Host Seeking and the Genomic Architecture of Parasitism Among Entomopathogenic Nematodes

Thesis by

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To my father Ray, my mother Annette, my stepfather Wallace, and my loving wife Beth, for nurturing my passion and enthusiasm for the natural world, and to those who have gone before, lighting the way with the knowledge of their discoveries.

"Doing what little one can to increase the general stock of knowledge is as respectable an object of life, as one can in any likelihood pursue."

—Charles Darwin

"To seek, to strive, to find, and not to yield."

—Lord Alfred Tennyson

Acknowledgments

The longer a person lives, the more friends, family, peers, acquaintances, and strangers affect their life. I have too many people to possibly thank for helping me get to this point and seeing it through successfully. While I certainly am pleased to be finishing my doctoral studies, by no means do I suppose that this is a task I have undertaken alone nor would I contend that this degree has been earned solely by my own work, but with considerable aid. I would like to thank all the teachers I have had over my many years in education, especially those who helped nurture my love of nature and biology, whether by teaching biology or science directly, or by teaching other subjects, thus helping highlight for me the importance and allure of biology. Although one might expect the acknowledgments of a doctoral thesis to focus primarily on the graduate school years, I feel it necessary to reach back into the recesses of the past and thank not only those who have helped me through these last five years, but also all those who have fed the insatiable flames of my passion for biology, thus helping me get to where I am now. Though I cannot remember all the teachers who have influenced me, I would like to begin by thanking Robert Mower and Dan Miller for teaching high school biology with such infectious enthusiasm that my burgeoning interest was greatly encouraged.

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Deciding where to go to graduate school and who to work for can be a very difficult decision. I do not think I could have chosen a better place than Caltech nor a better graduate advisor and mentor than Paul W. Sternberg. Most of my graduate school applications had already been sent off by the time Todd Ciche, then a postdoctoral researcher in the Sternberg lab, came to speak at BYU. He told his story about insect parasitic nematodes and what he was doing with them, and my imagination was sent into overdrive with the possibilities. Todd visited the Adams lab and I got to spend a couple of hours talking about his project and what else he was planning to do. Immediately following Todd’s visit, I researched more information about Caltech and Paul. I made up my mind to apply to Caltech and knew I wanted to work for Paul. Whereas all my

previous graduate applications had been to graduate schools, this application was different, because, although Caltech is among the best universities in the world, I was really only applying to work with Paul. When I came to interview I met with Paul and with several of his colleagues, including Sarkis Mazmanian and Judith Campbell. Of course they all approached the interview formally, as they might with any prospective graduate student. But when I made it clear that my intentions were to work for Paul Sternberg, the interviews became much less formal and they told me what a great person Paul was, how kind and considerate and genuinely interested in his students and postdocs. I thought it odd that none of his colleagues mentioned his scientific accomplishments and prowess but instead talked about what a charitable and genuinely kind person Paul is. I came to understand later that Paul's professional work speaks for itself but that what is remarkable about him is his seemingly endless reservoir of enthusiasm and excitement for research and his unassuming and approachable kindness. His door is always open, his scientific insight is unmatched, and his sincere kindness and concern for others is apparent to all who know him. It has been a pleasure and a privilege to work with such a brilliant and magnanimous man, and I am honored to have him as a mentor and colleague. He has nurtured my intellect, provided seemingly unlimited resources for my research, and helped to focus my enthusiasm and penchant for taking on too many projects.

Along the way I have met and befriended many graduate students and postdocs during my time at Caltech and would like to thank all of them for their friendship, encouragement, and advice. I want to thank the following individuals: Ted Ririe for helping me get started at Caltech and for his patience in showing me numerous molecular techniques; Cheryl Van Buskirk for her friendship, advice, and listening ear; Jagan

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I owe many thanks to the members of my thesis committee, Michael Dickinson, Bruce Hay, David Prober, Jared Leadbetter, James Baldwin, and Paul Sternberg for taking time out of their busy schedules to meet with me, read my work, and offer their constructive criticism and advice. I offer my thanks and gratitude to the Howard Hughes Medical Institute and the National Institutes of Health for generously funding much of the research that takes place in Paul's lab, making it easy to focus on the research and worry less about how to pay for it. I thank Caltech for allowing me to be part of such an amazing institution and for the wonderful research experience they have provided. I am convinced that I will never again find the unbelievable atmosphere of collaboration, scholarship, abundance of resources, and free-flowing creativity that is present in the Caltech community, and I am sad to see this chapter of my life close. What a truly marvelous place.

My family has been amazingly supportive of the time and effort I have put in to this program, and I am grateful to have their love, encouragement, and support, without which none of this work would have been possible. My parents, siblings, nephews, uncles,

aunts, cousins, grandparents, and wife have been there with encouragement and advice in all my trials, both professional and personal. What an invaluable blessing it is to have the uninhibited and unconditional support of family. It has made this journey so much easier and more enjoyable. I hope to be there for each of them when they need it most. Lastly, I offer a sincere and heartfelt acknowledgement to God, our Heavenly Father, the creator of all things, and His son and our Savior Jesus Christ, for all that I have and am. I am particularly grateful for the beauty and wonder of the natural world and our ability to understand what little of it we can. I have come to believe that scientific research at the highest level can draw one as close to God as many religious pursuits and that it can deepen one's reverence and awe for the Creator rather than dispel it.

Preface

The diversity of life on planet Earth is astounding, making it easy to become enthralled by the myriad different forms of all the organisms one encounters. However, when asked to define a narrowly focused research project, this wondrous diversity becomes challenging—even when a burgeoning biologist has some idea of the kinds of questions that pique his interest. How does one decide in which organism to study those questions? There are, of course, practical considerations such as the culturability of different organisms in the lab, how long they take to reproduce and develop, the cost of obtaining and growing them, the amount of space and resources they require, governmental regulations requiring specific care and treatment of some organisms (i.e., vertebrates), and many others. But there is also the matter of personal taste, passion, and interest. Some biologists absolutely love the organism they work with, while others see the organism as a tool or means to an end. As an undergraduate, I was counseled to become a 'question driven' biologist, meaning that I should not get too attached to any particular organism or technique, but that it should be the biological questions that drive the research, and with the questions well formed, one can then decide which organism and techniques are best suited to address those questions. While I appreciate the value of this counsel, I confess that I am completely enamored by nematodes. I have come to see in them an amazing model system where nearly any aspect of biology can be studied. They are particularly well suited as a model system of behavior, neurobiology, and genomics. Their central nervous system is relatively simple, their genomes are compact, and they are still capable of tremendously interesting behaviors, detailed in the thesis that follows.

Abstract

Nematodes represent an especially abundant and species-rich phylum, with many free-living and parasitic species. Among the diversity of parasitic species is a guild of specialists known as entomopathogenic nematodes due to their unusual ability to quickly kill their hosts with the aid of pathogenic bacteria. Herein I discuss in detail the hallmarks of entomopathogenic nematodes and how they are different from other insect parasites. Further I explore their host-seeking behaviors, demonstrating their ability to detect insect hosts in complex soil environments and assess their odor preference profiles. I show that CO_2 is a major driver of host seeking and that entomopathogenic nematodes detect CO_2 using the same pair of conserved neurons that the fruit-dwelling *Caenorhabditis elegans* uses to detect and respond to CO_2. I demonstrate dramatic differences in odor preference profiles and virulence capabilities, even between closely related nematodes. I discuss the role of genomic sequencing generally and more specifically in nematology, including how genomes are sequenced and analyzed and the types of characteristics that are most prominently assessed. This thesis concludes with a discussion of the genomic sequencing of entomopathogenic nematodes in the genus *Steinernema* and the clues these genomes provide regarding the genomic architecture of parasitism.

Contents

Chapter 1:

An Introduction to Nematodes and Entomopathogenic Nematodes*

* This chapter includes a quick guide first published in *Current Biology* in 2012 that was written by Adler R. Dillman and Paul W. Sternberg.

Abstract

Nematodes are amazing animals, both ancient and diverse. Among their diversity are many plant and animal parasites, many of which negatively affect humans. However, not all parasitic nematodes are bad and some are currently being used as organic alternatives to chemical pesticides for controlling damaging insect pests. Although there are many insect-parasitic nematodes, the entomopathogenic nematodes are the best studied of these and are remarkably different in their lifestyle and in their particular parasitism. Herein I discuss the difference between entomopathogenic nematodes and other insect parasites and what makes them so interesting and useful.

Introduction

In an effort to discern order amid the astounding diversity of life, humans have classified life into the following taxonomic rankings, in descending order: Domain, kingdom, phylum, class, order, family, genus, and species. Modern taxonomists and systematists use this conceptual hierarchy genealogically, grouping closely related species (singular: species) into genera (singular: genus), closely related genera into families, families into orders, orders into classes, classes into phyla (singular: phylum), phyla into kingdoms, and kingdoms into domains [1]. This classification scheme, or genealogy of life, was originally established by Carolus Linnaeus in the 1700s and has been modified to its current form by a host of scientists, reshaping this scheme according to newer findings, as our understanding of the relationships between organisms has increased. For instance, the ranking of domain was not introduced until 1990, and currently there only three recognized domains of life: Archaea, Bacteria, and Eukaryota

[2]. At present, there are at 35 recognized phyla in the animal kingdom, though this number may fluctuate with new discoveries and as our understanding of animal relationships increases. Most people are only familiar with a handful of these phyla, such as Chordata, which includes all vertebrates, encompassing virtually anything you would see at a zoo. Other more commonly known phyla include Arthopoda and Mollusca, which are made up of insects, crustaceans, arachnids, and cephalopods (e.g., squid and octopuses) and gastropods (e.g., snails and slugs). Nematoda is a phylum of roundworms that originated during the Precambrian or Cambrian explosion over 500 million years ago [3, 4]. Although fewer than 30,000 species of nematodes have been described, there are thought to be between 1 and 10 million species of nematodes on Earth, making Nematoda the most speciose (alluding to both their beauty and species-richness) phylum on the planet, even more so than Arthopoda [5–8]. This abundance of evolutionary time and their relatively simple body plan has allowed nematodes to adapt and occupy virtually every ecological niche and climate imaginable. Nematodes occupy marine, freshwater, and terrestrial environments from tropical and temperate environments to extremely dry and restrictively cold environments. Nathan A. Cobb, often considered the father of modern nematology, has written: “[Nematodes] occur in arid deserts and at the bottom of lakes and rivers, in the waters of hot springs and in the polar seas where the temperature is constantly below the freezing point of fresh water. They were thawed out alive from Antarctic ice in the far south by members of Shackleton’s expedition. They occur at enormous depths in Alpine lakes and in the ocean” [8]. To borrow another famous quote of his: “If all matter in the universe except the nematodes were swept away, our world would still be dimly recognizable, and if, as disembodied spirits, we could then

investigate it, we should find its mountains, hills, vales, rivers, lakes and oceans represented by a film of nematodes" [9].

Most nematodes are microscopic, varying from 0.5–2 mm in length, with the most heavily studied nematode, *C. elegans*, averaging 1 mm (Figure 1.1). Though rare, there are larger nematodes. The largest, *Placentonema gigantissima*, is a whale parasite that was recorded at over 8 meters in length. Their general body plan is highly conserved among species and relatively simple, essentially consisting of a round tubular body with a mouth on one end and an anus on the other, a digestive tract, and reproductive system [10]. Nematodes also have an excretory-secretory system and a complex nervous system, but no circulatory system. Though this general body plan is conserved, there is extensive morphological diversity of the mouth and cephalic appendages among many species, generally relating to feeding, habitat, and ecology.

