it was found that *S. riobrave* is the most efficacious and can result in > 90% control of *D. abbreviatus* (Duncan and McCoy, 1996; Shapiro and McCoy, 2000a,b). EPNs have been used commercially against *D. abbreviatus* for more than 20 years. Factors that have contributed to this success include: (i) a relatively high-value crop that is well irrigated and provides shade; (ii) a targeted area for application (EPNs only need to be applied to the understory and thus row middles are not sprayed, thereby substantially reducing the total cost per ha); (iii) once the weevil larvae are established in the soil there are no registered chemical insecticides that can be used effectively; and (iv) a high level of efficacy.

The peachtree borer, *Synanthedon exitiosa*, is another success story in that high levels of efficacy have been achieved and other parameters for widespread implementation are in place. *S. exitiosa* is an important pest of *Prunus* spp., including peach (Horton and Johnson, 2005). Moths emerge and mate during late summer and early fall, and mated females oviposit on or near the bark of host plants. Larvae bore into roots, where they will continue to feed and cause major damage. Numerous field applications have indicated that *S. carpocapsae* results in > 90% control when applied in the late summer or fall in prophylactic applications, which is similar to the timing of chemical insecticide applications; moreover, the level of control achieved by *S. carpocapsae* in prophylactic applications has consistently been equivalent to the standard recommended pesticide, chlorpyrifos (Shapiro-Ilan *et al.*, 2009, 2015a). Additionally, *S. carpocapsae* can achieve high levels of control (> 90%) when applied curatively in the spring, whereas spring applications of chlorpyrifos are ineffective (Shapiro-Ilan *et al.*, 2016b). Given that only the area around the base of each tree needs to be treated to control *S. exitiosa*, the amount of nematodes needed per hectare is greatly reduced relative to applications where the entire acreage must be treated. Thus, the cost is greatly reduced and not excessively more expensive than chlorpyrifos; currently the cost of chlorpyrifos is approximately US\$12.5/ha, and the cost of applying EPNs for *S. exitiosa* can be as low as US\$25/ha (treating the entire acreage with EPNs covering all space would be US\$250/ha, or more). Commercial application for control of *S. exitiosa* is still relatively low though adoption of the technology has been initiated. An aspect that is likely to assist wider-spread implementation is education and extension.

Conclusion

Due to their many attractive qualities, EPNs have been developed into commercially viable inundative biocontrol agents. As indicated, the processes for EPN production, application, and improvement can serve as a model for developing other organisms for inundative biocontrol.

When initiating a new biocontrol program for a target pest, the concepts discussed above should be applied. The first step should be to determine the biocontrol organism that is the best match for the target pest. For EPNs or other inundative biocontrol agents, this can consist of virulence screening among strains and species under laboratory conditions. One can compare virulence within a microbial group or between groups (fungi, EPNs, virus, etc.) depending on what candidates exist. The choice of candidates may depend on prior literature concerning the target pest or related pests. Laboratory tests should attempt to mimic natural field conditions in whatever manner possible (soil type, moisture levels, temperature, etc.). Once several candidates have been identified, then greenhouse and/or field testing is in order so that the most appropriate biocontrol agent can be identified. Conceivably, the initial screening process will result in sufficient efficacy and a new inundative biocontrol program can be launched. If efficacy is not satisfactory, or other factors (such as economics) prevent adoption of the biocontrol approach, mechanisms to enhance efficacy and reduce costs should be implemented, such as improving the organisms, production, formulation, or application system.

Among EPNs, and other microbial control agents that are used inundatively, various factors have prevented these products from reaching broader markets. Knowledge on optimum use of existing biopesticide products is lacking in many arenas (for example, proper matching of pathogen and host, or ensuring suitable environmental requirements), therefore improved education of the end-user is required. Advancements in strain improvement, formulation, and application have substantially improved application efficacy. Nonetheless, additional research focused on lowering product costs, increasing availability, and improving efficacy will result in wider use of EPNs. Development of new target pests for biocontrol as well as discovery of new EPN strains and species will also lead to expanded use. Leveraging chemical signaling and booster agents is likely to be a prime area of research with fruitful results. With research and development in these areas, EPNs will continue to contribute to inundative biocontrol in various systems and serve as a model for other organisms as well.

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13 Parasitic Nematodes: Model Systems for Studying Parasitism and Pathogenesis

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Introduction: Origin of Parasitism in Nematoda

Fossil records and phylogenetic hypotheses

Nematodes are undoubtedly one of the most omnipresent life forms on earth and have conquered all ecological niches and lifestyles from free-living to parasitic (Bernard, 1992; Ettema, 1998; Lambshhead *et al.*, 2003; De Ley, 2006; Powers *et al.*, 2009; Blaxter and Denver, 2012). Approximately 25% of the described species in Nematoda are parasites of plants and animals, including humans. Parasitic taxa have been the most extensively studied, due to the damage they cause to crops, livestock, and humans and their relevance to agriculture and to veterinary and human medicine (Wallace, 1963; Norton, 1978; Zuckerman and Rohde, 1981; Poinar, 1983; Anderson, 2000a).

Estimation of the origin of parasitism in this phylum is quite challenging, as fossil records are quite rare and, when they are found, it is difficult to assign them to an extant group (Poinar, 2015). According to fossil records, the earliest parasite record dates from the Devonian period (396 Mya) and it is represented by *Palaeonema phyticum*, a plant-parasitic fossil species in

the Enoplea (Poinar *et al.*, 2008). Various nematode stages, including eggs, juveniles, and adults, were found in the stomatal chambers of a land plant from this geological period (Poinar, 2015).

With respect to animal parasites, Poinar (2011) suggested that animal parasitism probably arose in invertebrate marine hosts in the Cambrian and later evolved to terrestrial invertebrates by the Ordovician period. A mermithid nematode, *Cretacimermis libani*, found in the body cavity of an adult midge (Diptera: Chironomidae) in Cretaceous Lebanese amber, is the earliest insect parasite (Poinar, 1994).

The oldest vertebrate parasites can be tracked to the Triassic in coprolites of cynodonts (mammalian-like reptiles) in Brazil (Da Silva *et al.*, 2014; Hugot *et al.*, 2014). Eggs on an Ascarididae nematode (*Ascarites rufferi*) and a member of the Oxyuridae (*Paleoxyuris cockburni*) represent parasitic associations at the reptile–mammal transition point, some 240 Mya (Da Silva *et al.*, 2014; Hugot *et al.*, 2014). According to Poinar (2015), oxyurids were perhaps the first nematodes to form parasitic associations with terrestrial animals, starting with invertebrates and then reaching to vertebrate hosts. Although it is not known when oxyurids invaded insects, it can be speculated that the association could date to Silurian, based on the earliest fossils of their

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millipede hosts (Wilson and Anderson, 2004). The oldest filarial nematode, *Cascofilaria baltica*, was found in Baltic amber and in association with a blackfly that could have served as its vector (Poinar, 2011).

The evolutionary history of nematodes has been controversial for a long time but, with the rise of molecular tools, a reappraisal of the evolution in this phylum was achieved in the late 1990s and early 2000s (Blaxter *et al.*, 1998; Dorris *et al.*, 1999; Park *et al.*, 2011). The current consensus recognizes three major groups: (i) Enoplia, comprising mainly free-living nematodes; (ii) Dorylaimia, with free-living and some parasitic forms (such as Trichinellida, Mermithida, Muspiceida, and Dioctophymatida); and (iii) Chromadoria, which encompasses some free-living forms and many parasites (De Ley and Blaxter, 2004).

A few studies support the idea that free-living nematodes are the most ancestral group (De Ley and Blaxter, 2004; Blaxter and Koutsovoulos,

2015) and that parasitism evolved convergently and independently in multiple lineages, arising at least five different times in the evolutionary history of the phylum (Blaxter *et al.*, 1998; De Ley and Blaxter, 2004; Blaxter and Koutsovoulos, 2015). This clearly suggests that not only were nematodes successful in overcoming obstacles as parasites, multiple times, but parasitism was a successful lifestyle (Crook, 2014; Blaxter and Koutsovoulos, 2015).

According to Blaxter and Koutsovoulos (2015), plant parasitism evolved at least three times, whereas invertebrate and vertebrate animal parasitism may have evolved at least ten times and five times for each group, respectively. In spite of this information, the basal speciation of nematodes remains unresolved, as current phylogenetic hypotheses based on ribosomal or mitochondrial genes appear too limited and insufficient for the Enoplea (Blaxter and Koutsovoulos, 2015; Coghlan *et al.*, 2019) (Fig. 13.1).

				Type of parasitism:		
						
Nematodes	Chromadoria	Clade V	Diplogasteromorpha	×		
			Bunonematomorpha			
			Rhabditomorpha	×	×	
			Strongylida			
		Clade IV	Tylenchomorpha	×		×
			Panagrolaimomorpha	×	×	
			Cephalobomorpha	×		
			Drilonematomorpha	×		
		Clade III	Ascaridomorpha		×	
			Spiruromorpha		×	
			Rhigonematomorpha	×	×	
			Dracunculoidea		×	
			Gnathostomatomorpha		×	
			Oxyridomorpha	×	×	
		n.a	Brevibuccidae			
			Myolaimina			
			Teratocephalidae			
			Plectida			
			Araeolaimida			
			Monhysterida	×		
			Desmodorida			
			Chromadorida			
	Enoplia	Clade II	Enoplida	×		
			Triplonchida			×
	Dorylaimia	Clade I	Trichinellida		×	
			Dioctophymatida		×	
			Mononchida			
			Mermithida	×		
			Dorylaimida			×

Fig. 13.1. Summarized SSU phylogeny of Nematoda and types of parasitic lifestyles.

Evolutionary trajectories of nematode parasites and bacteria symbioses

Through their evolutionary trajectories, many parasitic nematodes developed relationships with bacteria spanning from fortuitous to obligate and from beneficial to antagonistic or detrimental (Stock and Goodrich-Blair, 2008). Many parasitic groups developed intimate relationships with bacteria. Currently, four families, Onchocercidae, Steinernematidae, Heterorhabditidae, and Tylenchidae, are known to have associations with bacterial symbionts (Poinar, 1990; Ghedin *et al.*, 2008; Stock and Goodrich-Blair, 2008; Haegeman *et al.*, 2009; Taylor *et al.*, 2010; Brown *et al.*, 2016). These associations are considered mutualistic, as both the nematode hosts and the bacterial symbionts benefit from each other. Numerous studies have shown that the bacterial partners play key roles in the fitness of their nematode hosts (Hoerauf *et al.*, 2001; Murfin *et al.*, 2012, 2015; Grote *et al.*, 2017; McMullen *et al.*, 2017). For example, the discovery of a mutually beneficial association between *Wolbachia* bacteria and filarial nematodes spurred new insights into the pathogenesis and treatment of filariasis (Hoerauf *et al.*, 2001; Foster *et al.*, 2004; Allen *et al.*, 2008; Taylor *et al.*, 2010). Similarly, the nematodes offer shelter and nutrition to their bacterial symbionts, making the relationship indispensable for each other (Bouchery *et al.*, 2013; Lefoulon *et al.*, 2016; Stock, 2019). For instance, the infective juvenile stage of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) harbor the bacterial symbionts in a specialized receptacle in their intestine, where they provide essential nutrients for their multiplication and survival outside the insect host (Flores-Lara *et al.*, 2007; Kim *et al.*, 2012). Since many mechanistic aspects of nematode–bacterium interactions are conserved, these symbiotic partnerships are remarkable models to investigate basic and applied concepts of host–symbiont, host–pathogen, and host–parasite interactions with a multidimensional perspective (see Chapter 12, this volume).

Animal and human parasites: filarial nematodes

Filarial nematodes, or filariae, are members of the Onchocercidae family in the order Spirurida.

These are worldwide parasitic nematodes characterized by a broad range of hosts: squamates, crocodilians, amphibians, mammals, and birds (Bain, 2005). This family comprises eight subfamilies with more than 80 described genera and several are associated with relevance to human and veterinary medicine (Bouchery *et al.*, 2013). Since the first attempt at Onchocercidae classification by Wehr (1935), a clear distinction has been described between the spirurid nematodes that lay eggs expelled in the environment and the onchocercid nematodes that lay microfilariae larvae ingested with the host blood or lymph by vector. Study of the evolution of filarial nematodes was challenging for a long time, due to the pronounced regression of morphological features resulting from a parasitic lifestyle, as well as the absence of fossilized material (Chabaud and Bain, 1994). The classification of the Onchocercidae family was reconstructed several times until a more updated classification by Anderson and Bain (1976). The expansion of the filarial nematodes has been described as occurring during the Tertiary, based on their hosts groups (Chabaud and Bain, 1994).

With the rise of molecular techniques, molecular analyses of filarial nematodes has been proposed but for a long time biological material has been scarce and the markers used (mainly 12S rDNA and *coxI* genes) were not suitable to resolve the internal nodes describing the evolution of filarial nematodes (Ferri *et al.*, 2011). Among the nematode phylum, phylogeny based on 18S rRNA markers presents the filarial nematode belonging to Clade III grouped with the parasitic nematode orders Oxyurida, Rhigonematida, and Ascaridida (Blaxter *et al.*, 1998). More recently, multilocus phylogenetic analysis of filarial nematodes supports delimitation of four subfamilies suggested as ancestrally derived (Oswaldofilarinae, Waltonellinae, Icosiellinae, and Setariinae) but with doubt about the delimitation of three subfamilies (Splendidofilarinae, Dirofilarinae, and Onchocercinae) suggested as more recently derived and containing most of the agents of human and veterinary diseases (Lefoulon *et al.*, 2015).

Many filarial nematodes harbor *Wolbachia*, a Gram-negative intracellular α -proteobacterium (Rickettsiales). *Wolbachia* bacteria have been identified in three of the eight subfamilies of Onchocercidae: Onchocercinae, Dirofilarinae, and

Splendidofilariinae (Casiraghi *et al.*, 2004; Bain *et al.*, 2008; Lefoulon *et al.*, 2016). A few decades ago, it was hypothesized that *Wolbachia* infection was acquired as a single event in Onchocercidae (Bandi *et al.*, 1998; Casiraghi *et al.*, 2004), and that there was a congruency between the phylogenies of *Wolbachia* and the filarial host (Bandi *et al.*, 1998). However, more recent copylogenetic analyses have challenged these hypotheses and have shown evidence that the bacteria were acquired and lost multiple times in their evolutionary trajectory (Ferri *et al.*, 2011; Lefoulon *et al.*, 2016, 2020). Furthermore, recent studies have shown that not all filarial nematodes have *Wolbachia* endosymbionts and that approximately half of the studied filarid species lack symbionts (Ferri *et al.*, 2011; Lefoulon *et al.*, 2016). Genomics analyses of filarial nematodes identified as lacking *Wolbachia*, such as *Onchocerca flexuosa* and *Acanthocheilonema viteae*, have revealed the presence of *Wolbachia* sequences inserted into the nematodes' nuclear genome, suggesting that they may have lost their association during their evolution (McNulty *et al.*, 2010).

Insect parasites: entomopathogenic nematodes

Members in the Steinernematidae and Heterorhabditidae share many similarities in their life histories, morphological and ecological traits, and their symbiosis with enterobacteria. However, these similarities are considered the result of convergent evolution (Poinar, 1993). *Heterorhabditis* probably arose from a marine rhabditid such as *Pellioiditis marina* or a precursor that fed on free-living bacteria (Poinar, 1993). *Steinernema* shows no evidence of past association with a marine environment and, according to Poinar (1993), it probably evolved from a terrestrial proto-*Rhabditophanes* line which also gave rise to other insect parasites. With respect to their bacterial symbionts, both groups are quite distinct from each other, and the similarities observed are probably the results of convergent life histories (Poinar, 1993; Boemare, 2002).

Fossil records for EPNs are difficult to find because most infections occur in terrestrial larvae of holometabolous insects which infrequently turn up as fossils, given that diseased larvae are rarely fossilized (Poinar, 2003). However, the finding of a rove beetle (Coleoptera: Staphylinidae)

host in Burmese amber with nematodes associated with rod-shaped bacteria similar to those associated with *Heterorhabditis* is now considered the earliest fossil record for an ancestral species *Proheterorhabditis burmanica*, dating back to the early Cretaceous (Poinar, 2011).

Molecular-based phylogenetic studies have placed Steinernematidae as members of Clade IV, together with plant (orders Tylenchida and Aphelenchida) and animal parasites (family Strongyloidae) as well as with free-living rhabditids in the families Cephalobidae and Panagrolaimidae (Blaxter *et al.*, 1998; Dorris *et al.*, 1999; De Ley *et al.*, 2000). On the other hand, Heterorhabditidae have been grouped in Clade V together with Diplogasterida, other Rhabditina (including species such as *Caenorhabditis elegans*), and animal parasites (hookworms in the order Strongylida) (Blaxter *et al.*, 1998; Dorris *et al.*, 1999; De Ley *et al.*, 2000).

According to Poinar (1993), the bacterial symbionts of EPNs share many similarities with *Lucibacterium*, a luminescent marine genus, though, from an evolutionary standpoint, they are not closely related (Boemare, 2002). Poinar (2003) hypothesized that both *Xenorhabdus* and *Photorhabdus* coevolved with the nematodes, losing their ability to form resting stages that would enable them to survive in the environment because they were protected inside a nematode or insect.

The first molecular evolutionary framework to assess coevolution of *Steinernema* nematodes and *Xenorhabdus* symbionts was developed by Lee and Stock (2010) considering 30 nematode-bacterium pairs with a multilocus approach. This study showed that there were 12 cospeciation events in the evolutionary history of *Steinernema* and *Xenorhabdus*, each with 17 host-switching events and seven occurrences of sorting. Although overall cospeciation across all the taxa surveyed was not evident in this study, coevolution between *Steinernema* spp. and *Xenorhabdus* symbionts cannot be discounted (Lee and Stock, 2010).

Similarly, Maneesakorn *et al.* (2011) conducted a multigene analysis for assessing relationships between *Heterorhabditis* spp. and their *Photorhabdus* symbionts. This study revealed strong evidence for coevolution between these mutualistic partners. Host-switching events were only observed within *Heterorhabditis bacteriophora* strains (Maneesakorn *et al.*, 2011).

Plant parasites

Phylogenomic studies suggest that plant parasites had a long history with studies of *Wolbachia* symbionts and may represent the earliest branching clade in the evolutionary history of this bacterium (Brown *et al.*, 2016). For example, members of the Tylenchida such as *Radopholus similis*, a major parasite of banana, have an association with *Wolbachia* (Haegeman *et al.*, 2009).

More recently, a *Wolbachia* sp. was described in all stages of another plant parasite, the root-lesion nematode *Pratylenchus penetrans*, a migratory endoparasite of a wide range of crops and plant species. Brown *et al.* (2016) showed the presence of the bacteria by fluorescence *in situ* hybridization (FISH), demonstrating that the endosymbionts are widely distributed in the adults but with more dense packing in the females' ovaries, as well as in the anterior portion of the intestine and tail region (Brown *et al.*, 2016). Interestingly, the genome of this *Wolbachia*, wPpe, was the first complete genome produced from *Wolbachia* from a plant-parasitic nematode (Brown *et al.*, 2016).