While most species of nematodes are "free-living", there are also many parasites of plants and vertebrates. Most of these parasites are devastating and cause many well-known diseases, including elephantiasis, trichinosis, and river blindness. The World Health Organization estimates that more than two billion people are infected with nematodes (http://www.who.int/wormcontrol/statistics/). Though many parasites affect humans directly by causing disease, it is important to emphasize that vertebrate parasitic nematodes also affect humans indirectly by infecting livestock and pets [11]. There are many devastating plant-parasitic nematodes as well, causing an estimated 12.3% annual crop loss worldwide, effectively causing more than 77 billion dollars annually in lost crops [12].

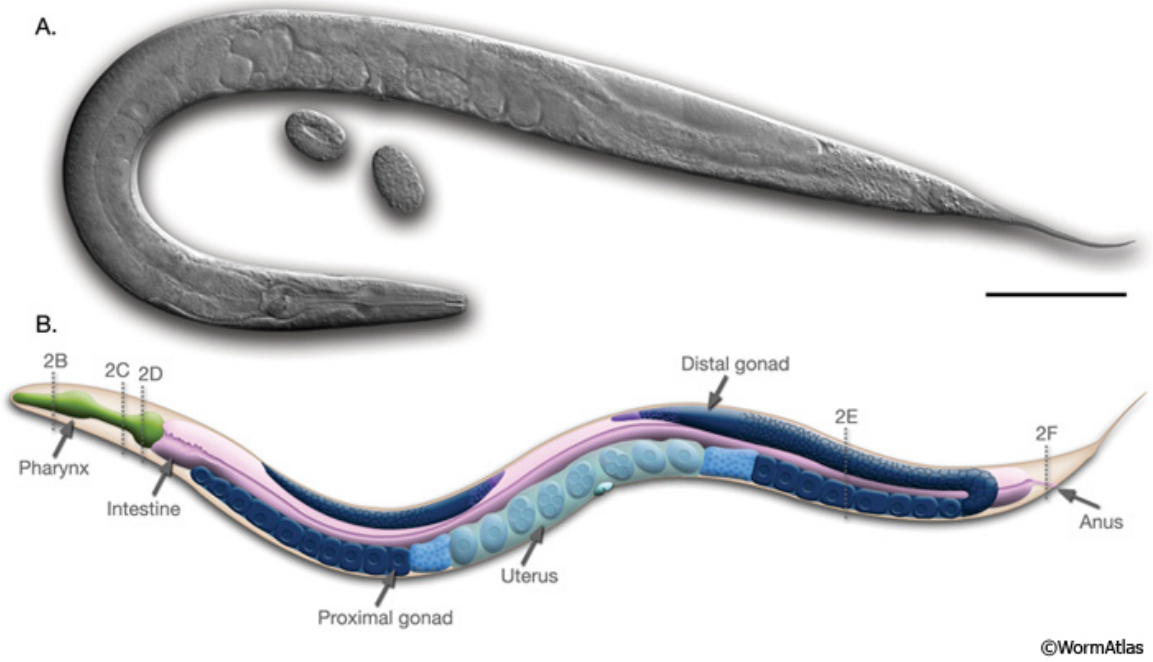

Figure 1.1 | Anatomy of an adult hermaphrodite *C. elegans*. A. DIC image of an adult hermaphrodite *C. elegans*, left lateral side. Scale bar is 0.1 mm. The two round shapes in the middle are recently laid eggs. **B.** Schematic drawing of anatomical structures. Dotted lines and numbers mark areas of additional detailed anatomical information that can be found at http://www.wormatlas.org.

While it is true that most parasitic nematodes affecting humans either directly or indirectly tend to have negative effects, there are some beneficial parasitic nematodes. Many insect-parasitic nematodes have been explored as potential alternatives to chemical pesticides for controlling harmful insect pests. Among these insect parasites, the entomopathogenic nematodes have been the most studied. What follows is taken from a "quick guide" published in *Current Biology* (see footnote in chapter heading), as a brief introduction to entomopathogenic nematodes.

What are entomopathogenic nematodes? Nematodes seem to have evolved to occupy nearly every niche imaginable, including a wide diversity of parasitic niches. Among the vast variety of parasitic nematodes, some have evolved an association with insect pathogenic bacteria. Together the bacteria and nematode are a lethal duo. These nematodes are called 'entomopathogenic nematodes' or EPNs for short. Essentially the nematodes serve as mobile vectors for their insect-pathogenic bacteria cargo, like little Typhoid Marys. The nematodes seek out and invade potential hosts and release their pathogenic payload into the nutrient-rich hemolymph. Infected insect hosts die quickly, the bacteria proliferate, and the nematodes feed on bacteria and insect tissues, and reproduce. When the host cadaver is depleted of resources, nematodes associated with pathogenic bacteria emerge and search for new hosts to infect (Figure 1.2). The cooperation with bacteria and the speed with which they kill sets EPNs apart from other nematode parasites.

How do they kill? The nematode and the pathogenic bacteria they carry contribute to varying degrees, depending on the combination. The known bacterial associates of EPNs, species of *Photorhabdus* and *Xenorhabdus,* are known to produce a toxic cocktail of secondary metabolites that are not only lethal to the insect hosts, but that prevent opportunistic bacteria and fungi from utilizing the nutrient rich cadaver, sequestering the resources for themselves and their nematode partners. The bacteria always contribute to the virulence of the duo, and usually contribute the lion's share. Some species of nematodes are thought merely to shuttle the bacteria, contributing very little to host death, while others are known to be lethal in their own right, producing a variety of secreted

protein products that degrade and digest host tissues, in addition to short-circuiting the host immune system. Even though some nematodes appear lethal on their own, no non-bacterial associated EPNs are known to exist.

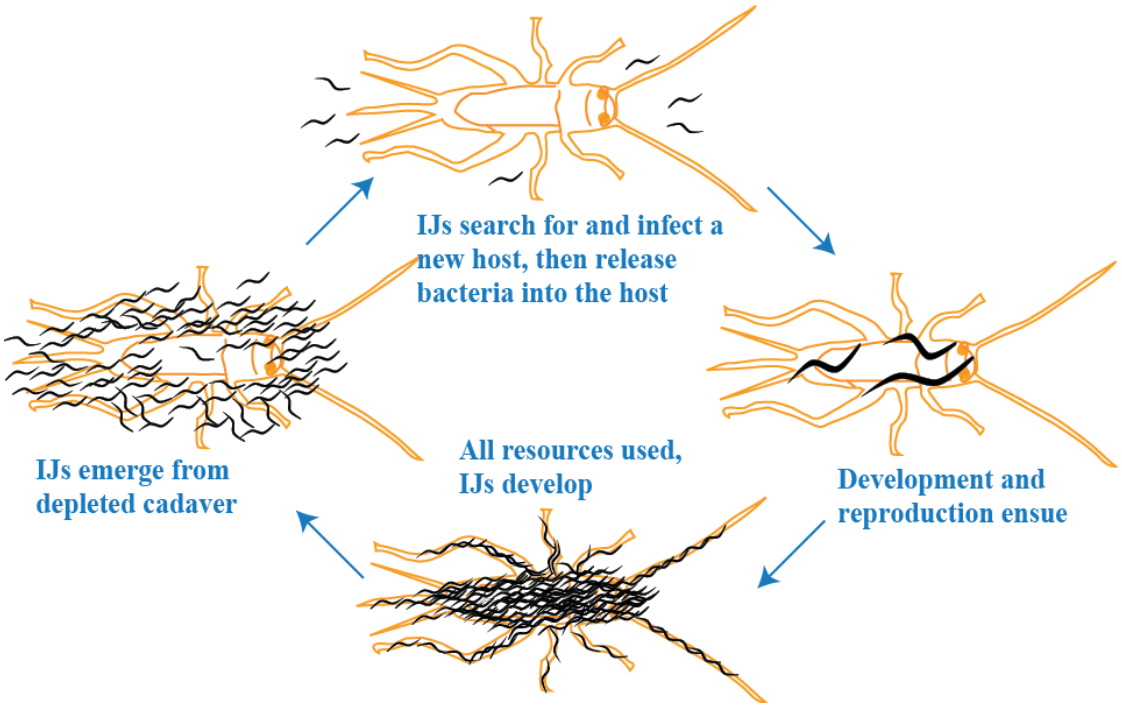

Figure 1.2 | Life cycle of entomopathogenic nematodes. The infective juvenile (IJ) stage seeks out a new host to infect, penetrating into the hemolymph and releasing the pathogenic bacteria it carries. The nematodes develop and reproduce in the nutrient-rich insect, going through several rounds of reproduction, depending on the size of the insect host. As resources deplete, a new generation of infective juveniles form and emerge, seeking new hosts to infect with the pathogenic bacteria they carry.

Are all stages infectious? The short answer is no. Only a modified third larval stage called the infective juvenile, analogous to the dauer juvenile stage in *Caenorhabditis elegans*, is infectious (Figure 1.3). In fact, infective juveniles are the only free-living stage of known EPNs, while all other developmental stages are only found inside infected

hosts. The infective juvenile is a stress tolerant, non-feeding, bacterial vectoring stage that seeks out insects to infect and kill.

Figure 1.3 | Entomopathogenic nematodes emerging from insects. Pictures showing entomopathogenic nematode infective juveniles emerging from *Galleria mellonella* waxworm larvae on the left and *Acheta domestica* crickets on the right

How did they get their name? The first entomopathogenic nematode was described by Gotthold Steiner in 1923; since then more than 75 species have been described, with more species being described every year. Most studies focus on EPNs from two genera: *Steinernema* and *Heterorhabditis*. It is through their association with insect pathogenic bacteria that they began to be called entomopathogenic nematodes. First the nematodes' bacterial partners were called entomopathogenic bacteria, because these bacteria have a median lethal dose or LD_{50} of ten thousand cells or less. This means that an inoculum of ten thousand bacterial cells or less, into the hemolymph, kills half of a tested population of insects. The term 'entomopathogenic' began to be applied to the nematodes themselves in the late 1980's and reinforces the link between nematology and insect

pathology. It is a useful technical epithet that differentiates them other types of parasitic nematodes, of which there are many.

Are they harmful to humans? While most parasitic nematodes might be seen as harmful, EPNs are beneficial to humans. Their potential as alternatives to chemical pesticides for controlling pesky insects was recognized early on and they have been subjected to extensive laboratory and field-testing. EPNs have been used in biological control since the 1930s and are currently used worldwide. For example, they have been used with high levels of success to control invasive species of mole crickets in Florida and continue to be used in orange groves in both Florida and California to control the citrus root weevil and other damaging crop pests. EPNs are even commercially available for pest control in home gardens and are commonly marketed as 'beneficial nematodes.'

Why are EPNs being studied? For starters, the symbiotic association with bacteria is highly specific in most cases and provides an excellent model for understanding the development and evolution of symbiosis. EPNs' potential as biological control agents continues to be evaluated with studies focusing on selection of desirable traits such as virulence, heat and stress tolerance, persistence, etc. Because at least two distantly related genera have evolved this specific type of parasitism (*Heterorhabditis* and *Steinernema)*, EPNs are an interesting system for the study of convergent and parallel evolution. Also, since they are odd intermediates between predators and parasitoids, there are many studies regarding their host-seeking behavior. They rely primarily on chemoreception for host seeking and some of them are capable of jumping, which is an extraordinary

behavior in nematodes that is unique to some *Steinernema*. Imagine, a 0.5–1 mm worm with no legs or hard body parts, and yet it is capable of jumping up to 9 times its body length.

What remains to be explored? There is much that remains unknown about EPNs, including: their global abundance and diversity, the extent of their host range and whether or not other arthropods or even non-arthropods are also infected, what has led to the specialization of some for certain hosts and not others, what drives niche partitioning within this guild, the molecular underpinnings of their symbiosis and parasitism, how they can survive carrying highly pathogenic bacteria, how they suppress or avoid host immunity, or just how genetically similar disparate species that have converged on this very particular lifestyle are. These and other questions remain underexplored, providing plenty of room for studying these fascinating, useful, and delightful worms.

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Chapter 2:

What Makes a Nematode Entomopathogenic*

* This chapter, first published in *PLoS Pathogens* in 2012, under the title "An Entomopathogenic Nematode by Any Other Name" was written by Adler R. Dillman, John M. Chaston, Byron J. Adams, Todd A. Ciche, Heidi Goodrich-Blair, S. Patricia Stock, and Paul W. Sternberg.

Abstract

Among the diversity of insect-parasitic nematodes, entomopathogenic nematodes (EPNs) are distinct, cooperating with insect-pathogenic bacteria to kill insect hosts. EPNs have adapted specific mechanisms to associate with and transmit bacteria to insect hosts. New discoveries have expanded this guild of nematodes and refine our understanding of the nature and evolution of insect-nematode associations. Here we clarify the meaning of 'entomopathogenic' in nematology and argue that EPNs must rapidly kill their hosts with the aid of bacterial partners and must pass on the associated bacteria to future generations.

Strangers, Acquaintances, and Enemies

Nematode-arthropod associations are plentiful and range from beneficial to antagonistic [1, 2]. These associations have been divided into at least four categories: 1) phoretic (nematodes are transported by an insect), 2) necromenic (nematodes obtain nutrition from insect cadavers), 3) facultative parasitism, and 4) obligate parasitism (see Sudhaus 2008 for a more detailed breakdown [3]). It is thought that insect parasitism evolves in this sequence, with parasites evolving from non-parasitic insect associates (Figure 2.1) [1, 3]. Nematodes also interact with bacteria in at least three ways: 1) trophism (nematodes eat bacteria), 2) parasitism (pathogens cause nematode diseases if not resisted), and 3) mutualism (nematodes and bacteria cooperate). Here we consider entomopathogenic nematodes, which employ bacteria to kill insects.

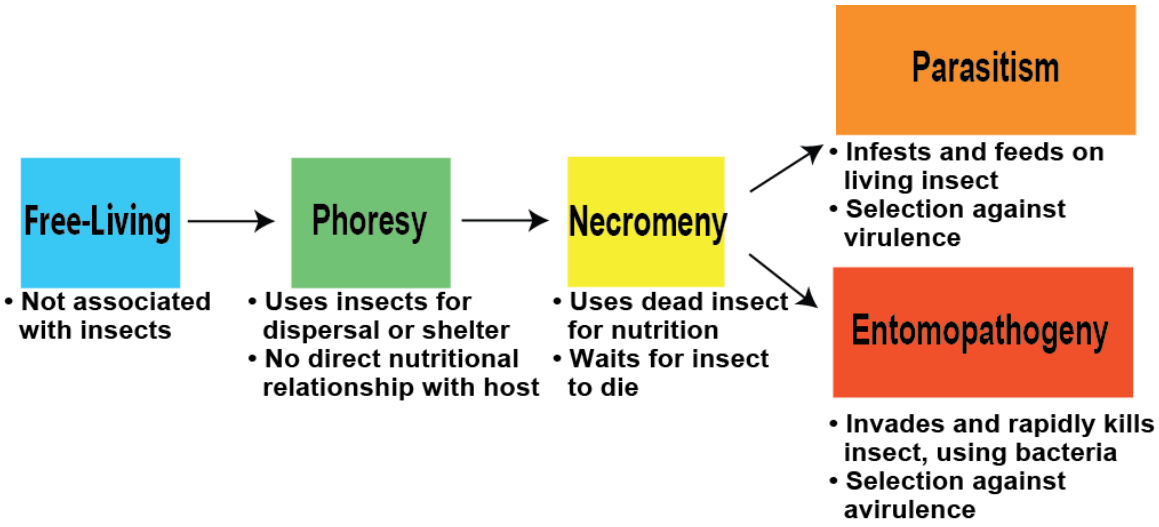

Figure 2.1 | Evolution of nematode-insect associations. The evolution of nematode-insect associations. **Free-living**: microbotrophic nematodes not known to associate with arthropods, vertebrates, plants, or fungi; only perhaps transiently associated with insects. **Phoresy**: a relationship where nematodes are adapted to use insects for dispersal or shelter but have no direct nutritional relationship to them. **Necromeny**: a relationship where nematodes are adapted to use saprophytic insect cadavers as a food resource but do not participate in insect death. **Parasitism**: a relationship where nematodes are adapted to use living insects directly for nutrition, likely inflicting some level of harm or even causing eventual death of the host. **Entomopathogeny**: a relationship where nematodes cooperate with insect-pathogenic bacteria to cause rapid insect disease and death and then feed and develop on the insect and bacterial resources. The distinction between parasitism and entomopathogeny is based on salient features including use of pathogenic bacteria and direction of selection (against virulence or avirulence), either making the nematodes more or less immediately harmful to their host.

Entomopathogenic Nematodes

The term ‘entomopathogenic’ is widely used in parasitology and pathology, usually referring “to microorganisms and viruses capable of causing disease in an insect host [4].” Nematodes in Steinernematidae and Heterorhabditidae associate with pathogenic bacteria to kill insect hosts, usually within 48 hours of infection. The hallmarks of this specific type of parasitism by nematodes, known as entomopathogeny, are 1) carriage of pathogenic bacteria by infective juvenile (IJ) nematodes (also known as dauer juveniles); 2) active host-seeking and -penetration by IJs; 3) release of the bacteria into the insect hemolymph; 4) death of the insect, and nematode reproduction and bacterial proliferation driven by cadaver-nutrient utilization; 5) reassociation of the pathogenic bacteria with new generations of IJs; 6) emergence of IJs from the nutrient-depleted cadaver as they search for new insect hosts (Figure 2.2) [5, 6]. Nematode parasites of this kind are known as “entomopathogenic nematodes” (EPNs).

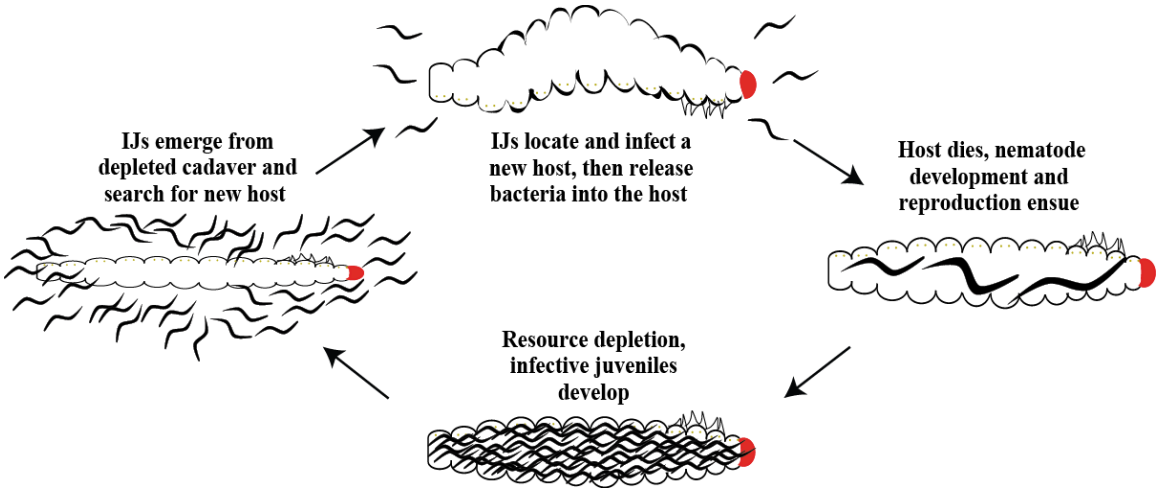

Figure 2.2 | Entomopathogenic nematode life cycle. The life cycle of entomopathogenic nematodes. The IJ stage is a developmentally arrested third larval stage and is the only free-living stage; all other stages exist exclusively within the host. EPN IJs carry symbiotic

bacteria and search for potential insect hosts. They enter a host, gain access to the hemolymph and release their bacterial symbiont. The symbiont plays a critical role in overcoming host immunity. The nematodes develop and reproduce in the resulting nutrient-rich environment until population density is high and resources begin to deplete, at which point new IJs develop and disperse, carrying the symbiotic bacteria to new hosts [5].

Recently, other nematode species have been shown to use pathogenic bacteria to parasitize insect hosts. Two *Oscheius* (=*Heterorhabditoides*) species, *Oscheius chongmingensis and O. carolinensis*, and *Caenorhabditis briggsae* have been identified as potential insect pathogens by baiting soil for nematodes using insect larvae as prey, a common approach used for finding EPNs [7–11]. All of these have been found to associate with insect pathogenic bacteria of the genus *Serratia*, while *O. carolinensis* may have additional associates [9–12]. *O. chongmingensis* and *C. briggsae* require their bacterial partners to cause host death, to grow and reproduce within killed insects, and emerging dauer juveniles are associated with the vectored pathogen [10, 11]. Ongoing studies suggest that these species are entomopathogenic nematodes, though their classification as entomopathogens has been contested both semantically and conceptually in the literature and scientific meetings (e.g., Nov. 2010 NemaSym NSF RCN meeting and the Jul. 2011 Society of Nematologists meeting) [13–15].

History, Context, and Formal Criteria

The term ‘entomopathogenic’ first appeared in the nematology literature, in reference to the bacterial symbionts of *Steinernema* and *Heterorhabditis* [16]. Bacteria are considered entomopathogenic when their LD_{50} is < 10,000 cells injected into the

hemocoel [17]. Some pathogens associated with *Steinernema* and *Heterorhabditis* have LD_{50}s < 10 cells when injected, but this varies with different hosts and these bacteria are not known to infect insects without the aid of their nematode partners [18]. 'Entomopathogenic' was applied to nematodes in 1981 and again in 1986 [19, 20], a use that gained momentum in 1988 [21]. This gradual, social use of the term 'entomopathogenic' without formal definition complicates its application to emerging nematode-bacteria partnerships. Indeed, the convenience of this descriptor is currently that it applies to both partners as a complex, rather than only the nematodes or bacteria. The only clearly identifiable EPN definition that we are aware of was proposed informally [4, 22]. This definition focuses on mutualism with bacteria and on the exclusivity of the IJ as the free-living stage. We find the use of these criteria incomplete since they do not consider rapid death, which is necessary to differentiate EPNs from phoretic, necromenic, or other less virulent forms of parasitism, and the inclusion of a stage-specific requirement in defining EPNs is unnecessary. Since convention provides no standard to assess classification of EPNs, and because 'entomopathogenic' was meant to differentiate insect-parasitic nematodes that serve as vectors of bacteria and to reinforce the link between nematology and insect pathology [2], we formally suggest two criteria: 1) the nematodes use a symbiotic relationship with bacteria to facilitate pathogenesis, which implies that the association is non-transient, though not necessarily obligate, and 2) insect death is sufficiently rapid that it can be unequivocally distinguished from phoretic, necromenic, and other parasitic associations (i.e., < 120 h), a time frame that also implies efficient release of the pathogen by the nematode vector [17]. These criteria are based on early investigations of EPNs and what we consider the

fundamental principles of the EPN lifestyle [1, 2]. We intend this discussion to provide a more thorough evaluation of the defining characteristics of EPNs, though our criteria overlap with but are not as restrictive as the previous definition [4, 22].