Various studies suggest that plant parasites were the first hosts and later re-invaded the nematodes (Brown *et al.*, 2016). Brown *et al.* (2016) also proposed that an earlier transition to mutualism took place before they switched to filarial nematode hosts. Alternatively, Nikoh *et al.* (2014) considered that mutualism in *Wolbachia* arose through horizontal gene transfer (HGT) from other endosymbionts.

The presence of *Wolbachia* in plant-parasitic nematodes is interesting from the perspective of their evolutionary history; in particular in relation to genes encoding enzymes involved in plant cell-wall degrading, which have been ascribed to HGT. In this light, the *Wolbachia* endosymbionts could be considered possible donors of horizontally transferred genes and may be key players in the parasitic lifestyle of these nematodes (Brown *et al.*, 2016).

Plant parasites have also developed associations with other bacterial species through their evolutionary histories. For example, five plant-parasitic taxa, including three cyst nematode species (*Heterodera avenae*, *H. glycines* and *H. goettingiana*), the potato cyst nematode (*Globodera rostochiensis*) and the root-lesion nematode (*Pratylenchus penetrans*), have associations with *Cardinium*, an endosymbiotic bacterium

that is known to play a critical role in the reproduction of arthropod hosts (Zchori-Fein and Perlman, 2004). There are at least 17 valid species of *Xiphinematobacter* and they all appear to be clustered by geographic location when analyzing 16S rRNA sequences, suggesting an ancient origin and intimate metabolic integration with their arthropod and nematode hosts (Lazarova *et al.*, 2016; Orlando *et al.*, 2016).

Members of the Dorylaimida, specifically *Xiphinema* species (commonly known as dagger nematodes), which are root ectoparasites of economic importance on crops, have also been reported to have an association with 'Candidatus *Xiphinematobacter*' (*Verrucomicrobia*) (Adams and Eichenmuller, 1963; Mankau, 1981; Orton Williams, 1984; Coomans and Claeys, 1998; Coomans and Willems, 1998). According to Brown *et al.* (2016) this is the oldest and most host-coevolved endosymbiont. Analysis of its genome strongly suggests a mutualist role in relation to supplementation of essential amino acid to its nematode host (Brown, 2018).

Parasitic Nematode Models

The success of a parasite depends on its ability to locate, invade, and successfully colonize host tissues and/or organs where it can develop, reproduce, and propagate. Parasitic nematodes have diverse life cycles and transmission modes. Some groups have a monoxenous life cycle (i.e., only one host) such as *Rhabdias*, *Strongyloides*, *Ancylostoma* (Rhabditida), and *Nippostrongylus* (Strongylida); other groups have heteroxenous life cycles with multiple hosts, including intermediate and definitive (Anderson, 2000a).

Here, we focus on key parasitic groups that associate in a transient or conserved manner with bacteria that are currently used as model systems or have the potential to become models for research in human medicine, agriculture, and other biological disciplines.

Animal and human parasites: filarial nematodes

Life cycle

These nematodes have complex heteroxenous life cycles that involve multiple hosts, including

hematophagous arthropods as vectors (black-flies, tabanid flies, mosquitoes, or ticks) and very diverse definitive terrestrial hosts ranging from reptiles and birds to mammals, including humans (Anderson, 2000b) (Fig. 13.2).

In a generalized life cycle, the first larval stage (stage 1 or L1), known as microfilariae, are taken up in the blood meal of the vector arthropod host. The microfilariae exsheath and migrate from the gut of the vector host to the hemocoel, where they molt twice to the infective stage (L3) in the arthropod tissues (depending on the species, it can be fat body, muscles, Malpighian tubules, or hemocoel) (Anderson, 2000b). The L3s migrate to the proboscis of the vector host and, during another blood meal, the infective larvae are passed to the dermis of the vertebrate's skin. Inside the vertebrate host the infective larvae molt into larval stage 4 (L4) and migrate to their definitive niche, through the lymphatic system, coelomic cavities, cardiopulmonary system, or connective tissues, where they molt one last time to become adults and reproduce (Bouchery *et al.*, 2013). An important trait of these nematodes is

that the microfilarial worms develop in the uterus of the female and are laid in the blood or skin of the definitive host. Each filarial species has a preferential site in their definitive host (Anderson, 2000b). Infection by filarial nematodes is often asymptomatic in wild fauna and the pathologies depend on the tissue localization of the nematodes and the vertebrate host's immune response (Bouchery *et al.*, 2013).

Eight filarial nematode species are parasites of humans and are causative agents of four widely spread diseases: (i) lymphatic filariasis, caused by *Brugia malayi*, *Brugia timori*, and *Wuchereria bancrofti*; (ii) onchocerciasis, caused by *Onchocerca volvulus*; (iii) loiasis, caused by *Loa loa*; and (iv) mansonellosis, caused by *Mansonella (Esslingeria) perstans*, *Mansonella (Esslingeria) streptocerca*, and *Mansonella (Mansonella) ozzardi* (Taylor *et al.*, 2010; Bouchery *et al.*, 2013). According to the World Health Organization (WHO), in 2019 alone, 120 million people were affected by lymphatic filariasis.

Lymphatic filariasis is transmitted by mosquitoes and can cause acute fever and chronic

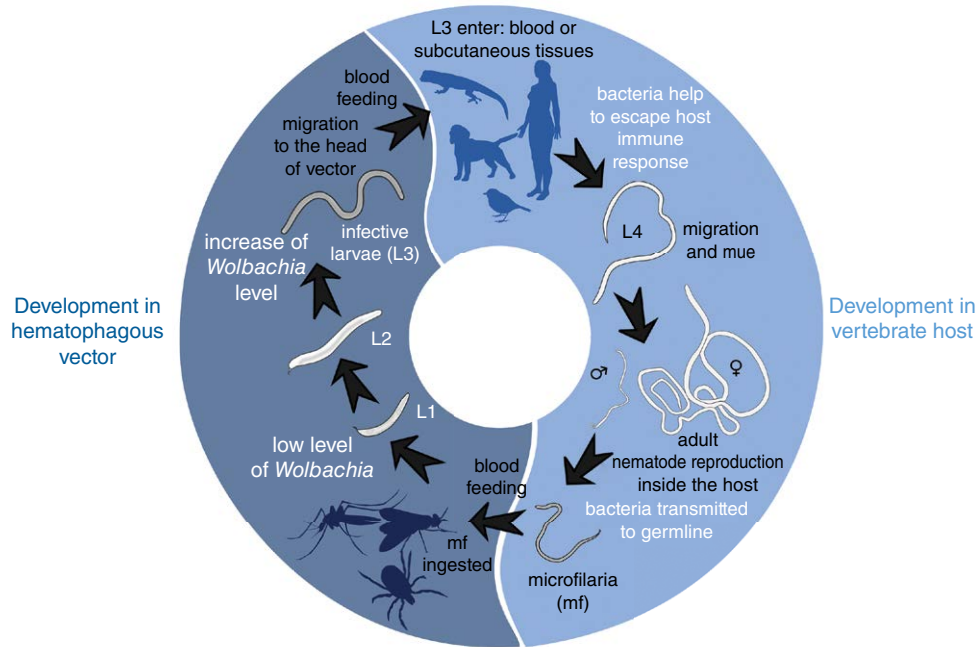


Fig. 13.2. Generalized life cycle of *Wolbachia*-filarial nematodes.

lymphedema (elephantiasis) (Bouchery *et al.*, 2013). Among other symptoms, eosinophilia is also common during acute stages of infection. In 2019, WHO reported over 150 million people in the world under treatment for onchocerciasis. This disease, also known as “river blindness”, causes ocular, cutaneous lesions such as chronic dermatitis and subcutaneous nodules formed around female worms.

Loa loa, known as the “tropical eye worm”, is transmitted by tabanid flies and is mainly characterized by migration of the adult worm in the eye and swellings of the body. This disease is currently not considered of major clinical significance and is not listed as a neglected tropical disease by WHO (Veletzky *et al.*, 2020). However, a recent study highlighted that the impact of loiasis is underestimated. For example, in Gabon, loiasis was found in association with a variety of symptoms as transient painful edema, arthralgia, and substantial morbidity (Veletzky *et al.*, 2020).

Human mansonellosis transmitted by biting midges is reported as mainly asymptomatic. Despite accumulating evidence of a high prevalence in endemic areas, there is currently no filariasis control program targeting this disease. A wide range of symptoms has been observed as angioedema, pruritus, fever, headaches, arthralgias, serositis, and neurologic manifestations (Coulibaly *et al.*, 2009). Similar to loiasis, mansonellosis is considered one of the most neglected tropical infectious diseases (Mediannikov and Ranque 2018).

As mentioned before, many filarial nematodes harbor endosymbiotic *Wolbachia* bacteria (Sironi *et al.*, 1995). *Wolbachia* are vertically transmitted through the female to the offspring and are present in all the stages of the filarial nematodes (Landmann *et al.*, 2010; Ferri *et al.*, 2011; Bouchery *et al.*, 2013) (Fig. 13.2). The association is essential to both the nematodes and the bacteria, and it is considered mutualistic (Taylor *et al.*, 2010) (Fig. 13.2). Depletion of *Wolbachia* sterilizes and disturbs the development of the filarial nematodes (Taylor *et al.*, 2010; Bouchery *et al.*, 2013). The mechanisms underlying this symbiosis remain poorly understood and most of the hypotheses presented have been developed by interpretation of genomics analyses and not by experimental means (Foster *et al.*, 2005; Darby *et al.*, 2012; Lefoulon *et al.*, 2020) (see Chapter 5, this volume).

Microfilariae–vector host interactions

As mentioned in the previous section, many parasitic nematodes use hematophagous vector hosts where the blood meal plays a key role in their life cycle. Numerous factors can influence the efficiency of the transmission of the microfilaria to the vector, such as the distribution and periodicity of the microfilariae in the final host, the duration of the blood-feeding, or even the frequency of contact between vector and final host (Nieguitsila *et al.*, 2013). Different filarial species are vectored by different types of hematophagous host, including biting midges, blackflies, fleas, horse and deer flies, mosquitoes, lice, louse flies, mites, and ticks (Anderson, 2000b). Most of the research on the impact that filarial nematodes have on their vectors has focused on human parasites and mosquitoes such as *Brugia malayi* or *Wuchereria bancrofti*. This is mainly because of the premise that, by controlling the vector, lymphatic filariasis could be reduced or eradicated (Bockarie *et al.*, 2009). A few studies suggest that filarial nematodes may have a negative impact on their vectors (Nieguitsila *et al.*, 2013; Somerville *et al.*, 2019). For example, Somerville *et al.* (2019) showed that *B. malayi* has a detrimental effect on the flight of *Aedes aegypti*, specifically on the speed and distance of its flight. In addition, *A. aegypti* mosquitoes that feed on blood from nematode-infected hosts have decreased energy reserves, including a decrease in glycogen and lipids levels, when compared with controls (Somerville *et al.*, 2019). A study by Gleave *et al.* (2016) showed that the presence of microfilariae negatively affects the fecundity of *A. aegypti* females, which produce fewer mature eggs. Similarly, *Ornithonyssus bacoti* mites that vector *Litomosoides sigmodontis* present a significant reduction of offspring; however, this has not been correlated with the L3 burden (Nieguitsila *et al.*, 2013). It has also been suggested that reduction of fitness in the mites may be linked to the response they have to the infection, such as a physiological modification or immune response that is detrimental to egg development.

Gleave *et al.* (2016) also demonstrated that *B. malayi* larvae have an effect on the behavior of female mosquitoes. Specifically, females that harbor early larval stages (L1 or L2) exhibit a reduced flight activation to the human host.

Contrastingly, a five-fold increase of convergence to the human host was observed when the mosquitoes carried infective larval stages (L3) (Gleave *et al.*, 2016). In general, cues toward the definitive host appear to be dependent on stage and density of *B. malayi* larvae present in mosquitoes.

Based on microfilarial periodicity, different physiological forms can be observed, including nocturnally periodic, nocturnally sub-periodic, and diurnally sub-periodic forms (Paily *et al.*, 2009). Periodicity is a phenomenon that was described in the mid-20th century (Lane, 1948; Hawking and Thurston, 1951) and it is correlated with transmission of the microfilarial larval stage by the vector host in relation to where the worms can be found in the definitive host. In the nocturnally periodic form, the microfilariae appear in the peripheral circulation of the host only during the night, whereas in the nocturnally sub-periodic and diurnally sub-periodic forms, the microfilariae remain in the peripheral blood all the time with a peak density at 12–20 h (Paily *et al.*, 2009). The different physiological forms are morphologically very similar but can be differentiated through microscopic examination of blood smear, whilst very challenging (Das *et al.*, 2011). Random amplified polymorphic DNA (RAPD) PCR amplification of *W. bancrofti* samples showed different RAPD profiles among nocturnally periodic and diurnally sub-periodic forms, suggesting genetic variation (Das *et al.*, 2011).

It has been suggested that the mechanism of microfilarial periodicity could be due to an accumulation of microfilariae in the lungs during the daytime, and their release to the circulating blood at night (Hawking and Thurston, 1951). The body temperature of the host, as well as a greater oxygen tension in the lungs during the day, stimulates microfilarial periodicity (Hawking *et al.*, 1966; Hawking *et al.*, 1981; Paily *et al.*, 2009). However, the exact mechanism of microfilarial periodicity remains not fully understood (Paily *et al.*, 2009). A few studies have demonstrated that microfilarial periodicity can be synchronized with feeding behavior of the vector mosquito, with a larger density of microfilariae in the peripheral blood observed during the peak biting time for *W. bancrofti* (Vanamail and Ramaiah, 1991; Weerasooriya *et al.*, 1998). This synchronicity is absent for other species such as *L. sigmodontis* or *A. viteae*, which still

have successful transmission of microfilaria to the definitive host (Reddy *et al.*, 1984).

Regulation of host's immune response

The ability of parasitic nematodes to confront host immunity is associated with the release of a large spectrum of immunomodulatory factors (Maizels *et al.*, 2018). For example, in filarial nematodes, immunomodulation is a mechanism used to alter the activation of the host immune response during the infection process that allows the nematodes to survive (Cooper and Eleftherianos, 2016). This complex mechanism is hard to generalize, because it varies greatly with the host species and the level of infection.

Invasion of the host tissues by the nematodes leads to recruitment of leukocytes enhancing the inflammatory response and release of cytokines (Cooper and Eleftherianos, 2016). Generally, in mammals, helminthic parasites induced T-helper type 2 (Th2) immune response while bacterial infection induced Th1 response (de Veer *et al.*, 2007; Allen *et al.*, 2008). Th1 response activates macrophages that attack bacteria, while Th2 response induces recruitment of mast cells and eosinophils. Mast cells and eosinophils are the most common cells to infiltrate the site of nematode infection; they contain granules with proinflammatory cytokines and chemokines making them effector cells (de Veer *et al.*, 2007). The capacity of the host to eliminate parasitic nematodes is connected to the ability of the nematodes to generate a polarized Th2 immune response. On the contrary, immunomodulation by excretory–secretory (ES) nematode products promotes parasite survival and longevity (Goodridge *et al.*, 2007). Interestingly, filarial nematodes have the highest average lifespan in their host's body among parasitic nematodes.

One of the front-line defenses of the innate system of definitive hosts are Toll-like receptors (TLRs) which are expressed by many cells of the immune system (dendritic cells (DCs), epithelial cells, fibroblasts, or leukocytes) present in tissues and respond quickly when the parasite enters the host (de Veer *et al.*, 2007). Binding of TLRs triggers a cascade of signaling events that activate the nuclear factor- κ B (NF- κ B) pathway inducing an inflammatory response (Venugopal *et al.*, 2009). Immunoregulation through acti-

vation of TLRs by filarial nematodes was demonstrated by Cooper and Eleftherianos (2016) and Venugopal *et al.* (2009). In *Acanthocheilonema viteae*, glycoprotein ES-62 was found to inhibit activation of B and T lymphocytes through TLR recognition (Goodridge *et al.*, 2007; Venugopal *et al.*, 2009).

Among antigen-presenting cells (APCs), dendritic cells (DCs) are one of the most powerful (de Veer *et al.*, 2007). During natural infection by filarial nematodes, the first immune cells to encounter the foreign antigen of infective larvae (L3) are the dendritic cells (Hoerauf *et al.*, 2005). For example, infective stages of *Brugia malayi* incubated with human Langerhans cells have a reduced ability to express major histocompatibility complex (MHC) class I and II, as well as IL-8 involved in antigen presentation, and decrease their capacity to stimulate T-helper cells (Semnani *et al.*, 2004). In addition, *B. malayi* microfilaria can interact with monocyte-derived dendritic cells forming cell–parasite aggregates that induce DC apoptosis, and decrease the production of IL-12 limiting T cell activation and proliferation (Semnani *et al.*, 2003). Interference with antigen processing can also be mediated by cysteine protease inhibitors or cystatins produced by parasitic nematodes (Cooper and Eleftherianos, 2016). For example, a cystatin secreted by *A. viteae*, Av-cystatin, was demonstrated to inhibit proinflammatory mechanisms of LPS-induced microglia cell via the anti-inflammatory cytokine IL-10 (Behrendt *et al.*, 2016).

Another way to modulate host immune response in filarial nematodes is the secretion of small RNA molecules, which was initially demonstrated for the human hookworm *Heligmosomoides polygyrus* (Buck *et al.*, 2014). The authors showed that parasite-derived microRNAs (miRNAs) and Y RNAs can be transported into mammalian host cells via exosomes regulating host genes associated with immunity and inflammation and suppressing the innate Type 2 response *in vivo* (Buck *et al.*, 2014). Similarly, immunomodulation has been identified in *B. malayi*, *O. volvulus*, *O. ochengi*, *L. loa*, and *L. sigmodontis* (Tritten *et al.*, 2014; Quintana *et al.*, 2015; Zamanian *et al.*, 2015). Some miRNAs are conserved among the filarial nematodes (in particular miR-71 and miR-100 family), suggesting some common circulating miRNA signatures (Quintana *et al.*, 2015). Exosome-like

vesicles (ELVs) containing miRNA were observed released from larval stage and adult of *B. malayi* (Zamanian *et al.*, 2015). The authors demonstrated that the ELVs can be internalized by macrophage host cell lines activating the host immune system (Zamanian *et al.*, 2015). These findings suggested that filarial nematodes used small RNA molecules as cell-to-cell effectors at the host–parasite interface to facilitate establishment or maintenance of the infection.