Koch's postulates can be used to establish pathogenicity of the nematode-bacterium complex or either partner alone, and we suggest that partner association across generations is particularly important in this evaluation [23]. To establish genetic heritability, genes must be passed through the F1 generation to the F2 generation; for example, a mule inherits, but does not pass on, traits inherited from its paternal donkey and maternal horse parents. Similarly, we argue that for an EPN association to be stable, nematodes must not only infect and kill an insect and produce progeny, but must also produce progeny that depart the carcass carrying the pathogenic bacteria. This does not require that the association be obligate—subsequent generations that thrive in non-insect environments may lose the symbiotic bacteria—but we believe it is crucial that symbiont transmission from the infecting parental generation to emerging nematodes from at least two subsequent insect infections be clearly established to distinguish nematode carriage of the bacteria or bona fide association from transient cuticle hitchhiking. Also, in associating, each partner must also benefit from the association. At a minimum the bacteria should increase overall nematode fitness by assisting in insect killing, nutrient liberation, or scavenger deterrence, and the nematodes should provide the bacteria with access to the insect host either by delivery to otherwise inaccessible host cavities or tissues, or by increasing dispersal range through direct carriage. Though EPNs must be capable of infecting and killing insect hosts, this does not preclude them from also, opportunistically, acting as scavengers or from competing with other EPNs for already

killed insects [24, 25]. An additional cautionary point here is that the symbiont transmission rate and the stability of nematode-bacterium associations themselves have been well characterized in representative taxa [26, 27], but these details are unclear in most of the 75 EPN species reported to date [7].

Insect host killing within five days of infection is an appropriate requirement and implies selection for virulence or at least selection against avirulence, differentiating entomopathogeny from other forms of parasitism such as those used by mermithids and allantonematids. 'Potentially pathogenic' bacteria, microbes that cause septicemia at low inocula when in the hemocoel but lack mechanisms for actively invading the hemocoel [17], usually cause death within two to four days in common laboratory larvae such as *Galleria mellonella*, though larger or adult insect hosts, such as mole crickets or *Manduca sexta,* take longer to succumb, depending on the size of the nematode founding population and which pathogenic bacterium is used [18]. Rapid death caused by EPNs reflects pathogenicity of the bacterial partner with possible contributions from the nematode and relies on efficient release of the bacteria into the hemolymph.

Specialization of EPNs

When considering appropriate criteria that define entomopathogenic nematodes it is tempting to use the particular details that are known for only a few representative taxa. Instead, we avoided specifics in favor of fundamental principles that underlie the associations, and observed that many interesting and often dogmatic EPN characteristics are less widespread than we expected. For example, specialization with particular bacteria is a hallmark EPN characteristic, and monospecificity between one nematode

and one genus of bacteria or even one symbiont species is commonly observed among these taxa [7]. However, growing evidence of promiscuous relationships between EPNs and their bacterial symbionts suggests that this may not be as common as originally thought (e.g., [28–30]). Although most *Heterorhabditis* and *Steinernema* symbionts localize to the nematode intestine, there are excellent examples of nematode-bacteria symbioses in other body sites (e.g., [31]). Of note, *Paenibacillus nematophilus* associates on the cuticle of *Heterorhabditis* spp., and, relevant to this discussion, *O. carolinensis* is associated with insect pathogenic *Serratia marcescens* on its exterior cuticle [12, 30]. Also, dogma dictates that these associations are obligate, since *Steinernema* and *Heterorhabditis* symbionts are generally not free-living, and *S. carpocapsae's* symbiont is auxotrophic for nicotinic acid which is not available in the environment [32]. However, *Photorhabdus asymbiotica* may be free-living (e.g., [33]). Also, most nematodes require their symbionts for growth and reproduction, but exceptions have been observed (e.g., [34, 35]). There are also differences between biological characteristics of the two nematode taxa. For example, *Heterorhabditis* maternally transmit symbionts by a sophisticated multistep process, while *Steinernema* have specialized host structures within which they carry their symbionts [28, 29]. Also, some *Steinernema* infect and kill insect hosts even in the absence of pathogenic bacteria, at least in laboratory conditions, but *Heterorhabditis* nematodes have not been reported to have this behavior. Finally, as we mentioned above, symbiont transmission to new generations varies widely in the few taxa where it has been studied from > 95% to ~ 10% [35, 36]. Together these findings reveal that *Steinernema* and *Heterorhabditis* are highly adapted to entomopathogeny and showcase adaptations likely to emerge as a result of long-term commitment to the

entomopathogenic lifestyle, even though the biological basis for their symbiotic association with bacteria differs significantly [5, 37]. The exceptions and differences that have been observed for all of these hallmark characteristics highlight why specializations should not be used to exclude newly described associations, and emphasize that applying observations from a few representative members to whole clades can be problematic. Indeed, few species in either genus have been thoroughly explored and we caution against assuming *a priori* these specializations to be true of all or even most steinernematids or heterorhabditids (e.g., [38]).

Classification of newly described associations

According to the standards we propose above, *C. briggsae* may not be an EPN. Infective juveniles recovered from dead insects seem able to re–infect new hosts but are less virulent in *G. mellonella* as a complex than injection of the bacteria alone, suggesting either inefficient release of the pathogen or some antagonism by the nematode vector. This may reflect that *C. briggsae* is somewhere between necromenic and entomopathogenic, that it is a nascent entomopathogen and not yet efficient, or that *G. mellonella* is a poor host. However, symbiont heritability has not been demonstrated, and the nature of *C. briggsae's* bacterial association remains unresolved [10, 11, 39]. Because *C. briggsae* has not met the suggested criteria it should not be considered an entomopathogenic nematode, facultative or otherwise, until heritability of the pathogenic bacteria is demonstrated and more is known about bacterial release and speed of host death. Our suggested criteria have been tested and met for both *O. chongmingensis* and *O. carolinensis* [9, 10, 12]. Therefore, these taxa should be considered EPNs even though

further research is required to determine the nature and heritability of their bacterial associations, and whether they are obligate or facultative EPNs.

Symbiosis and Entomopathogeny

Nematode-bacterium partnerships that do not explicitly fulfill the requirements to be classified as EPNs are still of extraordinary interest since they may represent developing, nascent partnerships, but they should not be considered entomopathogens. Our understanding of parasitism and its evolution is continually refined as biodiversity is explored and ecology and evolution become increasingly emphasized among established and satellite model systems. We have suggested specific and restricted use of the term 'entomopathogenic' in nematology, which will facilitate unambiguous communication. Among the twenty or more parasitic lineages of nematodes, entomopathogeny is a unique type of insect parasitism not found among vertebrate- or plant-parasitic nematodes. Recent work indicates that entomopathogeny has arisen at least three times within Nematoda, and that recently described species (*O. chongmingensis* and *O. carolinensis*) may represent nascent stages of EPN evolution. These developments emphasize the tremendous specialization exhibited by *Heterorhabditis* and *Steinernema* and increase their usefulness as models for the evolution of symbiosis and parasitism.

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Chapter 3:

A Sensory Code For Host Seeking in Parasitic Nematodes*

*This chapter, first published in *Current Biology* in 2011 and was written by Elissa A. Hallem[1], Adler R. Dillman[1], Annie V. Hong, Yuanjun Zhang, Jessica M. Yano, Stephanie DeMarco, and Paul W. Sternberg.

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Abstract

Nematodes comprise a large phylum of both free-living and parasitic species that show remarkably diverse lifestyles, ecological niches, and behavioral repertoires. Parasitic species in particular often display highly specialized host-seeking behaviors that reflect their specific host preferences. Many host-seeking behaviors can be triggered by the presence of host odors, yet little is known about either the specific olfactory cues that trigger these behaviors or the neural circuits that underlie them. *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* are phylogenetically distant insect-parasitic nematodes whose host-seeking and host-invasion behavior resembles that of some of the most devastating human- and plant-parasitic nematodes. Here we compare the olfactory responses of *H. bacteriophora* and *S. carpocapsae* infective juveniles (IJs) to those of *Caenorhabditis elegans* dauers, which are analogous life stages [1]. We show that the broad host range of these parasites results from their ability to respond to the universally produced signal carbon dioxide (CO_2) as well as a wide array of odors, including host-specific odors that we identified using TD-GC-MS. We show that CO_2 is attractive for the parasitic IJs and *C. elegans* dauers despite being repulsive for *C. elegans* adults [2, 3], and we identify an ancient and conserved sensory neuron that mediates CO_2 response in both parasitic and free-living species regardless of whether CO_2 is an attractive or a repulsive cue. Finally, we show that the parasites' odor response profiles are more similar to each other than to that of *C. elegans* despite their greater phylogenetic distance, likely reflecting evolutionary convergence to insect parasitism. Our results suggest that the olfactory responses of parasitic versus free-living nematodes are highly diverse and that this diversity is critical to the evolution of nematode behavior.

Results and Discussion

H. bacteriophora and *S. carpocapsae* are lethal parasites of insect larvae currently used as biocontrol agents for many insect pests. The two species are phylogenetically distant yet share similar lifestyles and ecological niches as a result of convergent evolution to insect parasitism (Figures 3.1A–C, 3.S1). Both species infect hosts only as infective juveniles (IJs), a developmentally arrested third larval stage analogous to the dauer stage of *C. elegans* [1, 4]. Both species are associated with symbiotic bacteria during the IJ stage [5, 6]. IJs live in the soil, where they actively seek out and infect hosts; all other life stages exist exclusively inside the host. IJs infect either by entering through a natural body opening or by penetrating through the insect cuticle. Once inside the hosts, IJs release their symbiotic bacteria, which helps them overcome the host immune system and results in rapid host death [7–10]. The nematodes reproduce inside the insect cadaver for 2–3 generations until resources are depleted, after which new IJs form and disperse into the soil (Figure 3.1C–G).

Despite their similar lifestyles, *H. bacteriophora* and *S. carpocapsae* are thought to use different strategies for host location: *H. bacteriophora* IJs are “cruisers” that move through the soil actively chemotaxing toward potential hosts, while *S. carpocapsae* IJs are “ambushers” that remain relatively stationary and stand on their tails, a behavior known as nictation, to facilitate attachment to passing hosts [11, 12]. Ambush foraging in *S. carpocapsae* also consists of an unusual jumping behavior in which the IJ nictates, curls into a loop, and propels itself into the air (Figure 3.1D). Jumping in nematodes is

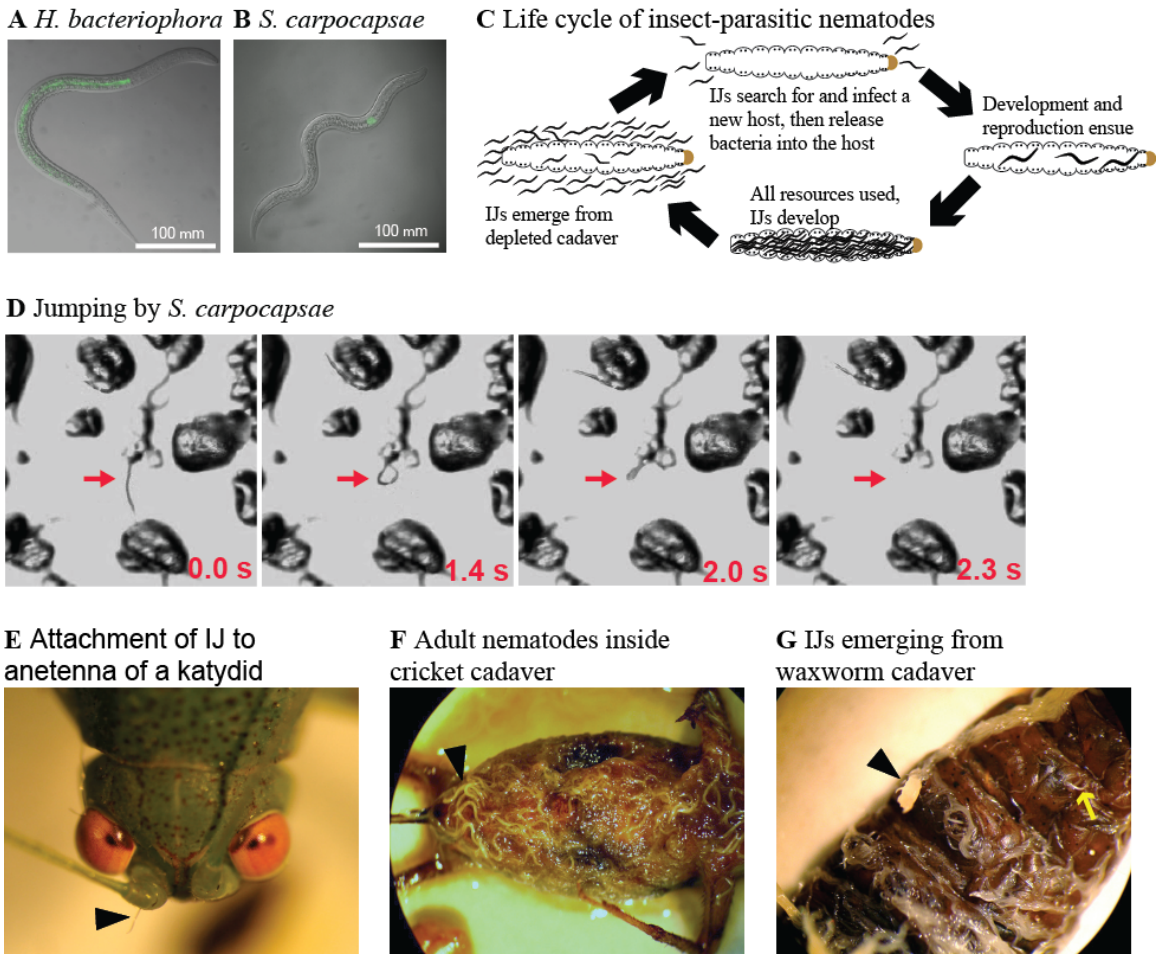

Figure 3.1 | Life cycles of insect-parasitic nematodes. A–B. Photomicrographs of an *H. bacteriophora* **(A)** and an *S. carpocapsae* (**B**) infective juvenile (IJ). Both species harbor a bacterial symbiont—*H. bacteriophora* harbors *Photorhabdus luminescens* and *S. carpocapsae* harbors *Xenorhabdus nematophila*—in the gut during the IJ stage. Nomarski images are overlaid with epifluorescence images; bacterial symbiont is labeled with GFP. In both cases, the anterior end of the worm is at the top. **C.** The life cycle of insect-parasitic nematodes. The IJ stage is a developmentally arrested third larval stage, and is the only free-living stage. IJs infect insect larvae by entering through a natural body opening, although *H. bacteriophora* can also penetrate directly through the larval cuticle. Following infection, IJs expel their symbiotic bacteria into the host, where it plays a critical role in overcoming the host immune system [5, 6]. The nematodes develop and reproduce inside the insect cadaver until the food is depleted, at which point new IJs

form and disperse into the soil in search of new hosts [13]. **D.** Jumping by *S. carpocapsae.* Still images of a jumping IJ. A standing IJ (0.0 s) curls (1.4 s) into a lariat structure (2.0 s) and propels itself into the air (2.3 s). Jumping was observed on an agar surface sprinkled with sand. Red arrows indicate the jumping IJ; time is recorded in the lower right. A single jump can propel the nematode nine body lengths in distance and seven body lengths in height, and can be elicited by chemosensory and mechanical stimuli [14]. **E–G.** Representative photomicrographs illustrating the insect-parasitic lifestyle. **E.** A Steinernematid IJ jumped onto and attached to a katydid antenna. Arrowhead indicates attached IJ. **F.** A cricket (*Acheta domesticus*) cadaver infected with steinernematids. Adult nematodes are visible beneath the cuticle throughout the cadaver; some of the most prominent nematodes are indicated by the arrowhead. **G.** IJs emerging from a depleted waxworm (*Galleria mellonella*) cadaver. Arrowhead indicates a clump of IJs; arrow indicates a single IJ.

unique to the genus *Steinernema* and is considered a specialized evolutionary adaptation that facilitates attachment to passing hosts as well as dispersal to new niches (Figure 3.1E) [15]. For both *H. bacteriophora* and *S. carpocapsae,* exposure to host volatiles can stimulate host-seeking behavior [16–19]. However, our understanding of how these parasites respond to specific olfactory cues is incomplete and nothing is known about the neural basis of these responses.

Parasitic IJs and *C. elegans* dauers are attracted to CO_2

To investigate how *H. bacteriophora* and *S. carpocapsae* IJs respond to host odors, we first examined responses to carbon dioxide (CO_2). CO_2 is emitted by all animals as a byproduct of respiration and is a host cue for a wide range of parasites and

disease vectors, including many parasitic nematodes [20–22]. We used a chemotaxis assay in which worms were allowed to distribute on a plate in a CO_2 concentration gradient (Figure 3.S2A). Parasitic IJs were strongly attracted to CO_2 across concentrations (Figures 3.2A, 3.S2C–D). To assay CO_2-evoked jumping, we developed a jumping assay in which standing IJs were exposed to a small puff of CO_2 from a syringe and given 8 seconds to jump in response to the puff (Figure 3.S2B). We found that CO_2 stimulates jumping by *S. carpocapsae* (Figures 3.2B, 3.S2E), demonstrating that CO_2 can evoke multiple host-seeking behaviors. CO_2 stimulated jumping at concentrations as low as 0.08%, which is ~twofold higher than atmospheric levels, indicating that jumping is highly sensitive to proximal levels of environmental CO_2 (Figure 3.S2E).

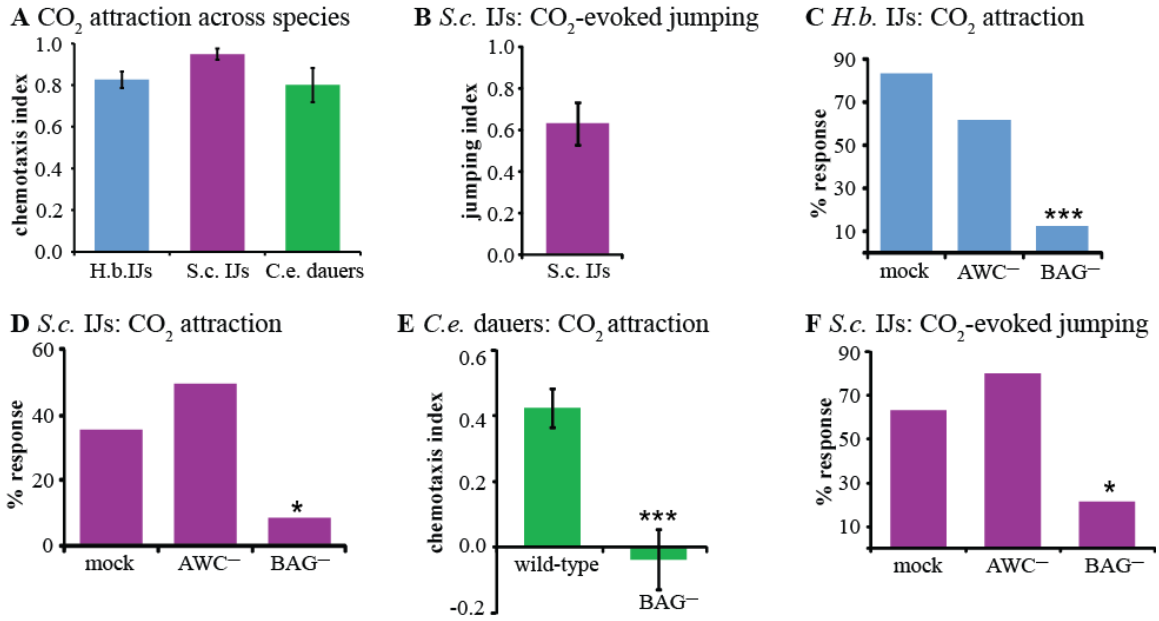

Figure 3.2 | BAG neurons are required for CO_2 response in free-living and parasitic nematodes. A. Parasitic IJs and *C. elegans* dauers are attracted to CO_2 in a chemotaxis assay (Figure S3A). n = 10–29 trials. **B.** CO_2 induces jumping by *S. carpocapsae* in a jumping assay (Figure S2B). n = 4–11 trials. **C–E.** BAG neurons are required for CO_2 attraction in *H. bacteriophora* and *S. carpocapsae* IJs, and *C. elegans* dauers. n = 12–34 worms for each

treatment (**C–D**) or n = 18–29 trials (**E**). **F.** BAG neurons are required for CO_2-evoked jumping by *S. carpocapsae* IJs. n = 10–18 worms for each treatment. ***, $P < 0.001$; *, $P < 0.05$, Fisher's exact test (**C**, **D**, **F**) or unpaired t test (**E**). Error bars represent SEM. For **C**, **D**, and **F**, y-axis values represent the percentage of worms that yielded a positive behavioral response; error bars are not present because each worm was scored once individually. AWC chemosensory neurons were ablated as a control. 10% CO_2 was used for all experiments.

The IJ stage of parasitic worms is analogous to the dauer stage of free-living worms*:* both are long-lived, non-feeding, developmentally arrested third larval stages [1], and conserved neurons and signaling pathways mediate exit from the dauer/IJ stage [23, 24]. *C. elegans* arrests development at the dauer stage when environmental conditions are unfavorable and develops to adulthood only after conditions improve; in nature, *C. elegans* is found primarily in the dauer stage [25]. We found that *C. elegans* dauers, like parasitic IJs, are attracted to CO_2 (Figures 3.2A, 3.S2F). By contrast, *C. elegans* adults are repelled by CO_2 [2, 3]. These results demonstrate that both dauers and IJs respond similarly to CO_2, and that *C. elegans* undergoes a developmental change in CO_2 response valence from the dauer to the adult stage. Why are dauers attracted to CO_2? Although the ecology of *C. elegans* is poorly understood, *C. elegans* dauers have been found in association with invertebrates such as slugs, snails, and isopods [26]. CO_2 attraction may enable dauers to migrate toward invertebrate carriers, thereby facilitating dispersal to new niches. CO_2 attraction may also serve as a means of locating bacterial food.

BAG sensory neurons are required for CO_2 attraction

To gain insight into the neural circuitry underlying host seeking, we leveraged the fact that neural anatomy and function are highly conserved across nematode species and life stages [23, 27–32]. In *C. elegans* adults, CO_2 repulsion requires a pair of sensory neurons called the BAG neurons [2]. We found that BAG neurons are easily identifiable in the parasitic IJs using the neuroanatomical map of *C. elegans* [33] (Figure 3.S2G; also see Methods). To investigate the role of BAG neurons in mediating CO_2 attraction, we ablated these neurons and examined CO_2 response. We found that parasitic IJs and *C. elegans* dauers that lack BAG neurons are not attracted to CO_2 (Figure 3.2C-E). In addition, *S. carpocapsae* IJs that lack BAG neurons do not exhibit CO_2-induced jumping (Figure 3.2F). Thus, BAG neurons are required for CO_2 attraction in both free-living and parasitic nematodes and contribute to both chemotaxis and jumping.