Wolbachia endosymbionts have also been shown to play an important role in aiding the nematodes to evade the immune response of their vertebrate hosts, and as such they have been defined as defensive mutualists (Hansen *et al.*, 2011). Indeed, several studies have shown that the bacteria facilitate the migration of larvae in the blood system, escaping degranulation by the eosinophils (Taylor *et al.*, 2010; Bouchery *et al.*, 2013). In another study, Hansen *et al.* (2011) demonstrated that *Wolbachia* symbionts of *Onchocerca ochengi* attract neutrophils in the vertebrate host, protecting the adult nematodes from eosinophils which can kill them by degranulation on their cuticle (Hansen *et al.*, 2011). Similarly, Tamarozzi *et al.* (2016) showed that *Wolbachia* can induce the formation of “neutrophil extracellular traps” (NETs) around *O. volvulus* nodules via lipoprotein ligation by two Toll-like receptor protein TLR2/6 which may decrease the penetration of immune cells on the cuticle surface.

In *B. malayi* and *L. sigmodontis*, *Wolbachia* endosymbionts release lipopolysaccharide-like molecules, and the surface protein (WSP) recognized by two Toll-like receptor protein TLR2/4, eliciting innate immune responses against microbial pathogens (Brattig *et al.*, 2000, 2004; Taylor *et al.*, 2000). Specht *et al.* (2011) also proved that presence of *Wolbachia* in the *L. sigmodontis* L3 can induce degranulation of mast cells associated with an increase of vascular permeability facilitating the larval migration. Altogether, these studies clearly highlight that *Wolbachia* symbionts are key players in the pathogenicity of their filarial hosts (Taylor *et al.*, 2010).

Experimental models

Development of filarid experimental models is challenging because of the difficulties in rearing the nematodes. Furthermore, rearing of their

natural hosts or vectors can also be problematic. In spite of these challenges, some initiatives such as the Filariasis Research Reagent Resource Center (FR3, Georgia, USA) have been established to make available different nematode stages of *Brugia* spp., *Dirofilaria immitis*, and *A. viteae* for research. Adaptation of small animal laboratory models has been essential for robustly evaluating *in vivo* candidate drugs at the pre-clinical stage. Both gerbils and mice models have facilitated studies on the biology of filarial nematode species but also have been used for confirmation of effectiveness in pre-clinical stages before informed decisions can be made for clinical development of treatments.

Rodents have been studied for over 70 years and have played a major role in the study of *L. sigmodontis* and *A. viteae* (Risch *et al.*, 2021). *L. sigmodontis* is one of the oldest developed experimental models (Bain and Philipp, 1991). The advantage of *L. sigmodontis* is that its natural host is the cotton rat (Murinae), so experimentation on laboratory-reared mice is possible and has often been used for funnel screening in early stages of the disease for drug development (Fig. 13.3) (Johnston *et al.*, 2014). *A. viteae*, which is a filarial worm with no association with *Wolbachia*, represents a useful model system that can be studied with rodent hosts. Furthermore, it can be used as a control for studies on filarid–*Wolbachia* symbiosis (Harnett *et al.*, 1990). However, maintenance of this species is challenging, as it requires the maintenance of populations of the soft tick, *Ornithodoros* sp., that vectors the nematodes. Recently, Lewis *et al.* (2020) developed an artificial membrane system to maintain colonies of the tick under laboratory conditions.

Alternative laboratory hosts have also been developed for studying lymphatic filariasis; indeed, rodent models have been adapted to carrying *B. malayi* (human parasite) and *Brugia pahangi* (parasite of cats and dogs) (Carraway and Malone, 1985; Kinnamon *et al.*, 1994). Nelson *et al.* (1991) developed an immuno-deficiency (SCID) mouse model which cannot generate functional B or T lymphocytes, allowing *Brugia* species development. Like *L. sigmodontis*, the early stages of these nematode species are often considered in drug development (Johnston *et al.*, 2014).

An obstacle in the study of *Onchocerca* species is the absence of good vertebrate models (Allen *et al.*, 2008). Bovine models have been

used to study *Onchocerca ochengi*, which is closely related to the human parasite *Onchocerca volvulus*. Similarly to *O. volvulus*, *O. ochengi* forms nodules that can be enumerated non-invasively or removed for analysis during immunological or chemotherapeutic studies (Allen *et al.*, 2008). However, experimentation on cattle can be very challenging, given that large quantities of test drugs are required (Halliday *et al.*, 2014). Halliday *et al.* (2014) adapted the SCID mouse to study *Onchocerca* filariasis, where they successfully implanted motile *Onchocerca* adult males into SCID mice and evaluated their survival. They also monitored viability *ex vivo* of male macrofilariae from rodent implants and reported that their motility was comparable to freshly isolated adult males for up to 7 days. Altogether this study provided a robust ‘pan-filarial’ small-animal model for research that has adequate capacity and throughput, to screen existing and future pre-clinical candidate macrofilaricides.

Another filarial worm, *Loa loa*, has been successfully maintained in the laboratory via experimental infection of splenectomized baboons (Orihel and Moore, 1975). Recently, experimental infections of lymphopenic mice deficient in interleukin (IL)-2/7 gamma-chain signaling also appear to be promising models for the study of microfilaraemic infections (Pionnier *et al.*, 2019).

Research on the canine heartworm, *Dirofilaria immitis*, which is responsible for severe disease and death in dogs in many parts of the world (McCall *et al.*, 2008), has also considered the establishment of infection in rodent models, to replace the use of dogs, cats, and ferrets to study this disease (McCall *et al.*, 2008).

Insect parasites: entomopathogenic nematodes

Life cycle

Nematodes in the Steinernematidae and Heterorhabditidae, generically known as entomopathogenic nematodes (EPNs), are mutualistically associated with gamma-proteobacteria (Enterobacteriaceae) (Boemare, 2002). *Steinernema* species associate with *Xenorhabdus*, while *Heterorhabditis* species partner with *Photorhabdus* (Boemare, 2002). This symbiotic partnership is antagonistic to a wide range of insects (Poinar, 1990; Grewal and Georgis, 1999; Stock, 2005). The third-stage

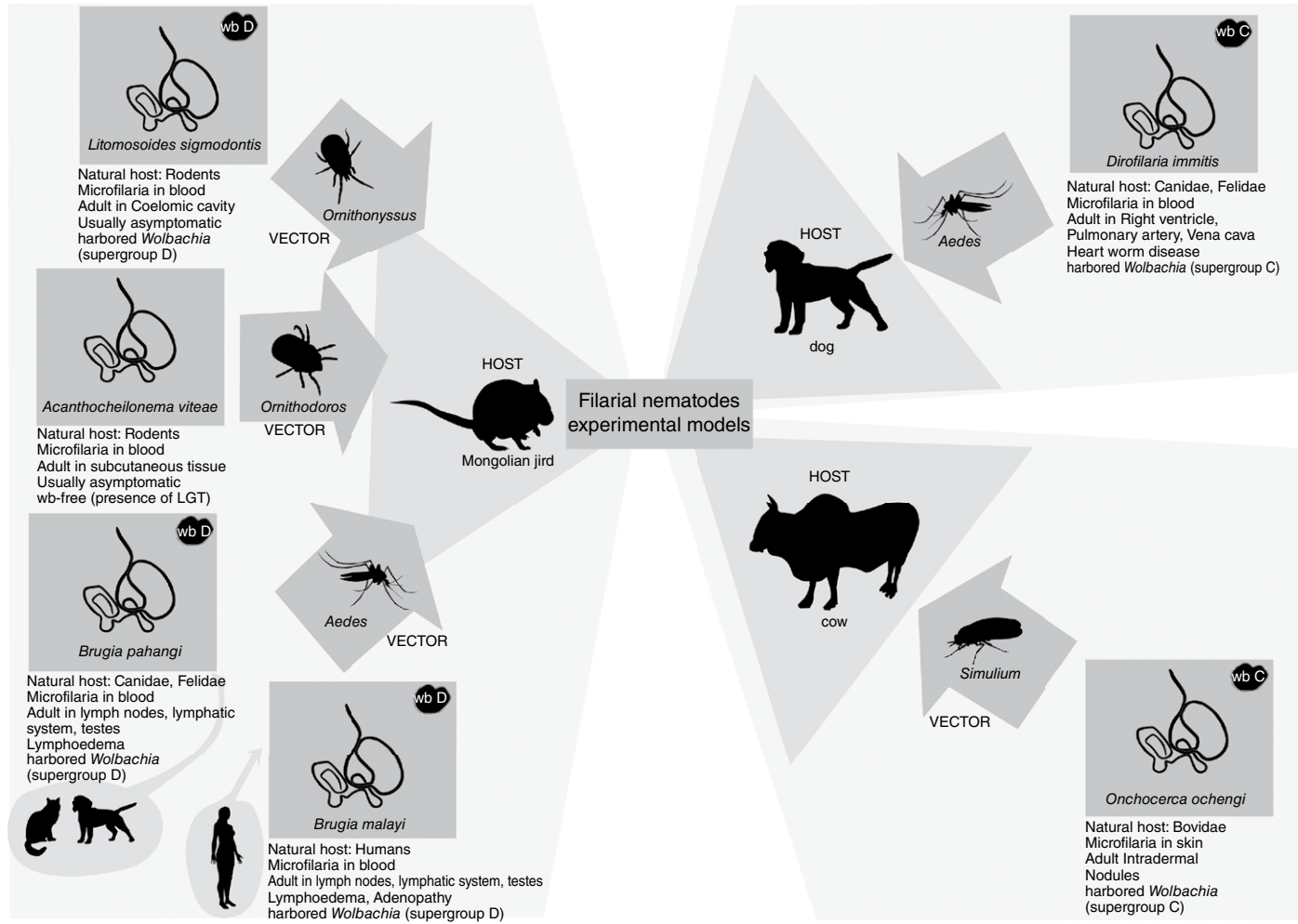


Fig. 13.3. Filarial nematodes as experimental model systems.

infective juvenile (IJ) is the only free-living stage in the nematodes' life cycle. This is an arrested stage, like the dauer stage in *C. elegans* that does not feed. This stage carries the bacterial symbionts in its intestine (Bird and Akhurst, 1983; Flores-Lara *et al.*, 2007; Kim *et al.*, 2012) and vectors the bacteria from one insect host to another. While *Xenorhabdus* bacteria are confined in a specialized receptacle in the most anterior portion of the intestine in *Steinernema* IJs, *Photorhabdus* are freely located in the intestinal lumen of *Heterorhabditis* (Boemare, 2002; Flores-Lara *et al.*, 2007; Kim *et al.*, 2012) (Fig. 13.4).

When IJs find a suitable insect host in soil they enter it through natural body openings (mouth, anus or spiracles) and make their way to the hemocoel. Once in the hemocoel, the IJs release their bacterial symbionts from the intestine on to the nutrient-rich insect hemolymph, where the bacteria proliferate. The insect host usually dies in 24–72 h due to septicemia (Nielsen-LeRoux *et al.*, 2012). The IJs recover from their arrested stage and start feeding on bacteria. The nematodes develop to adults and reproduce until the host resources are depleted. Usually, 1–2 adult generations develop in the

insect cadaver. Food scarcity and higher levels of ammonia due to crowding trigger the formation of the IJ stage (Fig. 13.4) (Forst and Neilson, 1996; Stock, 2005, 2015; Stock and Goodrich-Blair, 2008, 2012). The IJs abandon the insect cadaver and migrate to the soil in search of new hosts (Fig. 13.4).

The association between EPNs and their bacteria is mutually beneficial. The nematodes protect the bacteria from external environmental stressors and vector them into insect hemocoel. On the other hand, the bacteria are largely responsible for death of the insect, provide suitable nutrients for nematode growth and reproduction, and produce a wide range of antimicrobial compounds that offer protection against other competing microbes, creating a suitable environment for nematode reproduction (Akhurst and Boemare, 1990; Boemare, 2002; Forst and Clarke, 2002; Sicard *et al.*, 2004, 2006; Ciche *et al.*, 2006; Goodrich-Blair, 2007; Stock and Goodrich-Blair, 2008).

Xenorhabdus symbionts also play a role in other stages of the nematodes' life cycle, including adult development and maturation. For example, Roder and Stock (2018) showed that the bacterial

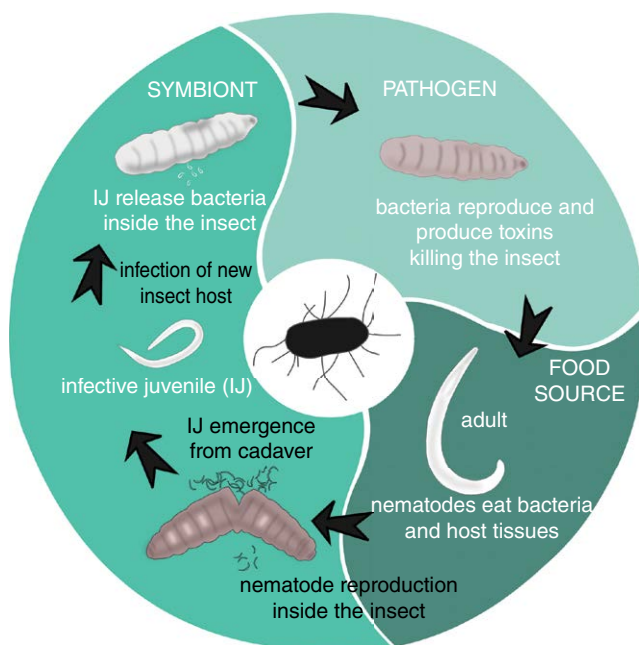


Fig. 13.4. Generalized life cycle of *Xenorhabdus*/*Photorhabdus*–entomopathogenic nematode associations.

symbionts, as a food source to the nematodes, play a role in gonad postembryonic development in first-generation adults. Specifically, the authors showed that when *Steinernema feltiae* nematodes were cultured *in vitro* with non-cognate symbionts, the size of both male and female gonads was significantly reduced when reared with non-cognate *Xenorhabdus* species. Contrastingly, in *Steinernema carpocapsae*, both males and females exhibited an enhanced gonad length when cultured with a non-cognate *Xenorhabdus nematophila* strain. Additionally, time to adult maturation as well as body and gonad size were directly influenced by the bacterial symbionts with which they were cultured (Roder and Stock, 2018).

In another study, Roder *et al.* (2019) demonstrated that first-generation adults of *S. carpocapsae* and *S. feltiae* produced slightly larger amounts of pheromones (signaling molecules also known as ascarosides) when both species were reared with non-native *Xenorhabdus* strains. However, lower pheromone amounts were observed when the nematodes were reared in non-cognate *Xenorhabdus* species. Pheromones have been shown to take part in a variety of sex-specific and social behaviors, including male attraction and aggregation in *C. elegans* (Srinivasan *et al.*, 2008, 2012). Thus, understanding the interaction of ascaroside production and food supply is critical to the unraveling of the life cycle of these entomopathogens and in the optimization of culturing methods for mass production systems of these insect parasites (Roder *et al.*, 2019).

Role of the infective juvenile stage

HOST LOCATION AND ATTRACTION. Perpetuity of EPN life cycles is entirely dependent on the IJs' ability to find a suitable host. Various studies have shown that IJs employ different strategies to find a host, including cruising, ambushing, or intermediate foraging behavior (Lewis *et al.*, 1993; Campbell and Gaugler, 1997; Gaugler *et al.*, 1997; Bal *et al.*, 2014). Cruisers such as *Steinernema glaseri* and *Heterorhabditis bacteriophora* are more efficient at targeting sedentary hosts, whereas ambushers such as *S. carpocapsae* sit and wait for the host and are more efficient at infecting mobile hosts (Campbell and Gaugler, 1997; Lewis, 2002). Intermediate foragers such as *Steinernema riobrave* and *S. feltiae* use both

strategies (Campbell and Gaugler, 1997; Gaugler *et al.*, 1997).

Host-seeking strategies are influenced by physical and chemical cues (Jones, 2001; Zhang *et al.*, 2021) as well as electrical and magnetic fields (Shapiro-Ilan *et al.*, 2009, 2012). EPNs use olfactory cues such as CO₂ and volatile chemicals produced by insects and damaged plants to find a suitable host (Zhang *et al.*, 2021). Most of the tested EPN species initiate host-seeking behavior in response to the olfactory cues. Paired BAG sensory neurons were demonstrated to be involved in the sensing of CO₂ levels in *C. elegans* (Bretscher *et al.*, 2008; Hallem and Sternberg, 2008). Similarly, removal of BAG neurons in *H. bacteriophora* and *S. carpocapsae* IJs stopped initiation of foraging in response to elevated CO₂ levels (Kooliyottill *et al.*, 2013). Additionally, IJs of *H. bacteriophora* devoid of BAG neurons were unable to locate *Galleria mellonella*, suggesting that host location is mediated through sensing CO₂ by BAG neurons in this nematode species. Contrastingly, ablated BAG neurons in *S. carpocapsae* did not affect host location, suggesting that this species uses alternative cues and/or other neurons for host location (Kooliyottill *et al.*, 2013).

EPNs also sense volatile chemicals emitted from insect-damaged plants to locate insect hosts (Rasmann *et al.*, 2005; Ali *et al.*, 2010; Le Vieux and Malan, 2014; Li *et al.*, 2015; Andaló *et al.*, 2017; Grunseich *et al.*, 2021). In most instances, chemotaxis to the volatiles is strain specific (Ali *et al.*, 2011; Laznik and Trdan, 2013, 2016a,b; Willett *et al.*, 2015).

EPNs also locate their insect hosts in response to the volatile compounds released from molted skins, feces, or exudates and pheromones (Schmidt and All, 1979; Shapiro *et al.*, 2000; O'Halloran and Burnell, 2003; Hallem *et al.*, 2011; Dillman *et al.*, 2012; Baiocchi *et al.*, 2017; Rivera *et al.*, 2017). For example, Hazir *et al.* (2016) showed that *S. riobrave* IJs are more attracted to the frass of *Helicoverpa zea* when feeding on tobacco than those that feed on tomato and eggplants.

With respect to electrical fields, Shapiro-Ilan *et al.* (2009, 2012) demonstrated directional movement in several EPN species with different host-foraging strategies in response to electrical fields, with ambusher species consistently moving to a lower electric potential, while

cruisers moved to a higher electric potential. The authors also reported that the lowest electric potential that elicited a response was 0.1 V, which is comparable to electrical potential associated with some insects and plant roots, and that the level of response to electrical potential diminished with nematode age (Shapiro-Ilan *et al.*, 2009).

Shapiro-Ilan *et al.* (2012) also tested the ability of *S. carpocapsae* to respond directionally to magnetic fields and reported a directional response of IJs. The authors concluded that *S. carpocapsae* uses electromagnetic fields as cues to aid navigation and foraging. The adaptive value of magnetoreception in entomopathogenic nematodes remains unclear but it can be speculated that this sensory response also contributes to navigation or foraging abilities.

Nematode pheromones (i.e., ascarosides) also play a role in the dispersal of EPN IJs in the soil as well as in interspecies communication (Kaplan *et al.*, 2012). Furthermore, recent studies have shown that ascaroside pheromone extracts can enhance the biological control potential of EPNs (Oliveira-Hofman *et al.*, 2019).

In summary, EPNs possibly use a combination of cues, including CO₂ and insect and/or plant volatiles as well as magnetic and electrical fields to find a suitable host. Despite this knowledge, further research is needed to characterize species-specific volatile chemicals involved in driving the foraging behavior of these nematodes, which is crucial to improve their efficacy.

HOST INVASION. Upon reaching the insect hemocoel, IJs exsheath and resume development, a process known as activation. Morphologically, activated IJs open the mouth, expand the anterior gut, and the pharyngeal bulb becomes activated (Lu *et al.*, 2017). IJs then start the release of the bacterial symbionts (Poinar and Thomas, 1967; Sicard *et al.*, 2004), which in steinerneematids is done through intestinal peristaltic movements until reaching the anus (Snyder *et al.*, 2007). Contrastingly, in heterorhabditids release of *Photorhabdus* is through the mouth via regurgitation (Ciche and Ensign, 2004).

Traditionally, symbiotic bacteria have been considered largely responsible for the death of the insects; however, recent studies suggest that EPNs play a key role in actively suppressing or

evading insect immune responses. For example, many studies have demonstrated that *S. carpocapsae* and *S. feltiae* IJs are able to kill *G. mellonella* when they are depleted of their *Xenorhabdus* symbionts (Poinar and Thomas, 1966; Ehlers *et al.*, 1997; Han and Ehlers, 2000). Wang *et al.* (1995) also showed that *S. glaseri* tolerates gut fluids in the scarab beetle *Popillia japonica* and can avoid the host immune response. In relation to this, Wang and Gaugler (1999) showed that this nematode uses an anti-immune protein (SCP3a) to defeat the host immune system, thereby protecting itself from encapsulation and apparently protecting the symbiotic bacteria from phagocytosis.

A few studies have shown that purified trypsin and trypsin-like secreted substances from *S. carpocapsae* exhibit immune-suppressive properties, and the lipidic moieties of cuticle fragments of *S. feltiae* inactivate the prophenoloxidase system (involved in protective response against invading microbes, fungi, and parasitoids) in *G. mellonella* (Brivio *et al.*, 2002, 2004; Balasubramanian *et al.*, 2009, 2010). In *S. carpocapsae*, many excreted/secreted products (ESPs) have been identified (Hao *et al.*, 2010). Activated IJs release ESPs involved in host immune suppression or evasion. For example, elastase-like serine protease may be involved in host tissue penetration and is upregulated during the initial parasitic stage (Hao *et al.*, 2009). Similarly, Sc-Sp-1 and Sc-SP-3 (serine proteases) ESPs are expressed only during the parasitic stage and cause midgut tissue damage, inducing cell apoptosis (Toubarro *et al.*, 2009, 2010). Another ESP, Sc-Sp-6, a serpin-like protein from *S. carpocapsae*, plays a role in impeding insect digestive enzymes and clot formation, which are important insect defense mechanisms (Toubarro *et al.*, 2013).

A BPTI–Kunitz family inhibitor (Sc-KU-4) is highly expressed during nematode invasion and inhibits aggregation of insect hemocytes and entrapment of foreign materials, thereby avoiding encapsulation (Toubarro *et al.*, 2013). Several aspartic proteases are also upregulated during the pathogenic phase in *S. carpocapsae* but their functional role in nematode parasitism has not yet been elucidated (Balasubramanian *et al.*, 2012a,b). Comparative transcriptomic studies of activated and non-activated IJs also revealed a whole suite of effector proteins secreted by

activated IJs in *S. carpocapsae* and *S. feltiae* with potential roles in immunomodulation and tissue damage of host insects (Lu *et al.*, 2017; Chang *et al.*, 2019).

Additionally, the IJ cuticle seems to protect the nematodes from host recognition (Dunphy and Webster, 1987). For example, it has been shown that injection of *S. carpocapsae* cuticle fragments into *Rhynchophorus ferrugineus* prevented the immunogenic response of this host (Mastore *et al.*, 2014; Brivio and Mastore, 2018). Alterations of the lipidic moiety of the cuticle in *S. carpocapsae* also resulted in loss of “disguise” of the IJs and caused encapsulation by hemocytes in *Rhynchophorus ferrugineus* (Mastore *et al.*, 2014; Brivio and Mastore, 2018).

Contrastingly, *Heterorhabditis* IJs seem to lack evasive properties and are more vulnerable to the immune system of various insect species (Han and Ehlers, 2000; Cruz *et al.*, 2001; Balasubramanian *et al.*, 2010). However, recently secreted proteins have been identified with roles in suppression of the host’s antimicrobial peptides (Kenney *et al.*, 2019). For example, Eliáš *et al.* (2020) identified ESPs with Pellino and CUE domains in *H. bacteriophora* which seem to inhibit the insect phenoloxidase system by modulating insect immune responses. Additionally, ecdysteroid glycosyltransferase, lysozyme, and serine carboxypeptidases were identified from the transcriptomes of insect hemolymph-activated IJs of *H. bacteriophora*, revealing further roles in immunomodulation in the insect host (Kenney *et al.*, 2020, 2021).

Altogether these studies provide strong evidence that EPNs exhibit insect immune suppression

and are capable of contending the host immune system in the initial stages of the infection prior to the release of the symbiotic bacteria.

Experimental models

Burnell and Stock (2000) were the first to recognize the potential of EPNs as tractable model systems. Their relatively short life cycle, ease of rearing under laboratory conditions (both *in vivo* and *in vitro*), and malleability for genetic manipulation make them excellent model systems for studying in diverse biological, ecological, and evolutionary processes (Table 13.1).

Indeed, for more than two decades, EPNs and their bacterial symbionts have been considered tractable model systems that are amenable for studying physiological, chemical, structural, and developmental aspects of beneficial symbiotic associations (Burnell and Stock, 2000; Stock, 2005; Stock and Goodrich-Blair, 2008). Furthermore, mutualistic relationships with enteric bacteria position them as excellent models to investigate host–symbiont, host–pathogen, and parasite–insect host interactions (Flores-Lara *et al.*, 2007; Murfin *et al.*, 2015; Ciezki *et al.*, 2017; McMullen *et al.*, 2017; Labaude and Griffin, 2018; Chang *et al.*, 2019; Stock, 2019) (Table 13.1).

EPNs have complex biotic and abiotic interactions in the soil and can also serve as models for understanding various ecological studies, including the effects of disturbance on ecological interactions in soil (Campos-Herrera *et al.*, 2012). The diversity of these insect parasites as well as their interactions with other organisms can be significantly altered by management

Table 13.1. Entomopathogenic nematodes as model systems: summary of key traits.

Rearing conditions	Room temperature <i>In vitro</i> : with or without their symbiotic bacteria <i>In vivo</i> : various insect hosts
Size and storage	Adults range from 1 mm to 6 mm in length Juveniles 0.5–2 mm in length
Morphology	Transparent: easy to directly observe Cellular changes can be observed with standard microscopy
Life cycle	Short (8–21 days)
Propagation	Simple, hermaphrodites and gonochoristic adults Inbreeding depression may occur
Cryopreservation	Yes
Genetic crosses	Yes, males exist
Genomes	Currently five <i>Steinernema</i> spp., four <i>Heterorhabditis</i> spp., but more are becoming available

practices such as irrigation, planting density, variety selection, tillage regime, fertility inputs, pesticide use, and various other factors (reviewed in Shapiro-Ilan and Gaugler, 2002; Barbercheck and Hoy, 2005; Stuart *et al.*, 2006). Research on EPNs can also provide insights into parasite–prey interactions, as these nematodes act as both parasites and prey in soil (Campos-Herrera *et al.*, 2012). Presence of suitable insect hosts is the primary biotic factor influencing the occurrence and persistence of these nematodes at a given location (Mráček *et al.*, 1999). Regulation of soil biota through predation is widespread in soil food webs and there are many examples of regulation of densities of soil animals and microbes by their consumers (reviewed by Wardle *et al.*, 2004). Predation on EPNs has not been studied extensively, but a wide range of soil organisms could play an important role in regulating EPN populations. EPNs have contributed substantially to population dynamics and ecosystem processes and as such can serve as great model systems in soil ecology.

As parasites and hosts of symbiotic bacteria, they can also serve as models in epidemiological research to study infectious diseases and the chain of infection (Fig. 13.5). Furthermore, the

tripartite symbiosis represented by EPNs, the bacterial symbionts, and the insect host is a malleable model system for studying interactions between microbes and their eukaryotic hosts. Additionally, the dual life of the bacterial symbionts, as mutualists of *Steinernema* and *Heterorhabditis* nematodes and pathogens of a wide range of insect species, offers also a platform for investigating host manipulation in microbial symbiosis. Many questions yet remain unanswered surrounding the evolution, ecology, and physiological capabilities of these insect pathogens. Much of the molecular underpinnings in this system await to be discovered, from understanding the basic ability of these nematodes to the ability to engineer them genetically to make more robust and specific biological control agents for application in the management of insect pests (Stock, 2019).

Plant parasites

Life cycle

A few plant-parasitic nematodes have been found to have symbiotic associations with bacterial

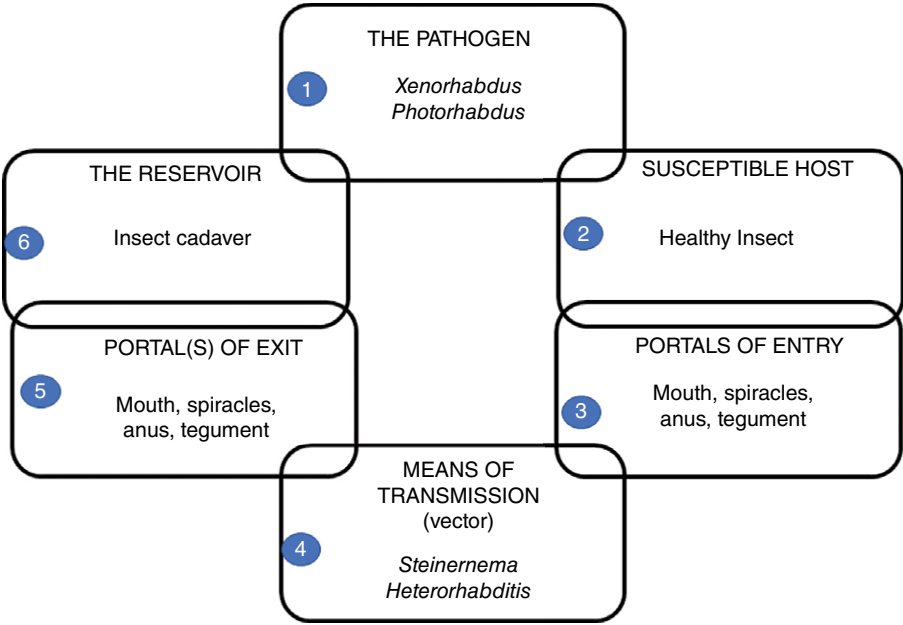


Fig. 13.5. Adaptation of the “Chain of Infection” based on the EPN–bacterial symbiont–insect tripartite system.

symbionts, including *Wolbachia*. In particular, members of the Tylenchida such as *Radopholus similis*, a major parasite of banana, has an association with *Wolbachia* (Haegeman *et al.*, 2009) (Fig. 13.6A). *R. similis* is a migratory endoparasite, and the adult stages live in the root and corm tissues of the host plants. Their life cycle can be completed in 20–25 days (O'Bannon, 1977; Gowen *et al.*, 2005). This species exhibits a pronounced sexual dimorphism where males have an atrophied stylet and are considered to be free-living. Juveniles and adult females are active and have mobile forms that may leave the roots in case of adverse conditions (O'Bannon, 1977). Migratory stages in the soil can easily invade new roots. The nematodes occupy an intercellular position in the cortical parenchyma where they feed on the cytoplasm of nearby cells, destroying them and causing cavities to develop (O'Bannon, 1977).

While analyzing the transcriptome of this species, Jacob *et al.* (2008) found that approximately 1% of the unigenes had similarity to *Wolbachia* sequences. At that time, it was not clear if these were nematode genes acquired by lateral gene transfer from an ancient and long-lost endosymbiont (Dunning-Hotopp *et al.*, 2007) or whether it was a true endosymbiont (Jacob *et al.*, 2008). It was not until later that Haegeman *et al.* (2009), using transmission electron microscopy (TEM), fluorescent immunolocalization,

and staining with DAPI, confirmed the presence of the *Wolbachia* endosymbionts within the reproductive tract of adult females. The authors also reported that another species, *Radopholus arabocoffeae*, carries *Wolbachia*-like endosymbionts (Haegeman *et al.*, 2009). Furthermore, phylogenetic analysis of 16S rRNA sequences conducted by these authors revealed that this symbiont formed a monophyletic cluster and was quite distantly related to other *Wolbachia* species; it could potentially be a novel species.

More recently, a *Wolbachia* sp. was described in all stages of another plant parasite, the root-lesion nematode *P. penetrans*, a migratory endoparasite of a wide range of crops and plants species. Brown *et al.* (2016) showed the presence of the bacteria by FISH, demonstrating that the endosymbionts are widely distributed in the adults but with more dense packing in the females' ovaries, as well as in the anterior portion of the intestine and tail region (Brown *et al.*, 2016). Interestingly, the genome of this *Wolbachia*, wPpe, was the first complete genome produced from a *Wolbachia* from a plant-parasitic nematode (Brown *et al.*, 2016).

Until now, five plant-parasitic taxa have been reported to have associations with *Cardinium*. This is an endosymbiotic bacterium that is known to play a critical role in the reproduction of arthropod hosts (Zchori-Fein and Perlman,

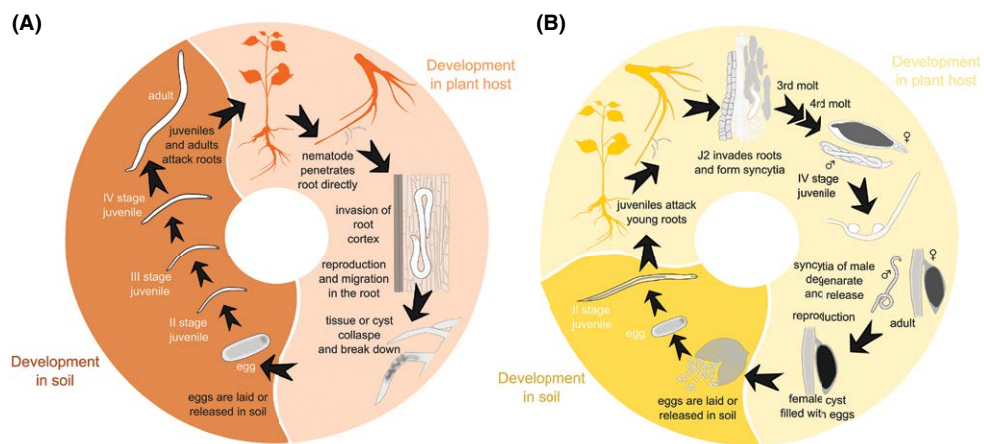


Fig. 13.6. (A) Generalized life cycle of plant-parasitic nematodes with bacterial symbiotic associations (*Wolbachia/Verrucomicrobia/Cardinium*). **(B)** Generalized life cycle of *Cardinium*-cyst nematodes symbiosis.

2004). The first record of *Cardinium*–nematode associations dates to the early 1970s, when Shepherd *et al.* (1973) reported the presence of rod-shaped “intracellular microorganisms” in the reproductive tract (ovaries, oviduct, and oocytes) of females of cyst nematode species (*Heterodera avenae*, *H. glycines*, and *H. goettingiana*). The authors described that the tissues containing *Cardinium* cells were unharmed, and that fertilized eggs were normal even when the ovary and oviduct were heavily infected, suggesting a commensal or facultative mutualist role (Shepherd *et al.*, 1973) (Fig. 13.6B).

Noel and Atibalentja (2006) also reported the presence of “*Candidatus* Paenicardinium endonii” in *H. glycines* (Tylenchida). The bacterium was observed in the pseudocoelom and intestine of juveniles and adults (females and males), in hypodermal chords of juveniles and males, in ovary walls and in oocytes in females, and in spermatozoa in males (Noel and Atibalentja, 2006).

In another study, Walsh *et al.* (1983a,b) identified *Cardinium* in a Bolivian strain of *G. rostochiensis* (potato cyst nematode) with a 100% prevalence. The bacteria were found in second-stage juveniles and adult stages (males) and were described as “rickettsia-like organisms localized intracellularly in most tissues but more common in muscle cells and in the esophageal glands and sperm in males” (Walsh *et al.*, 1983a,b).

More recently, Denver *et al.* (2016) discovered *Cardinium* endosymbionts in another plant parasite, the root-lesion nematode, *P. penetrans*, while conducting a “genome skimming” analysis to investigate pathogen function and biology of plant parasites from a phylogenetic angle. However, in another study, Wasala *et al.* (2019) found no *Cardinium* coinfections in field populations from California, Oregon, and Washington, USA.

Experimental models

As mentioned before, plant-parasitic nematodes display a wide variety of interactions with their hosts. Their small size, and the fact that many of the most important species are obligate biotrophs and cannot be cultured in large numbers, makes many of them difficult experimental organisms. However, the finding that *Arabidopsis thaliana* can be used as a suitable plant host for studying economically important species such as the root-knot (*Meloidogyne* spp.) and cyst (*Heterodera*

spp.) (Sijmons *et al.*, 1991) nematodes has expanded the consideration of plant parasites as model organisms. Since then, *A. thaliana* has become a model plant for research on plant–nematode interactions and other critical aspects such as the mechanisms by which the complex feeding structures induced by these nematodes are produced using the full scope of genomic resources (Jones *et al.*, 2013). Furthermore, the development of genomics tools and the accessibility to and affordability of genome sequencing have had a tremendous impact in the field of plant nematology (Chen *et al.*, 2005; Abad *et al.*, 2008; Jacob and Mitreva, 2011; Kikuchi *et al.*, 2011; Opperman *et al.*, 2008).

Because of their economic relevance, root-knot and cyst nematode parasites are the most widely studied plant parasites. Both parasitic groups are currently used as model systems to study the biology, behavior, and evolution of plant parasites (Dong and Opperman, 1997; Opperman and Bird, 1998; Tytgat *et al.*, 2000). They also serve as model systems in various disciplines, including plant cell biology and genetic engineering and molecular biology.

For now, only *Heterodera* species have been found to have symbiotic associations with bacteria. Perhaps this economically important parasite can be used as a model system to understand interactions between plant parasites and bacterial symbioses. Interestingly, genome data suggest that many genes present in plant-parasitic nematodes were horizontally transferred from bacteria, and many of these genes probably evolved into new genes that mediate interactions with the plant host and may also be involved in the regulation as important adaptations that allow nematodes to parasitize plants.