To further investigate the extent to which BAG neuron function is conserved throughout the phylum Nematoda, we examined a different nematode, *Pristionchus pacificus. P. pacificus* is a necromenic nematode that opportunistically feeds off insect cadavers and that is thought to represent an evolutionary intermediate between free-living and parasitic lifestyles [34]. Adult *P. pacificus* were previously shown to avoid CO_2 [2]. BAG-ablated *P. pacificus* adults do not avoid CO_2, indicating that BAG neurons are required for CO_2 repulsion by *P. pacificus* (Figure 3.S2H). The four species we have tested—*H. bacteriophora, S. carpocapsae, C. elegans,* and *P. pacificus*—display more molecular sequence divergence from each other than sea squirts do from humans [35]. Thus, BAG neurons play an ancient and conserved role in mediating CO_2 response in free-living and parasitic nematodes regardless of whether CO_2 is attractive or repulsive.

The fact that BAG neurons can mediate both attractive and repulsive responses is unusual for nematode sensory neurons, most of which are hard-wired for either attraction or repulsion. For example, the ASH sensory neurons play a conserved role in mediating repulsion to chemical and mechanical stimuli in free-living and parasitic nematodes [27, 29, 30], while the ADL neurons play a conserved role in mediating chemical avoidance [29]. The mechanism by which the BAG neuron can mediate either attraction or repulsion to the same stimulus is not yet understood.

BAG neurons are required for some but not all host-seeking behaviors

To test whether BAG neurons are required for host finding, we developed an assay in which headspace from a syringe containing insect larvae is used to establish a gradient of host odors. We examined responses to odors emitted by four insects that IJs are capable of using as hosts: waxworms (*Galleria mellonella*), superworms (*Zophobas morio*), mealworms (*Tenebrio molitor*), and crickets (*Acheta domesticus*). We found that *H. bacteriophora* and *S. carpocapsae* were attracted to all four insects (Figure 3.3A). Odors emitted by all four insects also stimulated jumping by *S. carpocapsae* (Figure 3.3B). The fact that *S. carpocapsae* chemotaxed toward host volatiles suggests that although these worms are generally considered ambushers, they are capable of utilizing a cruising strategy for host location. In contrast to the parasitic worms, *C. elegans* dauers were not attracted to these insects and in fact were repelled by mealworm odors (Figure 3.3A).

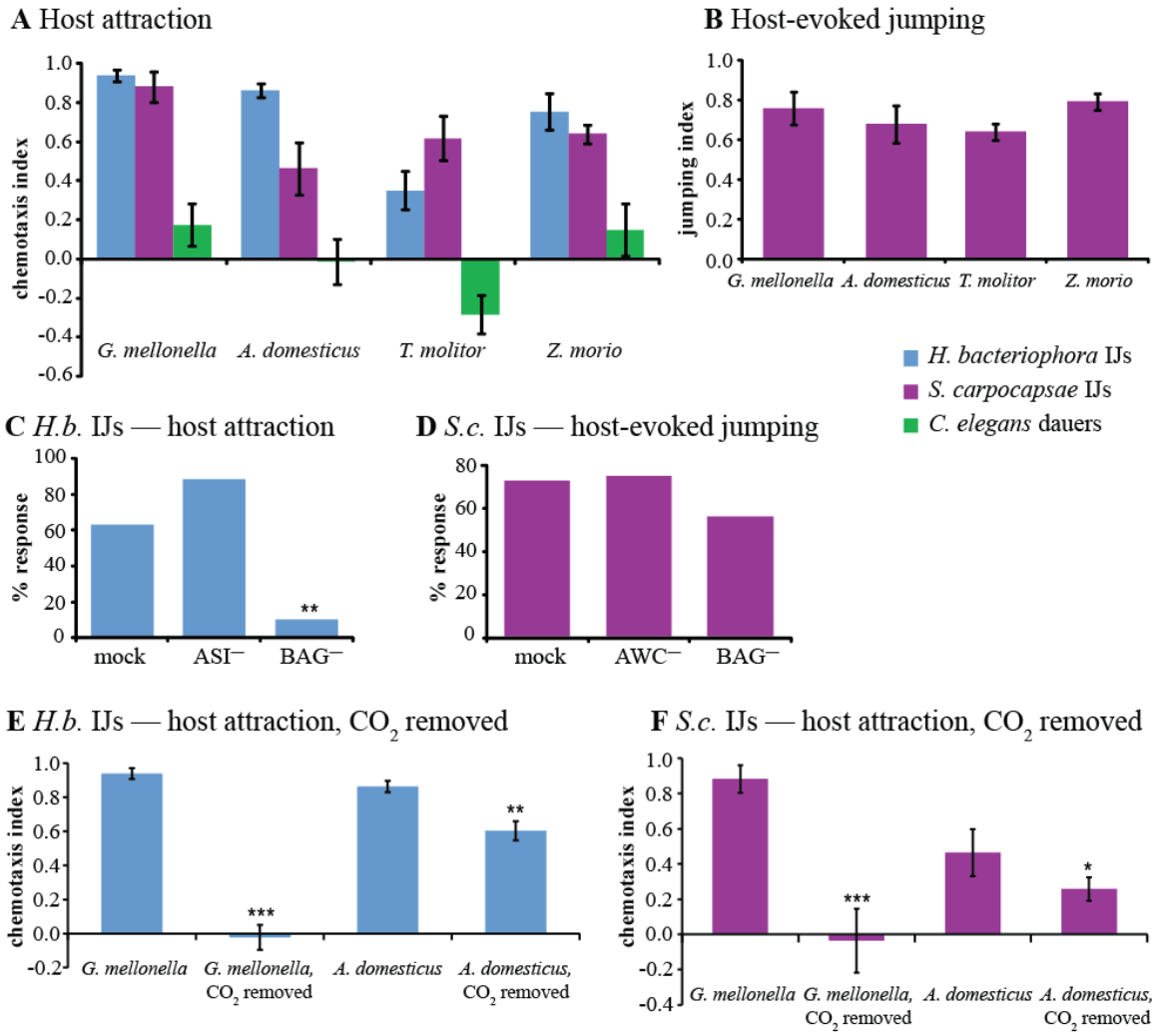

Figure 3.3 | BAG neurons are required for some but not all host-seeking behaviors.

A. Volatiles released by live waxworms (*Galleria mellonella*), crickets (*Acheta domesticus*), mealworms (*Tenebrio molitor*), and superworms (*Zophobas morio*) attract the parasitic IJs but not *C. elegans* dauers. n = 6–27 trials. **B.** Insect volatiles also stimulate jumping by *S. carpocapsae*. n = 3–11 trials. **, P < 0.01, one-way ANOVA with Dunnett's post-test. For **A–B**, error bars represent SEM. **C.** BAG neurons are required for chemotaxis toward waxworms in *H. bacteriophora*. n = 10–38 worms for each treatment. **, *P* < 0.01, Fisher's exact test. **D.** BAG neurons are not required for jumping evoked by waxworm odors in *S. carpocapsae*. n = 20–39 worms for each treatment. No significant differences were observed between treatment groups.

For **C–D**, values shown represent the percentage of worms that yielded a positive behavioral response; error bars are not present because each worm was scored once individually. AWC or ASI chemosensory neurons were ablated as controls.

We then examined host attraction in BAG-ablated animals. We focused on attraction to *G. mellonella* because it is the most commonly used laboratory host and IJs are capable of locating and infecting *G. mellonella* in complex soil environments [36, 37]. BAG-ablated *H. bacteriophora* IJs no longer chemotax to *G. mellonella* (Figure 3.3C), demonstrating a critical role for BAG neurons in host localization. Because BAG neurons are sensory neurons that detect CO_2 [38], our results suggest that CO_2 is an essential host cue for attraction of *H. bacteriophora* to *G. mellonella.* Insect-parasitic nematodes have a broad host range: they can infect a diverse array of insects and even some non-insect arthropods [39–41]. Our results suggest that *H. bacteriophora* may achieve this broad host range by relying primarily on CO_2 for attraction to some hosts. By contrast, ablation of the BAG neurons did not significantly affect the ability of *S. carpocapsae* IJs to jump in response to *G. mellonella* volatiles (Figure 3.3D), demonstrating that other neurons besides BAG and other host odors besides CO_2 are sufficient to mediate host-evoked jumping.

Host attraction involves responses to CO_2 as well as other host volatiles

To investigate the contribution of other host odors besides CO_2 to host attraction, we modified our host chemotaxis assay such that host volatiles were passed through a column of soda lime to chemically remove CO_2 (Figure 3.S3D). We found that removal

of CO_2 completely eliminated the attractive response to *G. mellonella,* consistent with our BAG-ablation results (Figure 3.S3E–F). By contrast, CO_2 removal reduced but did not eliminate attractive responses to *A. domesticus* (Figure S3E–F), demonstrating that other host volatiles besides CO_2 contribute to the attractiveness of some insect hosts.

Identification of volatiles emitted by insect larval hosts

To investigate the contribution of other odors to host-seeking behaviors, we used thermal desorption-gas chromatography-mass spectroscopy (TD-GC-MS) to identify odorants emitted by the four insects studied above. Overall, we identified eleven odorants that were given off in relatively high abundance by these hosts: hexanal and a-pinene from *G. mellonella* larvae; 2,3-butanedione and trimethylamine from *Z. morio* larvae; and acetic acid, 2-butanone, 3-hydroxy-2-butanone, dimethylsulfone, propanol, propionic acid, γ-terpinene, and trimethylamine from *A. domesticus* (Figure 3.S3). No abundant odorants were identified from *T. molitor* larvae using this technique (Figure 3.S3), suggesting that IJs may rely primarily on CO_2 to locate *T. molitor.*

Olfactory behavior in free-living versus parasitic nematodes

We constructed a panel of 57 odorants that included the identified host odorants, structurally related odorants, and other insect, plant, and bacterial odorants that nematodes are likely to encounter in their soil microenvironments. We then examined responses of *H. bacteriophora* IJs, *S. carpocapsae* IJs, and *C. elegans* dauers to these odorants. We found that all three species exhibited robust responses to many of the tested odorants (Figures 3.4A–B, 3.S4, and Table 3.S1). In the case of *S. carpocapsae,* we

found that many odorants differentially stimulated jumping and chemotaxis (Figure 3.4B), suggesting that different odorants are sufficient for different host-seeking behaviors. Five of the eleven host odorants that we identified—propanoic acid, hexanal, 2,3-butanedione, α-pinene, and γ-terpinene—stimulated jumping by *S. carpocapsae* (Figure 3.4B). By contrast, only one host odorant—1-propanol—was attractive to *H. bacteriophora* and none were attractive to *S. carpocapsae* in a chemotaxis assay (Figure 3.4A). Thus, the identified host odorants may function primarily in short-range host seeking. Two of the five host odorants that stimulated jumping are released by insect-damaged plants [42–44], raising the possibility that these odorants attract beneficial nematodes as a means of combating insect infestation. Such a strategy has already been documented for other species of insect-parasitic nematodes [45–47].