Certainly, sequencing of genomes of more plant-parasitic taxa will allow more detailed analyses and a better understanding of the organization of key genes within the genomes of this group.

Role of Symbionts in Disease Control

Filarial symbionts

As previously mentioned, several filarial nematode species are agents of human diseases but most of the disease control programs are focused

on the lymphatic filariasis and the onchocerciasis considered as presenting the more severe pathology. The WHO recently refined the goals for priority neglected tropical diseases (NTDs) with, among others, the elimination of onchocerciasis transmission and stopping lymphatic filariasis as a public health problem by 2030 (Risch *et al.*, 2021). Numerous mass drug administration (MDA) programs have been developed over the past decades, such as OCP (Onchocerciasis Control Programme), APOC (African Programme for Onchocerciasis Control), and OEPA (Onchocerciasis Elimination Program of the Americas) (Risch *et al.*, 2021). The mainstay of control remains through the MDA, which is based on large-scale treatment of endemic nematode populations using anthelmintic drugs: diethylcarbamazine, ivermectin, and albendazole (Hoerauf *et al.*, 2011). Specifically, diethylcarbamazine or ivermectin each given in combination with albendazole are used to treat lymphatic filariasis, while ivermectin as the sole drug administered annually or biannually is used to treat onchocerciasis (Taylor *et al.*, 2010). It has been observed that a single dose of diethylcarbamazine (6 mg/kg) or ivermectin (150–200 lg/kg) leads to sustained killing of blood and skin microfilariae (Hoerauf *et al.*, 2011). These treatments are efficient mainly on the first larval stage of the nematodes (microfilaria) and not on the adult stages, which require long treatment coverage to break the transmission cycle (10–14 years for onchocerciasis, 5–8 years for lymphatic filariasis) (Taylor *et al.*, 2014). Thus, the aim of these treatments is to decrease the prevalence of microfilariae-positive cases to reduce to levels at which transmission is no longer sustained (Hoerauf *et al.*, 2011). In the case of diethylcarbamazine, the activity of the drug is not well understood but appears to be associated with inducible nitric oxide synthase and the cyclooxygenase pathway leading to rapid sequestration of the microfilariae (McGarry *et al.*, 2005). However, use of diethylcarbamazine appears dangerous in the case of onchocerciasis because it often triggers intense inflammatory reactions around *Onchocerca* microfilariae in the skin and the eyes (Taylor *et al.*, 2010). Thus, diethylcarbamazine cannot be used for mass drug administration against onchocerciasis or against lymphatic filariasis in Africa where there is a risk of co-endemicity with onchocerciasis and loasis (Hoerauf *et al.*, 2011).

Indeed, it has been reported that individuals infected with high microfilarial loads of the filarial nematode *Loa loa* suffered from severe adverse events following intake of diethylcarbamazine or ivermectin during MDA programs (Gardon *et al.*, 1997; Taylor *et al.*, 2010; Risch *et al.*, 2021). So co-endemic areas remain a problem for the elimination of onchocerciasis and lymphatic filariasis. Albendazole is a broad-spectrum anthelmintic inhibiting the polymerization of β -tubulin and microtubule formation (Taylor *et al.*, 2010). Associated with diethylcarbamazine or ivermectin, it can reduce microfilarial loads in the periphery for longer (Taylor *et al.*, 2010). Currently, research is focused on the development and validation of novel macrofilaricidal treatment strategies such as flubendazole or oxfendazole (Risch *et al.*, 2021).

Concerns about ivermectin resistance, as well as safety constraints in areas of co-endemicism with *L. loa* (Hoerauf, 2008), make treatments that target *Wolbachia* with antibiotics such as doxycycline a safer alternative for the control of filariasis (Hoerauf, 2008). The filarial nematode–*Wolbachia* symbiosis represents a clear example of how the discovery of the bacterial symbionts shifted research for the treatment of onchocerciasis and lymphatic filariasis. In this respect, and over the past few years, anti-*Wolbachia* drugs have been considered as an alternative treatment strategy by various programs involved in the elimination of these diseases (Neglected Tropical Diseases Modelling Consortium Onchocerciasis Group, 2019). Unlike anthelmintic drugs, antibiotic therapy can stop the reproduction and kill the adult nematode stages, thus stopping the cycle (Hoerauf, 2008). Although treatment time has also been reduced, the process is still long-lasting, ranging from 4 to 6 weeks, and it is not recommended for pregnant women, or for children younger than 8 years (Taylor *et al.*, 2014).

A recent study by Gunderson *et al.* (2020) explored the long-term effect of antibiotic treatment on *B. pahangi*. Their results showed a rebound of *Wolbachia* titers 8 months after treatment, showing clusters of densely packed *Wolbachia* in the ovarian tissues. This study suggested that *Wolbachia* might persist in the nematode females in specific sites (Gunderson *et al.*, 2020).

A few studies have focused on computational analyses, to identify key genes in *Wolbachia* that

could be targeted to kill the filarial nematodes (Holman *et al.*, 2009). For example, it has been demonstrated that, in *B. malayi*, decreasing GT-Pase (guanosine triphosphate enzyme) activity (associated with *ftsZ* protein in *Wolbachia*) with berberine reduces the motility of adult female and microfilariae as well as reducing the production of microfilariae (Li *et al.*, 2011).

Genome analysis of the glycolytic/gluconeogenic pathway in the *Wolbachia* host of *B. malayi* (wBm) revealed the absence of pyruvate kinase (PK), suggesting that the bacterium may instead utilize the enzyme pyruvate phosphate dikinase (PPDK). Therefore, the absence of PPDK in mammals makes the enzyme an attractive *Wolbachia* drug target. Since there is no protein sequence homology between PPDK and PK, it is feasible that highly specific inhibitors of PPDK activity may be identified (Raverdy *et al.*, 2008). More recently, it has been demonstrated that inhibition of glycolysis using 3-bromopyruvate has a significant impact on bacterial and worm survival as well as on secretion of microfilariae (Voronin *et al.*, 2019).

Another strategy has focused on the screening of large-scale libraries to identify compounds affecting *Wolbachia*. This has been the strategy chosen by the "Anti-*Wolbachia*" (A-WOL) consortium (founded in 2007 and supported by the Bill & Melinda Gates Foundation), to discover new drugs targeting *Wolbachia* and to reduce treatment time (Taylor *et al.*, 2014). The collaboration of the A-WOL consortium with the pharmaceutical company AstraZeneca has allowed large-scale compound library screening (Clare *et al.*, 2019). As previously mentioned, filarial *Wolbachia* cannot be grown under laboratory conditions. However, *Wolbachia* strain wAlbB, host of the mosquito *Aedes albopictus*, can be grown in association with the C6/36 cell line and has been used as a laboratory model system (Xi *et al.*, 2005). The accessibility to C6/36 (wAlbB) cell line and *Wolbachia*-specific antibodies has facilitated high-throughput cytometric pre-screening of 1.3 million compounds (library AstraZeneca) (Clare *et al.*, 2019). This screening is the first step of the A-WOL for development of an anti-*Wolbachia* drug. The second step consists of *in vivo* tests using the murine experimental model *B. malayi* and *L. sigmodontis* (Johnston *et al.*, 2014). Two tylosin A analogs, A-1535469 and A-1574083, have been selected and represent

better results than second-generation tetracycline antibiotics on two murine models (*B. malayi* and *L. sigmodontis*) and one bovine model (*O. ochengi*) (Taylor *et al.*, 2019).

A-1574083 (now named ABBV-4083) has been selected for evaluation as a preclinical drug candidate (Taylor *et al.*, 2019). Another study has focused on quinazoline series present in the screening of 300,368 small molecules using primary cell-based high-content imaging screen associated with an orthogonal *ex vivo* validation assay (conducted on *B. pahangi*) (Bakowski *et al.*, 2019). Two clinical candidates, CBR417 and CBR490, have been identified as anti-*Wolbachia* quinazoline associated with *in vitro* safety profiles (Bakowski *et al.*, 2019). In summary, the past few years have seen a great deal of progress toward new candidate drugs targeting *Wolbachia*. However, key questions remain to be addressed, in particular regarding the underlying mechanisms of this obligate association, before clinical translation is achieved.

Entomopathogenic nematode symbionts

Photorhabdus and *Xenorhabdus* symbionts are known to produce a variety of compounds with insecticidal, acaricidal, antimicrobial, and nematocidal properties (Stock *et al.*, 2017; Dreyer *et al.*, 2018). Since the first discovery of antibiotics from these symbionts (Paul *et al.*, 1981), an increasing number of publications have arisen in relation to the discovery and bioactivity of various compounds from different species and strains (Webster *et al.*, 2002). Most of these compounds are produced either by non-ribosomal peptide synthetase (NRPS) or polyketide synthase (PKS) (Tobias *et al.*, 2017).

Bioactive compounds produced by *Xenorhabdus* include depsipeptides, xenocoumacins, peptide-antimicrobial-*Xenorhabdus* (PAX) peptides, indole-containing compounds, benzylideneacetone, and bacteriocins (Dreyer *et al.*, 2018). Several cell-free filtrates from *Xenorhabdus bovienii* YL002 culture resulted in more than 98% control of *Botrytis cinerea*, which causes grey mold rot in tomatoes, and *Phytophthora capsici*, which causes leaf scorch on peppers (Fang *et al.*, 2011). Similarly, cell-free conditioned medium of *Xenorhabdus budapestensis* and *Xenorhabdus szentirmai*

were proved effective against important agricultural pathogens, including *Xanthomonas* spp., *Erwinia amylovora*, *Pythium* spp., *Phytophthora* spp., and *B. cinerea* (Fodor *et al.*, 2012). Various species of *Xenorhabdus* and *Photorhabdus* were tested for their antifungal activity against *Monilophthora roreri*, the causal agent of frosty pod rot in cocoa. Exudates from some of the tested *Xenorhabdus* and *Photorhabdus* resulted in 70% antifungal activity, which is significantly high compared with commonly used commercial fungicides (San-Blas *et al.*, 2012). Recently, *Xenorhabdus* has been found to display miticidal activity against *Tetranychus urticae*. Cell-free supernatants of *Xenorhabdus szentirmaii*, *X. nematophila*, *X. bovienii*, *X. cabanillasii*, *Photorhabdus luminescens*, and *P. temperata* were screened against *T. urticae*, a plant-feeding mite, and, of all the tested bacteria, *X. szentirmaii* and *X. nematophila* resulted in a 90% reduction of the mites (Eroglu *et al.*, 2019). In some cases, the active compounds responsible for bioactivity against various agricultural pests were isolated and purified from *Xenorhabdus* strains. For example, a bioactive compound bicornitun, isolated from *X. budapestensis*, was found to be effective against *Phytophthora nicotianae* and *Erwinia amylovora* (Boeszoermenyi *et al.*, 2009). Xenorhabdins isolated from different *Xenorhabdus* strains resulted in 100% mortality of larvae of *Helicoverpa punctigera*, which is a major insect pest of cotton and other crops in Australia (McInerney *et al.*, 1991). In another study, indole derivatives and xenorhabdins were isolated from *X. bovienii* A2, indole derivatives effective against *B. cinerea* and *Phytophthora infestans* (Li *et al.*, 1995). A compound with antibacterial activity was isolated from *X. nematophila* K1 and it was found that the compound was benzylideneacetone, which has been commercially available from Sigma-Aldrich for a long time. Benzylideneacetone was active against important plant pathogens, including *Agrobacterium vitis*, *Pectobacterium carotovorum* subsp. *atrosepticum*, *Pseudomonas syringae* pv. *tabaci*, *Ralstonia solanacearum*, and *Pectobacterium carotovorum* subsp. *carotovorum* (Ji *et al.*, 2004). Two antibacterial peptides, GP-19 and EP-20, were isolated from *X. budapestensis* NMC-10. Among the two peptides, GP-19 had broad-spectrum activity against bacteria and fungi, including *Xanthomonas campestris* pv. *vesicatoria*, *Pseudomonas solanacearum*, and *Verticillium dahlia*, whereas EP-20

was very effective against *P. capsici* only (Xiao *et al.*, 2012). Another study, aimed at screening for antibiotics in *Xenorhabdus nematophila* YL001 against plant pathogens, resulted in identification of three compounds, of which nematophin displayed broad-spectrum antifungal activity and was found to be as effective as chemical fungicide (carbendazim) against *Rhizoctonia solani*, which has a wide host range and worldwide distribution (Zhang *et al.*, 2019).

Photorhabdus species are known to produce anthraquinones, stilbenes, indole, photobactin, carbapenem, and peptides (Bode, 2009; Stock *et al.*, 2017). Antimycotic activity of *Photorhabdus luminescens* was tested against 32 fungal species, of which *P. luminescens* was 100% effective against *B. cinerea*, *Ceratocystis ulmi*, *Ceratocystis dryocoetidis*, *Mucor piriformis*, *Pythium coloratum*, *Pythium ultimum*, and *Trichoderma pseudokoningii* (Chen *et al.*, 1994). Similarly, metabolites extracted from the cultures of *P. luminescens* effectively suppressed *Phytophthora cactorum* and *Fusicladosporium effusum*, which are fungal pathogens of pecan (Shapiro-Ilan *et al.*, 2009). The efficacy of bacterial metabolites is comparable with chemical fungicides, dodine and fenbuconazole (Shapiro-Ilan *et al.*, 2009). Trans-cinnamic acid (TCA) purified from *P. luminescens* arrested the complete growth of *F. effusum*, the causal agent of pecan scab, at a very low concentration of 64 µg/ml. Moreover, TCA was toxic to *Colletotrichum gloeosporioides*, *C. fragariae*, and *C. acutatum*, which are very important plant pathogens (Bock *et al.*, 2014). However, in a recent study, TCA alone was able to significantly inhibit the mycelial growth of *Pythium myriotylum* but not the zoospore germination of the pathogen (Shan *et al.*, 2020). A high-molecular-weight protein complex purified from *P. luminescens* was found to have insecticidal activity against *Manduca sexta*, *Galleria mellonella*, *Tenebrio molitor*, *Monomorium pharaonic*, *Blattella germanica*, and *Periplaneta americana* belonging to four orders (Bowen and Ensign, 1998). A very small compound benzaldehyde produced by *Photorhabdus temperata* M1021 displayed strong insecticidal activity against *G. mellonella* and antibacterial activity against *Phytophthora capsica*, *R. solani*, and *Corynespora cassiicola* (Ullah *et al.*, 2015). In addition to the metabolites with insecticidal and antimicrobial properties, 3,5-dihydroxy-4-isopropylstilbene (ST) and indole, which had

nematicidal activity, were purified from *P. luminescens* MD. ST resulted in 100% mortality of J4 and adult stages of *Aphelenchoides rhytium*, *Bursaphelenchus* spp., and *C. elegans*, but was not effective against *Meloidogyne incognita* (J2) or IJs of *Heterorhabditis megidis*. On the other hand, indole was lethal against J4 and adults of *Bursaphelenchus* spp., J2 of *M. incognita*, and IJs of *Heterorhabditis* spp. (Hu *et al.*, 1999). Additionally, 2-stilbenol isolated from *P. luminescens* was found to be more toxic to *Bursaphelenchus xylophilus* than ST (Hu *et al.*, 1996).

Along with the traditional identification and purification of secondary metabolites, with the advent of sequencing, genomes of various strains of *Photorhabdus* and *Xenorhabdus* became available. Analysis of these bacterial genomes revealed that more than 6% of their genomes contain gene clusters encoding secondary metabolites (Ogier *et al.*, 2010). Furthermore, comparative genomic analysis of seven *Photorhabdus* strains revealed the presence of up to 22 biosynthetic gene clusters (BGCs) encoding for several bioactive molecules in each strain (Bozhüyük *et al.*, 2017). A large-scale analysis linking the genomes and metabolomes of 25 *Xenorhabdus* strains and five *Photorhabdus* strains predicted the presence of BGCs ranging between 24 and 41 per each strain (Tobias *et al.*, 2017).

Despite discovering dozens of secondary metabolites with broad-spectrum bioactivity against important plant pests and pathogens from the symbionts of EPNs, not a single product is commercially available in the market for pest management (Stock *et al.*, 2017; Dreyer *et al.*, 2018). Decades of research on the bioactive molecules produced by *Xenorhabdus* and *Photorhabdus* established them as untapped solutions for pest and disease control in agricultural along with health care (Dreyer *et al.*, 2018). Along with the purification and characterization of bioactive molecules, another crucial requirement for commercialization is scaling-up the production to industrial scale. Some studies have been carried out in this regard by manipulating the growth conditions (Wang *et al.*, 2008; Sa-uth *et al.*, 2018), homologous expression in yeast (Schimming *et al.*, 2014), exchange of native promoters, and manipulating the regulatory proteins (Booyesen and Dicks, 2020). Exchanging the native promoter with arabinose-inducible

promoter P_{BAD} showed a dramatic increase in the production of bioactive compounds in *Xenorhabdus* and *Photorhabdus* (Bode *et al.*, 2015). In addition to promoters, knowledge on regulatory proteins modulating gene expression is necessary to increase the production of bioactive molecules. However, very limited research has been conducted in this regard in *Xenorhabdus* and *Photorhabdus* (Engel *et al.*, 2017; Booyesen and Dicks, 2020). In future, extending the testing of already discovered bioactive molecules' efficacy against a broad range of plant pathogens and pests in greenhouses and pilot field trials, as well as testing their biosafety against non-target organisms coupled with scaling-up the production, would result in sooner availability of these products.

Plant parasite symbionts

With respect to plant-parasitic nematodes and their bacterial endosymbionts, their role in host virulence yet remains to be answered. Furthermore, investigations should focus on the role of symbionts in the evolution of nematicide resistance and the effect of nematicides on both the nematodes and their symbiotic partners. There is also a dearth of knowledge about the roles of the endosymbionts of plant parasites in the context of complex soil communities. Understanding multitrophic interactions and the role of symbiont passage through plants deserves further investigation.

Emerging Tools for the Study of Parasitism and Pathogenesis of Nematode–Bacteria Partnerships

Model organisms play an essential part in research, in particular for the understanding of biological processes. In this respect, and over the past 50 years, the free-living soil bacterivore nematode, *C. elegans*, has been one of the best model organisms for understanding the biology of all animals, including humans. Furthermore, *C. elegans* represents a pillar for facilitating biomedical research (Baumeister and Ge, 2002; Wangler *et al.*, 2017). In addition to possessing all

model traits including a short life cycle and amenability to experimental and genetic manipulation, *C. elegans* is an outstanding model organism for research in the post-genomic era. For example, Sonnhammer and Durbin (1997) found that approximately 65% of the human genes involved in disease have a counterpart in this nematode. Studying genes simultaneously in humans and model organisms can provide insights into a particular disease and an understanding of the pathological processes (Bargmann, 2001). Phenotyping the morphology and behavior of mutants can also facilitate the establishment of cell lineage maps that can be used to study cancer, neurological disorders, and other diseases, and lead to medical diagnoses and to the discovery of treatments and therapies (Levitán *et al.*, 1996; Hengartner, 2001; Cheong *et al.*, 2015; Gahl *et al.*, 2016).

New technologies and increased affordability of next-generation sequencing have facilitated complete genome analyses of many nematode parasitic species. In the early 2000s, scientific initiatives such as WormBase, a central data repository for information about nematodes (<https://parasite.wormbase.org/> accessed 17 March 2022), generated 134 genomes of parasitic species (Harris *et al.*, 2004). Additionally, the 950 nematode genomes initiative was launched to share and collectively enter genomes for sequencing and make the data available for analyses (Kumar *et al.*, 2012).

More recently, large-scale whole-genome comparative analyses (including 36 published genomes and 31 new taxa assemblies) have provided a framework for identifying common strategies of infection in parasitic taxa (Coghlan *et al.*, 2019). Parasitic nematodes are particularly challenging to study, due to the complexity of their lifestyles; however, a few species have been developed as experimental models (Bain and Philipp, 1991; Hoffmann *et al.*, 2000; Stock, 2005; Allen *et al.*, 2008). For example, filarial species such as *B. malayi*, *B. pahangi*, *L. sigmodontis*, *O. ochengi*, *D. immitis*, and *A. viteae* (Fig. 13.3) have helped to improve our understanding of the diseases they cause and have also allowed identification of new drug candidates for cure or ease of the diseases (Hoffmann *et al.*, 2000; Allen *et al.*, 2008; Bouchery *et al.*, 2013).

Genome sequencing has generated unique insights into how parasitic nematodes have

adapted to their various niches and have become dependent on their hosts by surrendering their ability to synthesize nutrients *de novo* for less energy-intensive scavenging mechanisms (Ghedini *et al.*, 2007; Desjardins *et al.*, 2013).

However, there is a vast gap between the multi-omics approaches explored for *C. elegans* when compared with those available for parasitic species (Ma *et al.*, 2020). The increase of draft genomes and transcriptome datasets from parasitic nematodes available provides a solid base for mass spectrometry-based proteomic and lipidomic studies. For example, the recent characterization of glycosphosphatidylinositol (GPI) anchored proteins in the genome of *B. malayi* has provided a venue for studying their role in a wide variety of biological processes during the nematodes' life cycle (Mersha *et al.*, 2019).

Furthermore, over the past decade, metabolomics has emerged as a powerful tool to study parasites and host-parasite relationships (Ma *et al.*, 2020). This approach also holds promise for the development of biocontrol, therapeutics, and diagnostics. For example, genome-scale metabolic networks of *B. malayi* have been used to identify stage-specific metabolic dependencies and predict drug targets (Curran *et al.*, 2020).

Similarly, in the EPN *Steinernema*, a transcriptome analysis of genes involved in heat stress response has provided further insights into temperature adaptations in these nematodes. For example, a study by Yaari *et al.* (2016) showed a large fraction of downregulated genes in the desiccation-tolerant nematode *S. riobrave*, whereas a larger fraction of the genes in a heat-susceptible species, *S. feltiae*, were upregulated. This study also revealed many genes and metabolic cycles differentially expressed in the stressed nematodes, many of which are new and should be further investigated for their involvement in desiccation and heat tolerance (Yaari *et al.*, 2016).

Overall, the study of host-parasite interactions involves consideration of the complex interplay of the parasites, their hosts and, in many instances, other protagonists such as symbionts. Thus, consideration of omics-focused research opens the doors for an in-depth and multi-angle approach for studying parasitic nematodes and identifying novel markers of infections as well as for the discovery of new drugs and for the management of pests.

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14 Genetic Improvement of Beneficial Organisms

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Introduction

Global losses due to pests can vary from about 26% to 80%, depending on the type of crop (Oerke, 2006). Chemical pesticides are commonly used to fight this problem but they pose threats to humans and wildlife, and might have an adverse impact on soil fertility by killing beneficial microorganisms (Aktar *et al.*, 2009). Other strategies rely on biological control agents. Some key players in the biological control field are microbial agents, i.e., viruses, bacteria, fungi, protozoa, and nematodes (Hazir *et al.*, 2004; Barratt *et al.*, 2018; van Lenteren *et al.*, 2018).

Among the most potent biological control agents are the entomopathogenic nematodes (EPNs) from the families Steinernematidae and Heterorhabditidae. The EPNs are referred to as parasitic nematodes, because they invade and feed on their insect host. They have only one free-living stage that is adapted for survival outside of the insect host (Griffin, 2012). However, EPNs differ from other insect-parasitic nematodes due to their symbiotic relationships with insect-pathogenic bacteria, and the special mechanisms that enable them to associate with the bacteria and transmit them into the insect host. This association causes the insect's rapid

death, within 48–72 h (Dillman *et al.*, 2012). The unique life cycle of EPNs and their symbiotic bacteria has led to great interest in these organisms as microbial control agents. Their relevant attributes include ease of mass production, ease of application with standard spraying techniques, broad host range documented for more than 200 insect species, rapid killing of the host, non-toxicity to vertebrates, and low environmental risk. Alongside these advantages, there are some difficulties with the commercial production of EPNs, which could otherwise compete with chemical pesticides in terms of production costs and residual pesticide activity after application. These difficulties stem mainly from EPNs' sensitivity to heat and desiccation stresses (Salame *et al.*, 2010; Harvey *et al.*, 2016). Their lack of tolerance to extreme environmental conditions directly influences the shelf life, quality, and field performance of nematode-based products. Moreover, the application of nematode-based products is limited mainly to protected and hidden habitats, which are less exposed to harsh environmental conditions. Thus, despite numerous successes in biological control, the use of EPNs remains far less than competing tactics such as the application of chemical insecticides (Shapiro-Ilan *et al.*, 2020). Deficiencies in beneficial organisms have been addressed via

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genetic improvement techniques, which are the focus of this chapter.

The use of genetic manipulation to improve beneficial arthropods, such as silkworms and honeybees, has been conducted for hundreds of years (Yokoyama, 1973; Hoy, 1990).

During the past century, genetic improvement programs (GIPs) have provided innovative methods for controlling insect pests (Beckendorf and Hoy, 1985; Hoy, 1985, 1986). Beneficial insects, as well as other arthropods, used as biological control agents have been selected for climate tolerance (Wilkes, 1942; White *et al.*, 1970), host-finding ability, host preference (Allen, 1954; Box, 1956), improved sex ratio (Simmonds, 1947; Wilkes, 1947), increased fecundity (Wilkes, 1947; Ram and Sharma, 1977), and resistance to insecticides (Pielou and Glasser, 1952; Roush and Hoy, 1981; Hoy, 1984; Hoy *et al.*, 1988; Havron *et al.*, 1991a,b; Hoy and Cave, 1991).

As the use of EPNs for biological control of insect pests becomes more widespread due to improvements in production methods (Grewal *et al.*, 2005; Shapiro-Ilan *et al.*, 2012), the use of powerful genetic tools to improve their performance has been strongly advocated (see reviews by Gaugler, 1987; Fodor *et al.*, 1990; Segal and Glazer, 1998, 2000). However, unlike the long history and vast research on the use of beneficial insects for biological control, the use of EPNs is only in its third decade; consequently, research and development of GIPs for EPNs is in its infancy. Nonetheless, throughout the past decade nematodes have been used to study general environmental, biological, physiological, and molecular processes, as reflected in the various chapters of this volume; this vast knowledge can be utilized to improve the species of nematodes known to be beneficial for use in agriculture.

This chapter will summarize progress in genetic improvement in steinernematid and heterorhabditid EPNs and refer to technologies that may advance this approach. It will present the different approaches that can be used toward genetic improvement and then provide a series of case studies for improving EPN efficacy and commercial suitability. The chapter also includes a section on the importance of maintaining trait stability in the culturing and improvement of EPNs.

To consider a GIP, one must identify the traits that need to be improved. In general, two directions for EPN improvement have been suggested. The first is enhancement of EPN field efficacy; that is, by improving their infectivity to target pests or their ability to overcome environmental factors affecting their performance and biocontrol consistency (Gaugler, 1987; Segal and Glazer, 2000). The second is enhancement of their commercial suitability, i.e., increased production efficiency and consistency as well as shelf-life stability (Strauch *et al.*, 2004; Sumaya *et al.*, 2017). These goals need to be “translated” into defined procedures that can be used in a GIP. Knowledge of the genetic, molecular, and physiological architecture of particular traits related to the improvement can be an essential prerequisite to ensure successful GIP.

Approaches to Genetic Improvement

The goal of GIPs can be to enhance single or multiple traits. For example, in reference to EPNs, traits of interest include virulence, reproductive capacity, environmental tolerance, etc. Genetic improvement of targeted traits can be achieved through several approaches, including discovery, selection, hybridization, or transgenic manipulation (Gaugler, 1987; Shapiro-Ilan *et al.*, 2020). Given that all of these approaches have been successfully studied and implemented for EPNs supports the premise that this group of nematodes serves as a model for other GIPs to enhance other organisms.

Discovery

If the EPN strains or species that are available commercially or among laboratory collections do not provide sufficient biocontrol efficacy, then a simple approach is to discover new strains or species that possess superior biocontrol traits. Discovery of new strains or species can be accomplished through surveys using the insect-baiting technique (Bedding and Akhurst, 1975; Shapiro-Ilan *et al.*, 2003; Stuart *et al.*, 2004). In this approach, soil is collected and baited with host insects, such as the greater wax moth *Galleria mellonella*. Using a highly susceptible host, such

as *G. mellonella*, allows one to cast a wide net in isolating a broad range of EPNs. However, if one is seeking EPNs that are adapted and exhibit improved virulence to a particular target pest, it may be advantageous to use that host also as the bait insect (Shapiro-Ilan *et al.*, 2003).

Discovery of new EPN species has led to improved biocontrol efficacy. For example, relative to previously tested EPNs, the discovery of *Steinernema riobrave* greatly increased control levels of the citrus root weevil, *Diaprepes abbreviatus*, and plum curculio, *Conotrachelus nenuphar* (Lacey and Shapiro-Ilan, 2008). Beyond discovery of new EPN species, discovery of new strains (within a species) can be highly beneficial, as strains can vary substantially in virulence, environmental tolerance (e.g., temperature and UV tolerance), and other traits (Stuart *et al.*, 2004; Shapiro-Ilan *et al.*, 2014a, 2015). Additionally, EPNs with targeted improved traits can be sought by selecting the survey sites accordingly. For example, improved heat tolerance in EPNs was discovered in strains isolated from a hot and arid region of Israel (Glazer *et al.*, 1996). Survey and discovery can also be used to expand the genetic diversity of an EPN species (Stuart *et al.*, 2004) and thereby avoid genetic bottlenecks, improve the capacity for genetic selection, and enhance stability (see below).

Selection

Directed selection is one option for improving EPNs. The process entails exposing a diverse population to selection pressure for a particular desired trait. Selection has the advantage of being a simple and straightforward approach and is often effective. However, a potential drawback from the approach is reversion; a trait may revert back to its original state once selection pressure is removed (and thus this must be monitored). Also, it is possible that one beneficial trait is increased due to the selection regime, but another beneficial trait may inadvertently be reduced in potency (Gaugler *et al.*, 1990).

A variety of beneficial traits have been improved using the selection approach, including host-finding (Gaugler *et al.*, 1989, 1991; Salame *et al.*, 2010), efficacy (Tomalak, 1994a,b), nematicide resistance (Glazer *et al.*, 1997),

environmental tolerance to heat and or desiccation (Ehlers *et al.*, 2005; Salame *et al.*, 2010), sensitivity to volatile cues (Hiltpold *et al.*, 2010b), and dispersal (Bal *et al.*, 2014). More details on some of these studies may be found below under case studies.

Prior to any selection process for genetic improvement, it is essential to determine the genetic variation of the particular trait (expressed in terms of heritability, h^2) present in the population. Glazer *et al.* (1991) assessed the genotypic variation among infective juveniles (IJs) of *Heterorhabditis bacteriophora* strain HP88 under heat, desiccation, and ultraviolet light by comparing the performance of inbred lines of this nematode in laboratory assays. Considerable variation in all three traits was detected among the different inbred lines. The heritability values for heat and ultraviolet tolerance were high ($h^2 = 0.98$ and 0.66 , respectively), indicating that selection should be an efficient way of improving these traits in the population. The results for desiccation tolerance varied considerably within each line. The heritability value was low ($h^2 = 0.11$), indicating that the results were influenced mainly by environmental variation and suggesting that selective breeding for higher desiccation tolerance would be inefficient. Improvement through the induction of mutations might be a better approach.

In another example, to improve the production of *H. bacteriophora* in liquid culture, Johnigk *et al.* (2002) determined the heritability of the disposition to recover in 30 homozygous inbred lines, which were established by inbreeding over seven generations. The h^2 values of IJ recovery, as well as final yield, were determined in liquid culture. The proportion of IJs that recover from the infective stage to the developmental reproductive parasitic stage varies considerably in liquid culture, thus affecting the consistency of production. The calculated heritability for IJ recovery was low ($h^2 = 0.38$). No significant genetic variability could be detected for this trait. In contrast, high heritability ($h^2 = 0.90$) was found for the total number of IJs produced in the liquid medium. Also, Ehlers *et al.* (2005) attempted selection of *H. bacteriophora* for heat tolerance and cold activity. Analysis of heritability showed a high value ($h^2 = 0.68$) for heat tolerance and a low value ($h^2 = 0.38$) for activity at low temperature.

Hybridization

Hybridization is another non-molecular approach that can be utilized in GIPs. This approach entails controlled crosses followed by screening of the progeny for desired traits. The controlled crosses can be conducted under sterile conditions on artificial media (in the presence of the EPN symbiotic bacteria). Hybridization is an attractive tool for GIPs, because it is relatively simple and straightforward and results in fixed genetic traits. A downside is that improvement is limited to the extremes in beneficial traits that are present in the natural populations in hand (this is true for the selection approach as well). One can think of a classical breeding program akin to any other beneficial organism in agriculture (livestock, grain, fruits and vegetables, etc.). Conceivably, a comprehensive breeding/hybridization program could also be implemented for EPNs, though thus far such a scale has not been explored.

The first example of hybridization in EPNs was accomplished by Shapiro *et al.* (1997) in which heat tolerance was transferred from one *H. bacteriophora* strain to another. In heterorhabditids, because both hermaphroditic and amphimictic forms exist, precautions should be taken to ensure that controlled crosses result in progeny that resulted from mated individuals rather than self-replication. Shapiro *et al.* (1997) overcame this obstacle by using marker mutations to identify progeny that arose from two parents versus hermaphroditic selfing.

Steinernematids only possess amphimictic forms (with a lone exception in *Steinernema hermaphroditum*) (Stock *et al.*, 2004) and therefore hybridization is more straightforward in this genus. The first example of hybridization for genetic improvement in steinernematids was reported by Shapiro-Ilan *et al.* (2005); in this example, hybridization was used to combine virulence and environmental tolerance (to heat and desiccation) among *Steinernema carpocapsae* populations.

Mutagenesis and genetic engineering approaches

One of the most powerful tools for genetic analysis and improvement is the induction of mutations

(Fodor *et al.*, 1990). This approach is more complex than the genetic improvement approaches described above. However, unlike the other approaches (discovery, selection, hybridization), mutagenesis and genetic engineering have the advantage of extending the improvement program beyond the limits of the natural gene pool. The bulk of mutagenesis and genetic engineering studies have focused on *Heterorhabditis* spp. more so than on *Steinernema* spp. Advances made in mutagenesis and genetic engineering are captured in the remainder of this section.

The first induction and characterization of mutants of *H. bacteriophora* were reported by Zioni *et al.* (1992). A homozygous inbred line was used as the base population for mutagenesis and genetic analysis. Mutagenesis was induced by exposing young hermaphrodites to 0.05 M ethyl methanesulfonate (EMS), and a dumpy (dpy) mutant (*Hdpy-1*) was isolated. Morphological analysis revealed distortion of the head region in adults as well as in IJs. Backcrosses with the wild-type population and genetic analysis revealed that the mutation is recessive. Later on, more recessive dpy mutants (*Hdpy-2* and *Hdpy-3*) were isolated and characterized (Koltai *et al.*, 1994). Complementation tests indicated that each of the three mutations affects different genes. The *Hdpy-2* mutant was used as a genetic marker to validate crosses between heat-tolerant and heat-sensitive strains (Shapiro *et al.*, 1997) as described above.

Tomalak (1994c) described the first morphological and behavioral mutant in *Steinernema feltiae*. The mutation was spontaneous and occurred in a single gene locus designated *Sfdpy-1*. Action of the new allele was only clearly expressed in IJs. The resulting morphology was classified as “dumpy” due to the significantly reduced ratio of the nematode’s body length to maximum diameter. The identified gene was sex-linked and the new mutant allele remained recessive to the wild-type counterpart responsible for normal morphology. Aside from altering the morphology, pleiotropic action of the dpy allele affected nematode movement and infectivity to insect hosts. The mean dispersion distance of mutant juveniles and their infectivity to *G. mellonella* and *Lycoriella solani* larvae were significantly reduced compared with those of wild-type nematodes. Revertant individuals that were occasionally isolated from dpy populations

regained the ability to move quickly and dispersed even further than the juveniles from the parental strains. They were also more effective at penetrating the hemocoel of *L. solani* larvae. However, the numbers of infected insects did not significantly differ from those observed for wild-type ScP and SN strains. Following this study, Tomalak (1997) described morphological (Tomalak and Mráček, 1998) and genetic analyses of eight additional mutants of *S. feltiae*. These mutations were also found to be recessive and sex-linked.

Sumaya *et al.* (2017) reported on the use of EMS mutation to improve oxidative stress tolerance of *H. bacteriophora* IJs. The EMS mutant with high longevity was generated and one of the derived mutagenized inbred lines (MOX-IL6) survived 5.8 days and 28.4 days longer than its donor line (IL3) under oxidative stress and control conditions, respectively. A genetic cross between the mutagenized inbred line and its donor line (MOX-IL $\times$ IL3) still survived 2.5 days and 18.5 days longer than the donor line under oxidative stress and control conditions, respectively. Concerning virulence and reproductive potential, trade-off effects were not observed as a result of hybridization and mutagenesis.

In recent years, a new tool for the induction of mutations and genetic modifications has been developed, particularly for *Caenorhabditis elegans* research. Ciche and Sternberg (2007) developed the use of RNAi in heterorhabditid nematodes. This approach was further used by Moshayov *et al.* (2013) to silence gene that are presumably related to the recovery process from the infective to parasitic stage.

A method to edit the *C. elegans* genome using the clustered, regularly interspersed, short palindromic repeats (CRISPR) RNA-guided Cas9 nuclease has been developed (Dickinson *et al.*, 2013; Friedland *et al.*, 2013). Cas9 was able to induce DNA double-strand breaks with specificity for targeted sites, and these breaks could be efficiently repaired by homologous recombination. By supplying engineered homologous repair templates, the researchers generated *gfp* knock-ins and targeted mutations. The results outline a flexible methodology to produce essentially any desired modification in the nematode genome quickly and at low cost. This technology is an important addition to the array of available genetic techniques and can be utilized in EPNs.