Using hierarchical cluster analysis, we found that the odor response profiles of *H. bacteriophora* and *S. carpocapsae* are more similar to each other than to that of *C. elegans* (Figure 3.4C). This contrasts with the phylogenetic relationship among these species: *H. bacteriophora* and *C. elegans* are much more closely related to each other than to *S. carpocapsae* (Figures 3.4C and 3.S1). The fact that *H. bacteriophora* and *S. carpocapsae* show more similar odor response profiles thus suggests a key role for olfaction in their convergently evolved parasitic lifestyles. Our data also provide insight into the evolution of olfactory behavior in free-living and parasitic nematode lineages. The fact that CO_2 attraction at the dauer/IJ stage is conserved in phylogenetically distant nematodes and that conserved neural circuitry mediates these responses suggests that CO_2 attraction may be an ancestral feature of nematodes that precedes their divergence into free-living and parasitic lineages. By contrast, responses to other odorants differ

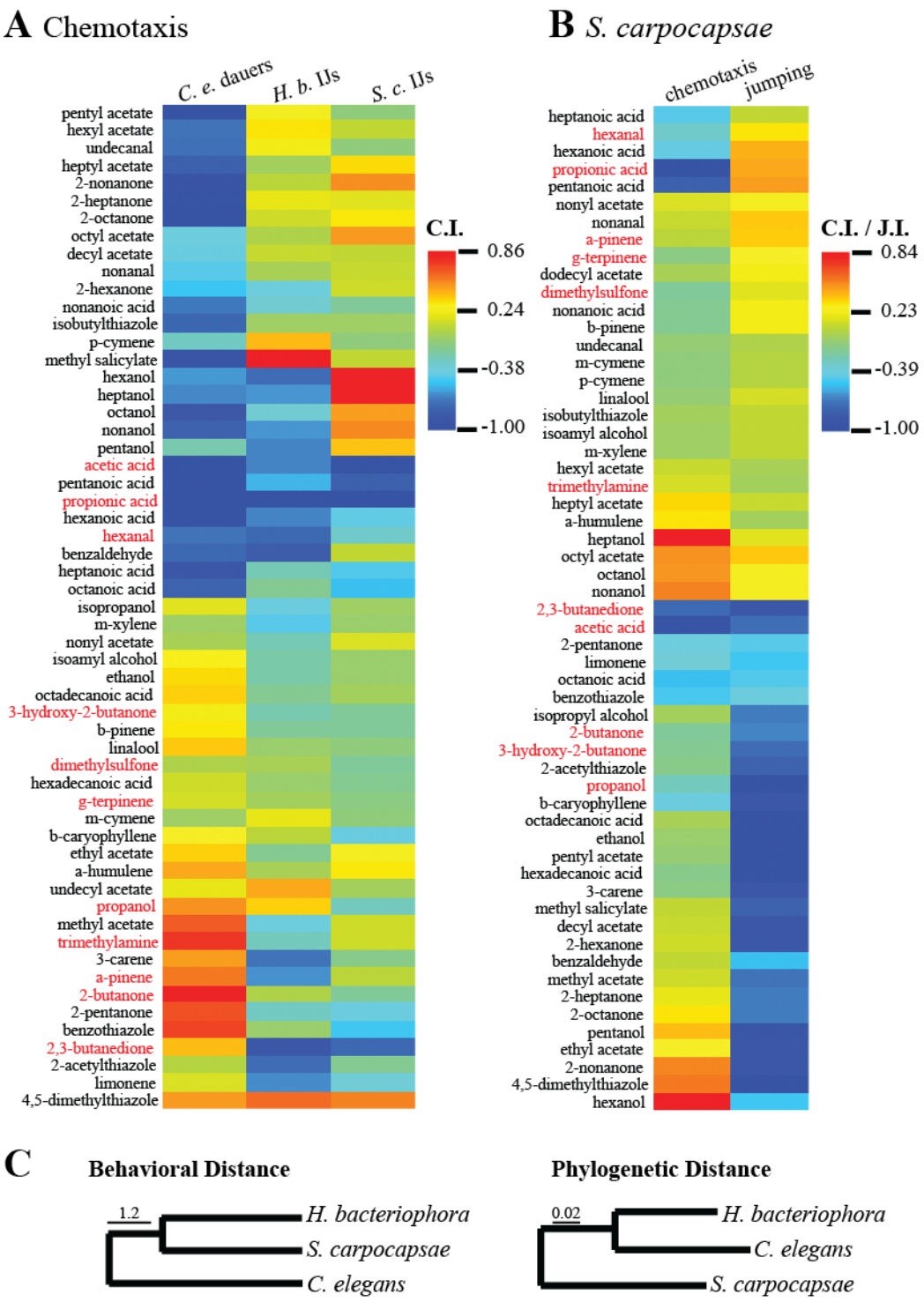

Figure 3.4 | Odor response profiles of free-living and parasitic nematodes. A. Odor response profiles of *C. elegans* dauers, *H. bacteriophora* IJs, and *S. carpocapsae* IJs. n = 5–33 trials for each odorant. **B.** A comparison of odorant-evoked chemotaxis and jumping by

S. carpocapsae. Both the chemotaxis index (C.I.) and the jumping index (J.I.) range from -1 to +1, with -1 indicating perfect repulsion and +1 indicating perfect attraction (Figures 3.S2B and 3.S8A). n = 5–8 trials for chemotaxis and 3–10 trials for jumping. Data for chemotaxis is from **A**. For **A** and **B**, response magnitudes are color-coded according to the scale shown to the right of each heat map, and odorants are ordered based on hierarchical cluster analysis. Host odorants identified by TD-GC-MS of insect headspace are highlighted in red. **C.** The odor response profiles of *H. bacteriophora* and *S. carpocapsae* are more similar to each other than to that of *C. elegans,* despite the fact that *H. bacteriophora* and *C. elegans* are more closely related phylogenetically. Left, behavioral dendrogram of olfactory responses across species. Behavioral distance is based on the Euclidian distances between species based on their odor response profiles. Right, phylogenetic neighbor-joining tree. Branch lengths in the phylogenetic tree are proportional to genetic distances between taxa; scale bar represents 0.02 nucleotide substitutions per site.

among species, suggesting that these responses may be more highly derived features that reflect niche-specific ecological requirements. Our discovery that BAG neurons mediate CO_2 response and host-seeking behavior in phylogenetically distant nematode species raises the possibility that compounds that block BAG neuron function may be useful for nematode control.

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Chapter 4:

Olfaction Shapes Host-Parasite Interactions in Parasitic Nematodes*

*This chapter, first published in *PNAS* in 2012 and was written by Adler R. Dillman, Manon L. Guillermin, Jooh Ha Lee, Brian Kim, Paul W. Sternberg, and Elissa A. Hallem.

Abstract

Many parasitic nematodes actively seek out hosts in which to complete their lifecycles [1]. Olfaction is thought to play an important role in the host-seeking process, with parasites following a chemical trail toward host-associated odors [2–7]. However, little is known about the olfactory cues that attract parasitic nematodes to hosts or the behavioral responses these cues elicit. Moreover, what little is known focuses on easily obtainable laboratory hosts rather than natural or other ecologically relevant hosts. Here we investigate the olfactory responses of six diverse species of entomopathogenic nematodes (EPNs) to seven ecologically relevant potential invertebrate hosts, including one known natural host and other potential hosts collected from the environment. We show that EPNs respond differentially to the odor blends emitted by live potential hosts as well as individual host-derived odorants. In addition, we show that EPNs use the universal host cue carbon dioxide (CO_2) as well as host-specific odorants for host location, but the relative importance of CO_2 versus host-specific odorants varies for different parasite-host combinations and for different host-seeking behaviors. We also identify novel host-derived odorants by gas chromatography-mass spectrometry, and find that many of these odorants stimulate host-seeking behaviors in a species-specific manner. Taken together, our results demonstrate that parasitic nematodes have evolved specialized olfactory systems that likely contribute to appropriate host selection.

Introduction

Many parasitic nematodes actively seek out hosts using sensory cues [8]. Host seeking is a complex behavior that involves chemosensory, thermosensory,

hygrosensory, and mechanosensory cues [1, 2, 8, 9]. Olfaction is a critical component of host-seeking behavior: many parasitic nematodes use carbon dioxide (CO_2) and other host volatiles for host location [1, 4, 8, 10–12]. However, little is known about how parasites respond to host-derived odors.

Entomopathogenic nematodes (EPNs) are powerful models for the study of odor-driven host-seeking behavior. EPNs comprise a guild—a group of phylogenetically divergent species that exploit the same class of resources in a similar way [13]—that includes the genera *Heterorhabditis, Steinernema,* and *Oscheius* [14, 15]. EPNs are parasites of insects that infect and kill insect larvae [14, 15]. They offer a number of advantages as model systems including small size, short generation time, and amenability to laboratory culturing and behavioral analysis [3, 16]. In addition, they resemble skin-penetrating human-parasitic nematodes in that they actively seek out hosts using olfactory cues [1, 3–5, 17, 18]. EPNs are also of interest as biocontrol agents for insect pests and disease vectors, and are currently used throughout the world as environmentally safe alternatives to chemical insecticides. The three genera of EPNs are phylogenetically distant but have highly similar lifestyles as a result of convergent evolution to insect parasitism [19].

EPNs are thought to engage in host-seeking behavior only during a particular life stage called the “infective juvenile” (IJ), a developmentally-arrested third larval stage analogous to the dauer stage of some free-living worms [20]. After long-range host location, IJs are thought to use short-range sensory cues for host recognition [21]. IJs then infect either by entering through natural orifices or by penetrating through the insect cuticle [22]. Following infection, IJs release a bacterial endosymbiont into the insect

host and resume development [23–25]. The bacteria proliferate inside the insect, producing an arsenal of secondary metabolites that lead to rapid insect death and digestion of insect tissues. The nematodes feed on the multiplying bacteria and the liberated nutrients of broken-down insect tissues. They reproduce in the cadaver until resources are depleted, at which time new IJs form and disperse in search of new hosts [26].

EPNs utilize a wide range of host-seeking strategies. Some are "cruisers" that actively seek out hosts, while others are "ambushers" that remain stationary and infect passing hosts. However, these strategies represent endpoints along a continuum, and many species are "intermediates" that are capable of utilizing both cruise and ambush strategies for host location [27, 28]. In addition, some EPNs of the genus *Steinernema* exhibit jumping, a rare behavior among soft-bodied, limbless organisms [29, 30]. Among EPNs, jumping is a highly specialized ambushing behavior in which the IJ propels itself into the air [3, 29, 31]. Jumping is thought to be a short-range host-seeking strategy that facilitates host attachment when the host is in close proximity [29, 32, 33]. In general, cruisers are most effective at infecting stationary hosts, while ambushers are most effective at infecting fast-moving hosts [34]. Previous studies have demonstrated that EPNs are attracted to CO_2 as well as to a number of other odorants [3, 5–7, 17, 35]. However, little is known about how EPNs respond to host odors, or how olfactory responses contribute to differences in host-seeking strategy.

Here, we show that EPNs respond differently to different potential hosts and host-derived odorants, and that olfactory responses differ even for closely related EPNs. We also identify host-derived odorants that stimulate host-seeking behaviors in a

species-specific manner. Our results suggest that parasitic nematodes have specialized olfactory systems that contribute to differences in host preference and host-seeking strategy among species.