Genomic and bioinformatic tools are now available for whole-genome analysis. Bai *et al.* (2007, 2013) compared *H. bacteriophora* GPS11 expressed sequence tags (ESTs) with the ESTs of animal-parasitic, human-parasitic, plant-parasitic, and free-living nematodes: 127 of them were identified as previously undescribed ESTs, of which 119 had homologs in ESTs and eight had homologs in proteins of free-living nematodes. These ESTs were assigned putative functions in transcription, signal transduction, cell-cycle control, metabolism, information processing, and cellular processes, thereby providing better insight into *H. bacteriophora* metabolism, sex determination, and signal transduction. In addition, 36 *H. bacteriophora* ESTs had significant similarities to ESTs of parasitic nematodes, but not to ESTs or proteins of free-living nematode species. Among these were ESTs encoding a centrin, an ankyrin-repeat-containing protein, and a nuclear hormone receptor. The analysis also revealed that parasitic nematode-specific ESTs in this *H. bacteriophora* dataset have more homologs in animal-parasitic nematodes than in those parasitizing humans or plants.

Rougon-Cardoso *et al.* (2016) obtained a high-quality draft of the *S. carpocapsae* genome comprising 84,613,633 bp in 347 scaffolds, with an N50 of 1.24 Mb. To improve annotation, they sequenced both short and long RNA and conducted shotgun proteomic analyses. *S. carpocapsae* shares orthologous genes with other parasitic nematodes that are absent in the free-living nematode *C. elegans*; it has ncRNA families that are enriched in parasites, and expresses proteins putatively associated with parasitism and pathogenesis, suggesting an active role for the nematode during the pathogenic process.

Dillman *et al.* (2015) sequenced and analyzed the draft genomes and transcriptomes of the entomopathogenic nematode *S. carpocapsae* and four congeners (*Steinernema scapterisci*, *S. monticolum*, *S. feltiae*, and *S. glaseri*). They used these genomes to establish phylogenetic relationships, explore gene conservation across species, and identify genes uniquely expanded in insect parasites. Protein domain analysis in *Steinernema* revealed a striking expansion of numerous putative parasitism genes, including certain protease and protease-inhibitor families, as well as fatty acid-binding and retinol-binding

proteins. Stage-specific gene expression of some of these expanded families further supports the notion that they are involved in insect parasitism by *Steinernema*. They identified a set of expanded gene families that are likely to be involved in parasitism, as well as a set of non-coding motifs associated with groups of orthologous genes in *Steinernema* and *Caenorhabditis* involved in neurogenesis and embryonic development that are likely part of conserved protein–DNA relationships shared between these two genera.

Tyson *et al.* (2012) conducted a molecular analysis of desiccation-tolerance mechanisms in the anhydrobiotic nematode *Panagrolaimus superbus* using ESTs, and revealed a series of candidate genes that may have an important role in stress-tolerance mechanisms (see also Chapter 9, this volume, on stress tolerance of nematodes). In addition, transcriptomic analysis of *H. bacteriophora* (Adhikari *et al.*, 2009) and *S. carpocapsae* (Hao *et al.*, 2010) listed genes with relevance to stress tolerance. Yaari *et al.* (2015) analyzed the transcriptomes of various steinernematid species with various tolerance capabilities to desiccation and heat stresses. This accumulated information will provide the basis for improvement of EPNs by genetic engineering.

Hashmi *et al.* (1995, 1998) used genetic engineering as a means of improving heat tolerance of *H. bacteriophora*. They reported the first successful transformation of an EPN. Foreign genes were introduced into *H. bacteriophora* strain HP88 by microinjection using vectors carrying the *C. elegans* genes coding for the roller phenotype and the 16 kDa heat shock protein (*hsp16*). A translational fusion made by inserting *lacZ* in-frame into *hsp16* was expressed in the body musculature, hypodermis, and pharyngeal muscles. Transcription of the *hsp16/lacZ* transgenes resulted in the rapid synthesis of detectable levels of β -galactosidase. In another study (Hashmi *et al.*, 1998), successful transformation of the *hsp70* gene was confirmed by Southern blot hybridization and polymerase chain reaction (PCR). Blot studies showed that the transgenic nematodes contain five to ten copies per genome of the introduced *hsp70*. Transcripts of *hsp70* mRNA were detected in both wild-type and transgenic nematodes. Transcripts increased several-fold in transgenic nematodes upon heat shock. IJs of both transgenic and wild-type nematodes were exposed to a sublethal

heat treatment (35°C) for 2 h followed by a normally lethal heat treatment (40°C) for 1 h. More than 90% of the transgenic nematodes survived the heat treatment, compared with 2–3% of the wild-type strain. Overexpression of *hsp70* resulted in an enhanced thermotolerance in the transgenic nematodes, which displayed normal growth and development. Furthermore, the transgenic strain was released in turf grass field microplots in the spring, summer, and fall of 1996 (Gaugler *et al.*, 1997), in accordance with the regulatory procedures at the federal, state, university, and local levels needed for field release in the USA. As predicted, persistence of the transgenic and wild-type strains did not differ. This risk-assessment study supports the view that the transgenic nematode strain is an unlikely environmental threat.

Vellai *et al.* (1999) reported successful transformation of the yeast desiccation-related gene encoding trehalose-6-phosphate synthase into the nematode *S. feltiae*. The transformed lines were able to survive well in increased concentrations of NaCl in M9 storage solution, while rapid mortality was recorded in the wild-type population. This study demonstrated the ability to transform steinernematids. However, the bioassays verifying the increase in osmotic-pressure tolerance were performed with a population of adult nematodes, not with IJs. The authors also suggested using the LEA-encoding genes (Browne *et al.*, 2002; Gal *et al.*, 2004) as candidates for transformation, or the gene promoter, to enhance desiccation tolerance (Vellai *et al.*, 1999).

Case Studies: Improvement of Nematode Efficacy

The ability of steinernematids and heterorhabditids to achieve high levels of biocontrol efficacy is complex and relies on a variety of factors. Successful biocontrol applications are dependent on the EPN's ability to survive in the environment, locate and invade the insect, and overcome the host immune system (Shapiro-Ilan *et al.*, 2020). Each of these steps, and possibly some secondary ones, should be considered for improvement and must be studied to determine their effect on the overall efficacy process, before a GIP is initiated.

It is assumed that rapid location, invasion, and establishment of nematodes in their target host will increase overall efficacy and minimize the exposure of the nematodes to environmental hazards after application in the field.

The first target addressed for improvement was the host-finding ability of EPN. Gaugler *et al.* (1989, 1991) showed that sufficient phenotypical differences exist for host finding between geographic isolates of *S. carpocapsae* to expect a strong response to selection for this trait. Genetic variation was maximized by hybridizing ten genetically diverse isolates to create a foundation strain for selective breeding. Thirteen rounds of selection resulted in a 20- to 27-fold increase in host finding. Moreover, the proportion of IJs initiating positive chemotaxis increased from less than 33% to more than 80%. Nematodes failing to migrate out of the inoculation zone declined from 33% to 8% after six rounds of selection. Relaxing the selection pressure produced a gradual decrease in host finding. This regression, coupled with the high realized heritability for enhanced host finding (0.64), suggested that wild-type populations take a passive approach to host finding. The improved host finding in the new "G-13" strain was later shown to be correlated with improved chemotaxis to CO₂ (Gaugler *et al.*, 1991).

The selected strain for improved host finding (G-13) was compared with two wild-type strains—the "All" strain and the foundation strain from which the G-13 strain was derived—for changes in fitness (Gaugler *et al.*, 1990). Acquisition of enhanced host-finding abilities did not appear to be correlated with a serious reduction in overall fitness. Selection did not affect pathogenicity, mobility, sex ratio, or morphology. However, strain G-13 did show a gain of fitness with regard to host penetration and reproductive potential, and a loss of fitness for storage stability.

Tomalak (1989, 1994a) selected *S. feltiae* strain PL for improved efficacy against *Lycoriella solani* (Winn.) (Diptera: Sciaridae) by repeated passage through this fly's larvae *in situ* in compost. There was a fourfold improvement in infectivity of the selected strain (ScP) to *L. solani* in laboratory tests after 33 rounds of selection. The control potential of the genetically selected strain ScP was further evaluated for the management of *Lycoriella mali* (Fitch) (Diptera: Sciaridae) (Grewal *et al.*, 1993). Trials were conducted at

two commercial mushroom farms with high and low levels of fly infestation. The efficacy of strain ScP was compared with that of *S. feltiae* strain SN and the chitin-synthesis inhibitor diflubenzuron. At low densities of *L. mali*, the two strains did not differ in efficacy, both causing 85–94% reduction in fly populations. At high fly densities, and a mixed infestation with the phorid fly, *Megaselia halterata* (Wood) (Diptera: Phoridae), strain ScP caused 56–83% reduction in *L. mali* populations, whereas strain SN caused 51–73% reduction. Strain ScP persisted longer than strain SN. Tomalak (1994b) also reported on the genetic improvement of *S. feltiae* for control of the western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae). In this study, partially inbred lines and recombinant congenic strains were used. They were selected for high infectivity and small body diameter, showing a substantial increase (> 40%) in infectivity and efficacy.

Hiltpold *et al.* (2010a) selected the EPN *H. bacteriophora*'s higher responsiveness towards (*E*)- β -caryophyllene (E β C), a sesquiterpene that is emitted by maize roots in response to feeding damage by the western corn rootworm (WCR). E β C is normally only weakly attractive to *H. bacteriophora*, which is one of the most infectious nematodes against WCR. By selecting *H. bacteriophora* to move more readily along an E β C gradient, they obtained a strain that was almost twice as efficient in controlling WCR population in fields planted with an E β C-producing maize variety.

Salame *et al.* (2010) selected heterogeneous populations of *S. feltiae* for enhanced downward dispersal by forcing IJs to move through a sand column to reach larvae of last-instar *G. mellonella* placed at the bottom of the column. After 25 selection cycles, the majority (> 75%) of these nematodes were found at the layer close to the insects (27% in the non-selected wild-type). No reduction in fitness was detected in the selected populations. Santhi *et al.* (2016) used a genetic improvement approach to enhance the host-seeking ability of *S. carpocapsae* and *H. bacteriophora* IJs. The IJs were allowed to move through a 45 cm L-shaped tube toward red palm weevil larvae. The IJs that reached within 5 cm of the insect larvae were collected and reared. Selection cycles were repeated 15 times. The host-seeking ability of *S. carpocapsae* IJs was enhanced 11-fold (from 3.7% to 39.8% of all IJs

reaching proximity of the larvae) and 8.5-fold (from 2.3% to 19.7%) for *H. bacteriophora* after ten cycles of selection. Further selections (cycles 11–15) had no significant impact on improving the IJs' attraction to the target host. The selection also enhanced infectivity to *Rhynchophorus ferrugineus*, *G. mellonella*, and *Spodoptera littoralis*. Nevertheless, the selection process did not affect the fitness, measured by stress tolerance and reproduction. After selection for enhanced responsiveness to E β C response, a small but significant negative effect on infectiousness of the selected strains was observed. However, this drawback was readily outweighed by the improved ability to locate hosts in the field.

Case Study: Improvement of Survival and Persistence

Once EPNs are released into the field environment, they encounter numerous factors—including physical, chemical, and biological components—that affect their survival and activity as biological control agents (Shapiro-Ilan *et al.*, 2020). It is assumed that during the course of evolution, EPNs, like other terrestrial organisms, adopted unique survival mechanisms to resist environmental extremes. As the importance of EPNs as biological control agents rises, substantial information regarding their survival mechanisms is being published. Two traits in particular are considered to be most important: heat tolerance and desiccation (Glazer, 2002). For example, EPNs can only persist for 2–3 weeks in dry soil (Kaya, 1990; Kung and Gaugler, 1990) (see Chapter 9, this volume, for detailed studies on desiccation tolerance of EPNs, as well as molecular and genetic knowledge). Several researchers noted that enhancement of their ability to survive the environmental factors increased their efficacy (Gaugler, 1987; Segal and Glazer, 1998, 2000) (see also Chapter 9, this volume, on survival mechanisms of nematodes), and genetic improvement has been suggested as a means to achieve such enhancement.

Shapiro *et al.* (1997) demonstrated the ability to transfer heat tolerance between two strains of *H. bacteriophora*. Transfer of this trait was accomplished by mating the heat-tolerant strain IS-5 (Glazer *et al.*, 1996) with the laboratory

strain HP88. The hybrid nature of the progeny was confirmed with a mutant of the HP88 strain (*Hp-dpy-2*) as marker (Koltai *et al.*, 1994) and by backcrossing. Progeny from the cross were screened for heat tolerance by measuring survival after 2 h exposure to 40°C. After six passages through last-instar larvae of the wax moth *G. mellonella*, survival of the hybrid nematodes was significantly greater than that of strain HP88 and similar to that of strain IS-5. At 32°C, strain IS-5 and the hybrid killed *G. mellonella* larvae at a faster rate than strain HP88. Both strain IS-5 and the hybrid exhibited sensitivity to cold storage at 10°C. No differences were detected in reproductive potential.

In an attempt to increase heat tolerance of *H. bacteriophora*, Ehlers *et al.* (2005) conducted four rounds of selection, which increased the mean tolerated temperature from 38.5°C to 39.2°C. The mean temperature at which the IJs of *H. bacteriophora* were active could be reduced from 7.3°C to 6.1°C after five rounds of selection. However, for unknown reasons, the mean temperature of IJ activity rose during five additional rounds of selection to 7.1°C. Screening of different isolates of the symbiotic bacterium *Photobacterium luminescens* for growth at low temperatures resulted in several cold-adapted strains from North America, which reached considerable cell density at 6°C.

One of the major obstacles to using EPNs in commercial pest control is their limited shelf life (Strauch *et al.*, 2004). The longevity of the nematode IJs can be prolonged by inducing a quiescent state in which their metabolic activity is much reduced. In general, this is done by storage at low temperature (Grewal *et al.*, 2005). However, EPNs are often exposed to higher temperatures during transportation (Mukuka *et al.*, 2010b), which reduces their viability. Selective breeding for enhancement of desiccation and heat tolerance as a means of prolonging their shelf life, and their capacity for storage and transportation, has been suggested.

To extend the shelf life of a commercial strain of *H. bacteriophora*, Strauch *et al.* (2004) determined the genetic variability of its desiccation tolerance and exploited it for enhancement of this trait by breeding. A hybrid strain resulting from crosses of eight *H. bacteriophora* isolates from different geographic origins was used. The desiccation stress was induced by hygroscopic

polyethylene glycol (PEG 600) solutions, which lowered the water activity (a_w) of this solution, causing removal of water from the IJs. In this study, the influence of an adaptation phase on desiccation tolerance was also investigated. The lowest mean tolerated a_w value (0.85) was achieved with an adaptation phase of 72 h at an a_w value of 0.96. The variance of the desiccation tolerance increased with a reduction in a_w value during adaptation. The heritability of the trait, determined by using homozygous inbred lines, was 0.46 for non-adapted populations (directly exposed to low a_w values) and 0.48 for adapted ones. A negative heterosis effect could be observed for the desiccation tolerance, because nearly all of the inbred lines had higher tolerance to desiccation stress than the hybrid strain. Improvement of desiccation tolerance by breeding was only obtained when the adaptation process was included in the selection process, which was related to higher phenotypic variance in the populations after adaptation. A total of eight rounds of selection and breeding were carried out. Without previous adaptation, the mean tolerated a_w value remained almost constant between 0.94 and 0.93. In contrast, when the IJs were adapted prior to exposure to desiccation stress, the tolerated a_w values dropped continuously from 0.89 to 0.81.

Mukuka *et al.* (2010a,b,c,d,e) initiated a GIP for the improvement of heat and desiccation tolerance in *H. bacteriophora*. To enhance heat tolerance, they first characterized the diversity of this trait among 36 populations of *H. bacteriophora* isolated from diverse environments across the globe, as well as 18 hybrid or inbred strains of these bacteria (Mukuka *et al.*, 2010a,b). Five strains of *Heterorhabditis indica* and one of *H. megidis* were also included. Nematodes were tested with or without prior adaptation to heat at 35°C for 3 h. The mean tolerated temperature ranged from 33.3°C to 40.1°C for non-adapted populations, and from 34.8°C to 39.2°C for adapted ones. *H. indica* was the most tolerant species, followed by *H. bacteriophora* and *H. megidis*. No correlation was found between the assessed tolerance levels with and without adaptation to heat, implying that different genes are involved. Correlation between a strain's heat tolerance and the mean annual temperature in its place of origin was weak. A high variability in tolerance among strains and the relatively high heritability

($h^2 = 0.68$) of the heat tolerance recorded for adapted *H. bacteriophora* provide an excellent foundation for future selective breeding for improved heat tolerance in *H. bacteriophora*.

Desiccation tolerance was evaluated in 43 strains of *Heterorhabditis* spp. and 18 hybrid/inbred strains of *H. bacteriophora* (Mukuka *et al.*, 2010b). Dehydration conditions, measured as a_w values, were produced by treating IJs with different concentrations of the non-ionic polymer PEG 600. Significant interspecific variation was recorded between nematode strains and species. The mean tolerated a_w value (MW_{50}) ranged from 0.90 to 0.95 for non-adapted nematode populations and 0.67 to 0.99 for adapted ones. For selective breeding, only the 10% most tolerant individuals were used. The lowest a_w value tolerated by this 10% of the population (MW_{10}) ranged from 0.845 to 0.932 for non-adapted nematode populations and 0.603 to 0.950 for adapted ones. Adaptation significantly increased the desiccation tolerance and a weak correlation was recorded for tolerance with and without adaptation. The nematode strains that were most tolerant to heat or desiccation formed the basis for the foundation of a parental stock produced by cross-breeding and subsequent genetic selection for enhanced tolerance (Mukuka *et al.*, 2010c). In this study, *H. bacteriophora* heat tolerance and desiccation tolerance were significantly increased by cross-breeding tolerant parental strains and successive genetic selection. During the latter process, the selection pressure was constantly increased and only the most tolerant 10% of the nematode populations were propagated for further selection steps. Assessment of tolerance and selection for both traits were performed with and without prior adaptation to the stress conditions. Eleven rounds of selection were performed to increase heat tolerance. A final overall increase in mean heat tolerance of 5.5°C was achieved when the nematodes were pre-adapted to the heat stress. For non-adapted tolerance, an increase of 3.0°C (from 40.1°C to 43.1°C) was recorded. For comparison, a commercial strain had a mean tolerated temperature after adaptation of 38.2°C and of 36.5°C without adaptation. To assess the desiccation tolerance, the mean tolerated a_w value of a population was measured. Cross-breeding the most tolerant strains reduced the a_w value from 0.67 to 0.65 after adaptation, and from

0.9 to 0.7 without prior adaptation. A subsequent six rounds of selection could not increase the tolerance, regardless of whether the nematode had been adapted to the stress.

Monitoring beneficial traits such as infectivity is essential in attempts to genetically improve other traits by crossing tolerant strains or using selective breeding. The fitness of the above-described selected heat- and desiccation-tolerant hybrid strains was evaluated following the selection period (Mukuka *et al.*, 2010d,e), in terms of virulence, host penetration, and reproductive capacity, compared with the commercial strain EN 01 of *H. bacteriophora*. Only the heat-tolerant strains were superior or similar in fitness to strain EN 01. The strains with increased desiccation tolerance were generally less fit, possibly reflecting a trade-off effect of selection for desiccation tolerance. Hybrid strains selected for enhanced tolerance to a stress after adaptation to that stress generally ranked better in terms of fitness than those that were not adapted prior to stress exposure. This could be a result of pleiotropy. The commercial strain had the highest reproduction rate per mean number of nematodes penetrating the host insect, a result of automatic selection of inbred lines with high reproductive potential during the commercial production process in liquid culture.

The effect of stress exposure on the infectivity of heat- and desiccation-tolerant hybrid strains of *H. bacteriophora* was assessed against last instars of *G. mellonella* (Mukuka *et al.*, 2010e). Nematode IJs were exposed to desiccation stress at an a_w value of 0.85 for 24 h or to a temperature treatment at 40°C or 0°C for 24 h prior to inoculation of five IJs per insect. Hybrid strains resulting from crosses of the three very best heat- or desiccation-tolerant strains and crosses of heat-tolerant with desiccation-tolerant strains were compared with a commercial strain of *H. bacteriophora*. Exposure to desiccation stress caused a significant reduction in infectivity of all strains, not surpassing 25% mortality, except one strain that was not affected and achieved 37.5% mortality. Infectivity of untreated IJs of desiccation-tolerant hybrids differed significantly, with a mean infectivity of 54%, ranging from 33.8% to 89.6%. The mean mortality from infection with heat-tolerant hybrids was significantly higher (78.2%). Infectivity of the commercial strain and two other hybrids

was not affected by the heat treatment. Consequently, the authors concluded that the infectivity of heat-tolerant strains is not necessarily affected by low-temperature stress.

In order to provide fundamental data required for a genetic program to produce hybrids with improved stress tolerance and prolonged shelf life and soil persistence, Sumaya *et al.* (2017) evaluated the relation between dauer juvenile (DJ) oxidative stress tolerance and longevity in *H. bacteriophora* at 25°C and 7°C. A strong and significant correlation between DJ oxidative stress tolerance and longevity during storage in Ringer's solution was recorded. Phenotyping of these traits was performed for 40 *H. bacteriophora* wild-type strain and inbred line collections. At 25°C, the mean time survived (MTS₅₀) in Ringer's by 50% of the DJs ranged from 21 to 57 days, whereas under oxidative stress, survival was from 3 to 22 days. At 7°C, a maximum MTS₅₀ of 94 days was assessed when DJs were stored in Ringer's, while the maximum MTS₅₀ was only 25 days with oxidative stress induction. The heritability of DJ tolerance to oxidative stress, determined by using homozygous inbred lines, is high ($h^2 > 0.9$), an indication of a high probability for successful selective breeding. In a subset of preselected *H. bacteriophora* inbred lines, DJ oxidative stress tolerance correlated with the DJ survival (persistence) after application to sand ($R = 0.719$).

In an additional study by this group (Sumaya *et al.*, 2017) the improvement of *H. bacteriophora* DJ longevity was achieved by hybridization and mutagenesis. A hybrid pool deriving from two oxidative stress-tolerant and long-living parental strains was generated. This hybrid AU1 × HU2 survived 2.6 days and 18 days longer than its best parent under oxidative stress and control conditions, respectively. In addition to the natural genetic variability, an EMS-mutant pool (M-OXI) with high longevity was generated and one of the derived mutagenized inbred lines (MOX-IL6) survived 5.8 days and 28.4 days longer than its donor line (IL3) under oxidative stress and control conditions, respectively. A genetic cross between the mutagenized inbred line and its donor line (MOX-IL × IL3) still survived 2.5 days and 18.5 days longer than the donor line under oxidative stress and control conditions, respectively. Concerning virulence and reproductive potential, trade-off effects

were not observed as a result of hybridization and mutagenesis.

Salame *et al.* (2010) bred a heterogeneous population of the EPN *S. feltiae* for tolerance to both rapid and slow desiccation. The nematodes were selected for tolerance of rapid desiccation by exposing IJs to ambient conditions (22–25°C; 50–65% relative humidity (RH)) for 100 min. A survival rate of 80–90% was reached after ten selection cycles. To select for tolerance of slow desiccation, the IJs were exposed to 97% RH for 72 h, followed by exposure to 85% RH for an additional 72 h. A high survival rate (> 85%) was obtained after 20 selection cycles (10–15% survival of the non-selected wild-type). No reduction in fitness was detected in the selected populations. Nevertheless, the population selected for slow desiccation was more tolerant of heat stress than the foundation population.

H. bacteriophora was also subjected to selection for enhancement of nematocidal resistance (Glazer *et al.*, 1997). This is because, when applied to the soil, the IJs of this nematode may encounter residual nematicides that will hamper their survival and efficacy. The nematicides used were fenamiphos (an organic phosphate), oxamyl (a carbamate), and avermectin (a biological product). Estimates of heritability (h^2 values) for the three nematicides were 0.31, 0.71, and 0.46, respectively. After 11 rounds of selection for resistance to nematicides, resistance increased dramatically. For fenamiphos and avermectin, an eight- to ninefold increase in resistance was recorded, and a 70-fold increase was recorded for oxamyl. When selection was relaxed, resistance to oxamyl and avermectin was stable while a decrease was recorded with fenamiphos. Fitness was retained in all selected populations when evaluated for infectivity. Cross-resistance was displayed for some, but not all of the nematicides tested.

Trait Stability

A critical issue in any EPN biocontrol program or GIP is the maintenance of beneficial traits. Repeated culture of invertebrate or microbial biological control agents in laboratory or industrial settings can lead to deterioration of beneficial traits; these trait changes can arise due to

genetic processes (for example, inadvertent selection, inbreeding, or drift), or from non-genetic factors (for example, nutrition or disease) (Tanada and Kaya, 1993; Hopper *et al.*, 1993; Chaston *et al.*, 2011). Beneficial trait deterioration has been reported in EPN populations (Shapiro *et al.*, 1996; Stuart and Gaugler, 1996; Wang and Grewal, 2002; Bilgrami *et al.*, 2006). Shapiro *et al.* (1996) reported that the heat-tolerance trait in newly isolated *H. bacteriophora* strain IS-5 essentially remained stable after 12 passages of the culture in *G. mellonella*; however, the level of the trait was dependent on environmental conditions (rearing temperature). Wang and Grewal (2002) reported rapid deterioration of environmental tolerance (to heat, desiccation, and UV) and reproductive potential for *H. bacteriophora* strain GPS11 during maintenance in the laboratory.

In selection programs, genetic stability of genetically selected lines has been questioned and evaluated from the earliest attempts to improve nematode performance by genetic means. Gaugler *et al.* (1989), who selected *S. feltiae* for host finding, indicated that “relaxation of selection pressure produced a gradual decrease in host-finding”. Similarly, in other studies where trait stability was evaluated after relaxation of the selection regime, a reduction or reversion was reported (Burman and Pye, 1980; Anbesse *et al.*, 2013a).

Bilgrami *et al.* (2006) conducted an extensive study on trait stability in *H. bacteriophora* and *S. carpocapsae*; they reported that 20 serial passages in *G. mellonella* resulted in impaired performance of both nematode species. Virulence, heat tolerance, and fecundity deteriorated in all experimental *H. bacteriophora* lines, and four out of five experimental lines deteriorated in host-finding ability. All *S. carpocapsae* experimental lines deteriorated in heat tolerance and nictation, and four out of five experimental lines showed decreased reproductive capacity, whereas virulence declined in two experimental lines. They tested whether trait deterioration was due to changes in the nematode, bacterium, or both symbiotic partners by exchanging nematodes or bacteria from control populations with nematodes or bacteria from the most deteriorated experimental lines and assessing trait recovery. The source of deterioration varied according to the trait, but only the bacterial partner played a role in trait reduction for every

trait and species, whereas the nematode was the main source only for *S. carpocapsae* nictation. Deterioration of the bacterial symbionts when cultured alone was confirmed by Wang *et al.* (2007).

The cause for trait deterioration in EPNs (when using *H. bacteriophora* as a model) was found to be genetically based, with inbreeding depression being the predominant factor (Bai *et al.*, 2005; Adhikari *et al.*, 2009; Chaston *et al.*, 2011). Another factor leading to trait deterioration is “cheaters” (Shapiro-Ilan and Raymond, 2016). Cheaters are population members that do not contribute to the collective virulence of the group; as the number of cheaters increases in the population relative to the number of co-operators (those contributing to collective virulence) then the virulence trait deteriorates. The level of cheaters in a population is correlated to relatedness, which also corresponds to the pathogen load infecting the host (Shapiro-Ilan and Raymond, 2016). If there are too many invaders in a host (leading to a lack of relatedness) this in turn leads to an increased number of cheaters. Thus, from a practical side, when culturing EPNs, over-infection (infecting with too many IJs) should be avoided.

Some initial studies have begun to elucidate the molecular basis of trait deterioration. Adhikari *et al.* (2009) generated transcriptional profiles of two experimental lines of *H. bacteriophora*, identified the differentially expressed genes (DEGs), and validated their differential expression in the deteriorated line. The expression-profiling study was performed between experimental lines L5M and OHB of *H. bacteriophora* with probes for 15,220 ESTs from the *H. bacteriophora* transcriptome. Microarray analysis showed 1185 DEGs, comprising 469 down- and 716 upregulated genes, in nematodes with deteriorated traits. Analysis of the DEGs showed that trait deterioration involves massive changes in the transcripts encoding enzymes involved in metabolism, signal transduction, virulence, and longevity. We observed a pattern of reduced expression of enzymes related to primary metabolic processes and induced secondary metabolism. Expression of 16 DEGs in deteriorated-trait nematodes was validated by quantitative reverse transcription-PCR, which revealed similar expression kinetics for all of the genes tested, as shown by microarray. The authors suggested that the study provides an attractive short-term

application for use of a model organism to better understand interspecies interactions and to enhance our understanding of the mechanisms underlying trait deterioration in biological control agents. This information could also be used to improve the beneficial traits of biological control agents and better understand fundamental aspects of nematode parasitism and mutualism.

Based on the well-documented concerns regarding trait stability (as indicated above), methods to stabilize beneficial traits in EPNs are imperative. Improved cryopreservation techniques in liquid nitrogen may help in reducing trait deterioration (Bai *et al.*, 2004). However, cryopreservation is not a standalone solution and can be problematic. The ability of EPN commercial producers or researchers to effectively improve cryopreservation is limited, because EPN populations vary in their adaptability to cryogenic storage, the approach is costly, and human error, mechanical failure, or neglect can result in complete loss of genetic material (Shapiro-Ilan *et al.*, 2014b).

As a solution to the problem of trait deterioration, Bai *et al.* (2005) suggested stabilizing beneficial traits through the creation of genetically homozygous inbred lines. The inbred lines were created through controlled selfing on solid media according to Glazer *et al.* (1991). Using *H. bacteriophora* as a model, trait stability was evaluated following serial culturing of three inbred lines and the foundation population in *G. mellonella* (Bai *et al.*, 2005). Laboratory data indicated that serial culture of the foundation population (16 passages) resulted in an over 30% loss in traits deemed beneficial for biological pest suppression, i.e., virulence to an insect host (*Diaprepes abbreviatus*), reproductive capacity, heat tolerance (at 38°C), and host-seeking ability. In contrast, the inbred lines were impervious to declines in all beneficial traits. A greenhouse test targeting *D. abbreviatus* provided additional evidence that the biocontrol efficacy of the inbred lines remains stable during serial culture.

In addition to the Bai *et al.* (2005) approach, Anbesse *et al.* (2012, 2013a,b) reported that multiple heterorhabditid inbred lines can be created during liquid culture. In liquid culture, all heterorhabditid progeny are produced by selfing (via hermaphrodites) (Ehlers and Shapiro-Ilan, 2005), and thus multiple inbred lines

are automatically created. Both inbred line approaches on solid media (Bai *et al.*, 2005) and liquid media (Anbesse *et al.*, 2013a,b) have merit, but only the former can be applied to steinernematids.

Future Prospects of Genetic Improvement in EPNs

The only nematodes used for beneficial purposes in biological control are the entomopathogenic nematodes, steinernematids and heterorhabditids. The vast amount of knowledge, techniques and material developed for nematode research can be utilized for improvement of EPNs. Studies have clearly shown that the use of classical and advanced genetic techniques can significantly enhance EPN performance.

Figure 14.1 summarizes the various steps and components for successful genetic improvement of these beneficial organisms.

Unlike many other beneficial organisms that are subjected to genetic improvement, such as agricultural plants and animals, the tools for EPN improvement are still at a relatively early stage. Additional tools are needed for characterization of heterogenicity/homogeneity of a population (RFLP, AFLP, SNPs, SSRs) (see general review by Freeland, 2005), markers to follow transfer or enhancement/degradation (molecular, morphological and QTLs) (Freeland, 2005) of traits as well as identified beneficial genes that can be transferred between populations by crosses or molecular means. Advances in this direction were recently reported by Levy *et al.* (2020), who studied the osmotic desiccation tolerance among natural populations of *H. bacteriophora* for genetic diversity. This was done by exposure of the IJs to PEG 600. Then, gene expression patterns and genomic variation were studied following osmotic desiccation stress. Transcriptomic study enabled identification of genes and molecular markers as genetic selection tools for enhancement

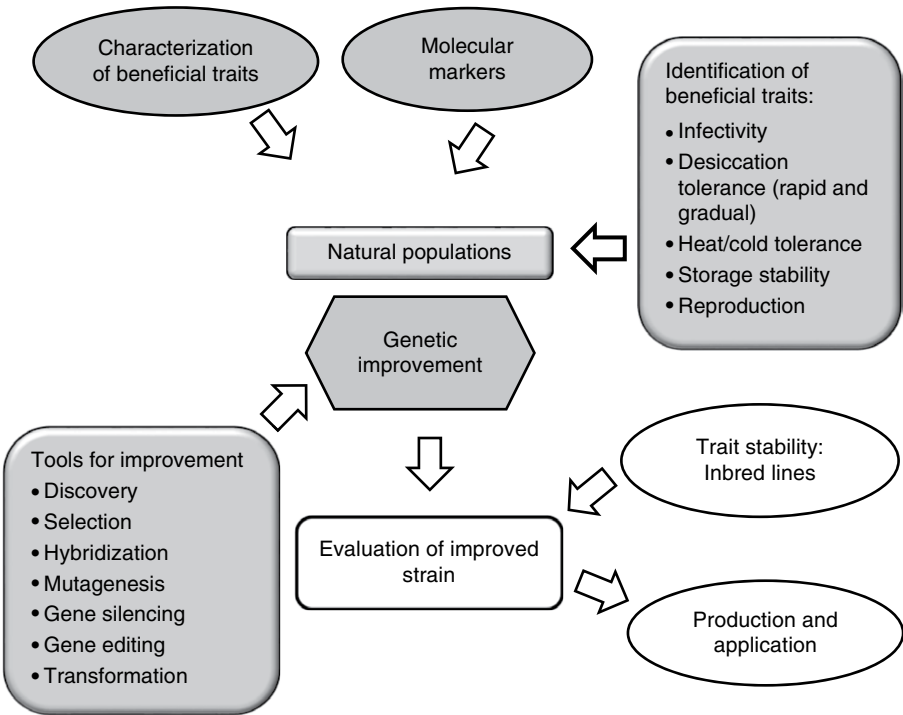


Fig. 14.1. Scheme 1: steps and components for successful genetic improvement of these beneficial organisms.

of EPN ability to withstand environmental stress conditions.

Fundamental research is needed into the genetic architecture of key traits, such as infectivity, stress tolerance and reproduction. The new genomic, proteomic, and bioinformatic technologies should be adapted for EPN genetic research (Dillman *et al.*, 2012). Considering that the genome of *H. bacteriophora* (TTO1 strain) has been sequenced (Bai *et al.*, 2013), new tools for genome editing (RNAi, CRISPR-Cas9) may be used for research as well as for the development of genetically improved nematodes. The present review shows that the development of genetically improved “products” stops, for the most part, in the laboratory, with only very few being tested in field trials (Grewal *et al.*, 1993; Gaugler *et al.*, 1997; Sharifi-Far *et al.*, 2018). Thus, the perception is that more products of GIP have yet to be proven under natural conditions.

As we have outlined in this chapter, GIP can be achieved using various approaches, including

discovery, selection, hybridization, and transgenics. In recent years, combinations of these approaches have proved to be powerful mechanisms for improving EPNs. For example, Mukuka *et al.* (2010c) combined selection and hybridization to effectively improve environmental tolerance in *H. bacteriophora*. Sharifi-Far *et al.* (2018) used a combination of the discovery approach and selection of superior inbred lines to improve cold tolerance and efficacy of *H. bacteriophora* for control of the cabbage maggot, *Delia radicum*. Choosing the most promising lines from among an array of homozygous inbred lines can be a solid approach to developing EPN products that are superior in beneficial traits and are innately stable (Bai *et al.*, 2005; Sharifi-Far *et al.*, 2018). In future research, combinations of GIP approaches along with incorporation of advanced molecular techniques will likely bring about the greatest achievements in EPN improvement. Comprehensive improvement programs are needed that encompass diverse beneficial traits.

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Nematodes as Model Organisms

Edited by Itamar Glazer, David I. Shapiro-Ilan, and Paul W. Sternberg

Nematodes are small multicellular organisms that have been used as biological models since the 1960s. For example, *Caenorhabditis elegans* is a free-living nematode worm, about 1 mm in length, that lives in temperate soil environments. It is made up of about 1000 cells, and has a short life cycle of only two weeks. It was the first multicellular organism to have its whole genome sequenced.

The book summarizes the importance of nematodes as model organisms in the fields of genetics, developmental biology, neurobiology, pharmacology, nutrition, ecology and parasitology.

Of interest to a broad audience across a wide spectrum of disciplines, this book is useful for biologists working on comparative studies to investigate biological processes across organisms; medical scientists and pharmacologists for exploration of drugs and medicine (including the use of genome editing to eliminate diseases); ecologists considering nematodes as indicators for environment changes; and parasitologists for host–parasite interactions. Many other researchers can use this book as a benchmark for the broad implications of nematology research on other aspects of science.

Cover image: Desiccated infective juvenile of *Steinernema carpocapsae*. Credit: Jayashree Ramakrishnan