Results

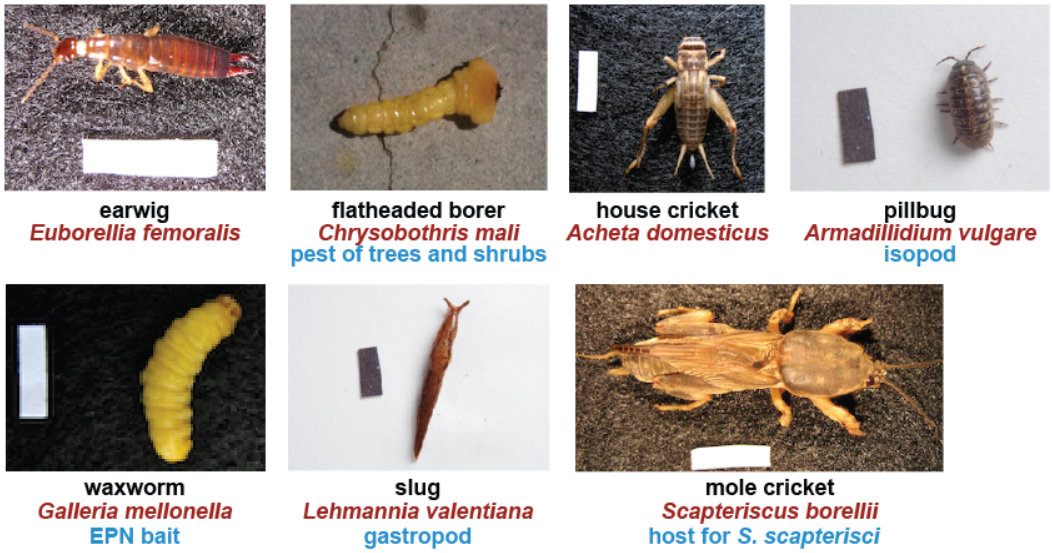
We examined the odor-evoked host-seeking behaviors of six different EPNs in response to seven potential invertebrate hosts. The EPNs—*Heterorhabditis bacteriophora, Steinernema carpocapsae, Steinernema scapterisci, Steinernema riobrave, Steinernema glaseri,* and *Oscheius* carolinensis—were chosen based on both their phylogenetic and behavioral diversity (Figure 4.S1). These species vary greatly in their host-seeking strategies: *H. bacteriophora* and *S. glaseri* are cruisers, *S. carpocapsae* and *S. scapterisci* are ambushers, and *S. riobrave* employs an intermediate host-seeking strategy. In addition, *S. carpocapsae, S. scapterisci,* and *S. riobrave* display jumping as well as chemotaxis behavior. The host-seeking behavior of *O. carolinensis,* a recently discovered EPN and the closest known EPN relative of *C. elegans* [25], has not yet been characterized.

These six EPN species were also chosen due to their differing host ranges. *H. bacteriophora* and *S. carpocapsae* are thought to have very broad host ranges, with *S. carpocapsae* capable of infecting over 250 different species of insects from 13 orders under laboratory conditions [36, 37]. By contrast, *S. scapterisci* is an orthopteran specialist with a much narrower host range than most EPNs; its only known natural host is the mole cricket [38–40]. *S. glaseri* has a somewhat broader host range; it is capable of infecting insects in several orders but is thought to prey primarily on

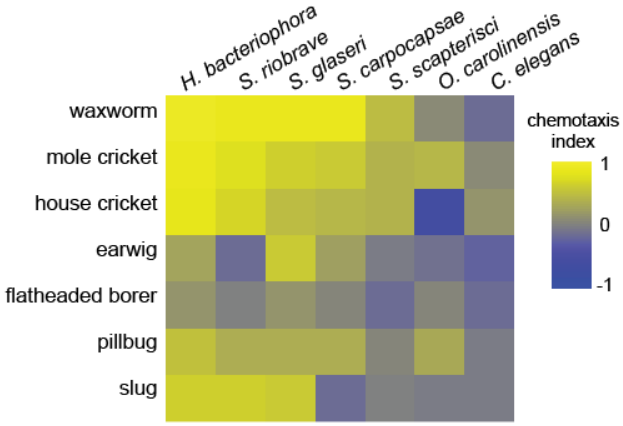
sedentary subterranean larvae, such as those of beetles [36, 41, 42]. *S. riobrave* has not been as thoroughly tested, but it is presumed to have a fairly broad host range and it has been used successfully as a biocontrol agent against both lepidopteran and coleopteran hosts [43, 44]. The host range of *O. carolinensis* has not yet been tested [45]. Little is known about the natural hosts of EPNs. Of the six EPN species used in this study, natural hosts are known for *H. bacteriophora*, *S. carpocapsae*, *S. scapterisci,* and *S. glaseri* and are *Heliothis punctigera* (Lepidoptera: Noctuidae) [46], *Cydia pomonella* (Lepidoptera: Nocteuidae) [47], *Scapteriscus vicinus* and *Scapteriscus borellii* (Orthoptera: Gryllotalpidae) [39, 48], and *Popillia japonica* (Coleoptera: Scarabaeidae) [49], respectively. Whether these represent true natural hosts or merely opportunistic hosts remains unclear for all but *S. scapterisci*, which has been used for decades to successfully control invasive species of mole crickets [38].

The seven potential invertebrate hosts—the mole cricket *Scapteriscus borellii*, the house cricket *Acheta domesticus*, the earwig *Euborellia femoralis*, the waxworm *Galleria mellonella,* the flatheaded borer *Chrysobothris mali*, the pillbug *Armadillidium vulgare*, and the slug *Lehmannia* valentiana—were also chosen based on their phylogenetic and ecological diversity (Figure 4.1A). Mole crickets are the only known natural host for *S. scapterisci* [38], and house crickets are related to mole crickets and can serve as laboratory hosts for both *S. scapterisci* and *S. carpocapsae* [50]. Earwigs were chosen because some earwig species are thought to be preferred natural hosts for *S. carpocapsae* [37].

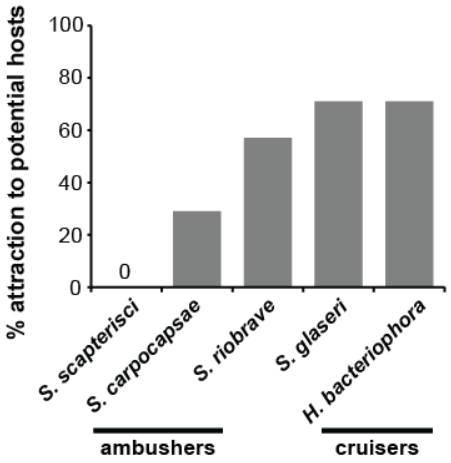
A Potential hosts



B Chemotaxis to hosts



C Host attraction reflects host-seeking strategy



D Jumping to hosts

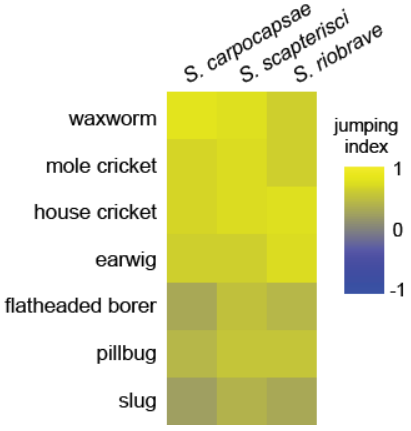


Figure 4.1 | EPNs respond differently to different potential hosts. A. Potential invertebrate hosts tested. Mole crickets, earwigs, flatheaded borers, pillbugs, and slugs were collected from the greater Los Angeles area. Waxworms and house crickets were purchased commercially. Scale bars are 1 cm x 2.5 mm. **B.** Chemotaxis of EPN IJs and *C. elegans* dauers to volatiles released by live potential hosts. The order of both the nematodes and the hosts in the heat map was determined by hierarchical cluster analysis (Ward's method). EPNs respond differently to different hosts ($P < 0.0001$), different hosts evoke different overall responses from EPNs ($P < 0.0001$), and different EPNs show different odor response profiles ($P < 0.0001$) (two-factor ANOVA with replication, with a Bonferroni post-test). n = 6–30 trials for each EPN-host combination. Mean, n, and SEM values for each assay are given in Appendix B; *P* values for each post-test are also given in Appendix B. **C.** Chemotaxis behavior reflects host-seeking strategy such that cruisers display more overall attraction to hosts than ambushers. The y-axis indicates the percentage of hosts that were strongly attractive (as defined by a chemotaxis index of ≥ 0.5). *S. scapterisci* and *S. carpocapsae* are cruisers, *S. glaseri* and *H. bacteriophora* are ambushers, and *S. riobrave* employs both cruising and ambushing strategies for host seeking. The responses of the ambushers *S. scapterisci* and *S. carpocapsae* cluster separately from the responses of the cruisers *S. glaseri* and *H. bacteriophora* and the ambusher/cruiser *S. riobrave* by k-means cluster analysis and hierarchical cluster analysis (Ward's method, coph. corr. = 0.85). **D.** Jumping of EPNs in response to volatiles released by live potential hosts. The order of the nematodes in the heat map was determined by hierarchical cluster analysis (Ward's method); the order of the hosts is the same as in **B**. EPNs respond differently to different hosts ($P<0.0001$) and different hosts evoke different overall responses from EPNs ($P < 0.0001$) (two-factor ANOVA with replication, with a Bonferroni post-test). However, different EPNs do not show significantly different odor response profiles (two-factor ANOVA with replication). n = 2–13 trials for each EPN-host combination. Mean, n, and SEM values for each assay are

given in Appendix B; *P* values for each post-test are given in Appendix B. For **B** and **D**, response magnitudes are color-coded such that a chemotaxis index or jumping index of +1 is yellow, -1 is blue, and 0 is grey.

Waxworms were selected because they are a common laboratory host for EPNs and are typically used as bait when collecting EPNs from soil; thus, many described EPNs are attracted to waxworms, even in complex soil environments [51, 52]. However, waxworms are damaging residents of beehives and are not likely to encounter soil-dwelling EPNs under natural conditions. Similarly, larval flatheaded borers are not likely to be encountered by EPNs, as they develop under the bark in the phloem of host plants [53]. They represent non-natural but potential hosts of EPNs, ones that EPNs have not evolved to find or infect. By contrast, pillbugs and slugs are non-insects that are similar in size to many potential insect hosts of EPNs and are often in the same or overlapping communities with EPNs. Pillbugs belong to the same phylum as insects (Arthropoda) but a different order (Isopoda), while slugs belong to a different phylum (Mollusca) and are much more distantly related to insects. Both pillbugs and slugs have been explored as potential alternative hosts for EPNs and found to be non-hosts or dead-end hosts for several EPNs [54–58]; however, the potential for EPNs to utilize isopods and gastropods as alternative or reservoir hosts when insects are scarce has not been fully explored, and whether EPNs display any behavioral preference for isopods and gastropods had not yet been tested. Mole crickets, earwigs, flatheaded borers, pillbugs, and slugs were collected from their natural habitats in the greater Los Angeles area and were tested within a few weeks of collection (Figure 4.S2).

EPNs respond differently to different host odors

We examined EPN responses to odors emitted from live hosts using both chemotaxis and jumping assays [3]. We found that all six EPNs responded significantly more to some potential hosts than others, and some potential hosts were significantly more attractive overall than others (Figure 4.1B, Appendix B). In addition, odor response profiles differ for the different EPNs such that some hosts are more attractive to some EPNs than others (Figure 4.1B, Appendix B). Overall, we found that host attraction reflects host-seeking strategy, with cruisers showing more host attraction than ambushers in our chemotaxis assay (Figure 4.1C). Thus, the host-seeking behavior of EPNs likely reflects their ability to respond differentially to odors emitted by different potential hosts. For comparison, we also examined the responses of *C. elegans* dauers to the potential host odors; the Hawaii strain was used for this comparison because it most closely resembles wild *C. elegans* strains [59]. We found that all of the invertebrate odors were neutral or repulsive (chemotaxis index < 0.2) for *C. elegans* dauers (Figure 4.1B, Appendix B). Thus, the host attraction we observe is specific to the EPNs.

Jumping behavior in response to potential hosts also varied for different EPNs and different hosts (Figure 4.1D, Appendix B). EPNs showed significantly higher rates of jumping in response to some potential hosts than others, and some potential hosts evoked significantly higher rates of jumping overall than others (Figure 4.1D, Appendix B). However, the three jumping EPN species did not show species-specific jumping profiles: the relative responses elicited by the different potential hosts did

not vary significantly across species (Figure 4.1D, Appendix B). These results suggest that chemotaxis behavior may display more species specificity than jumping behavior.

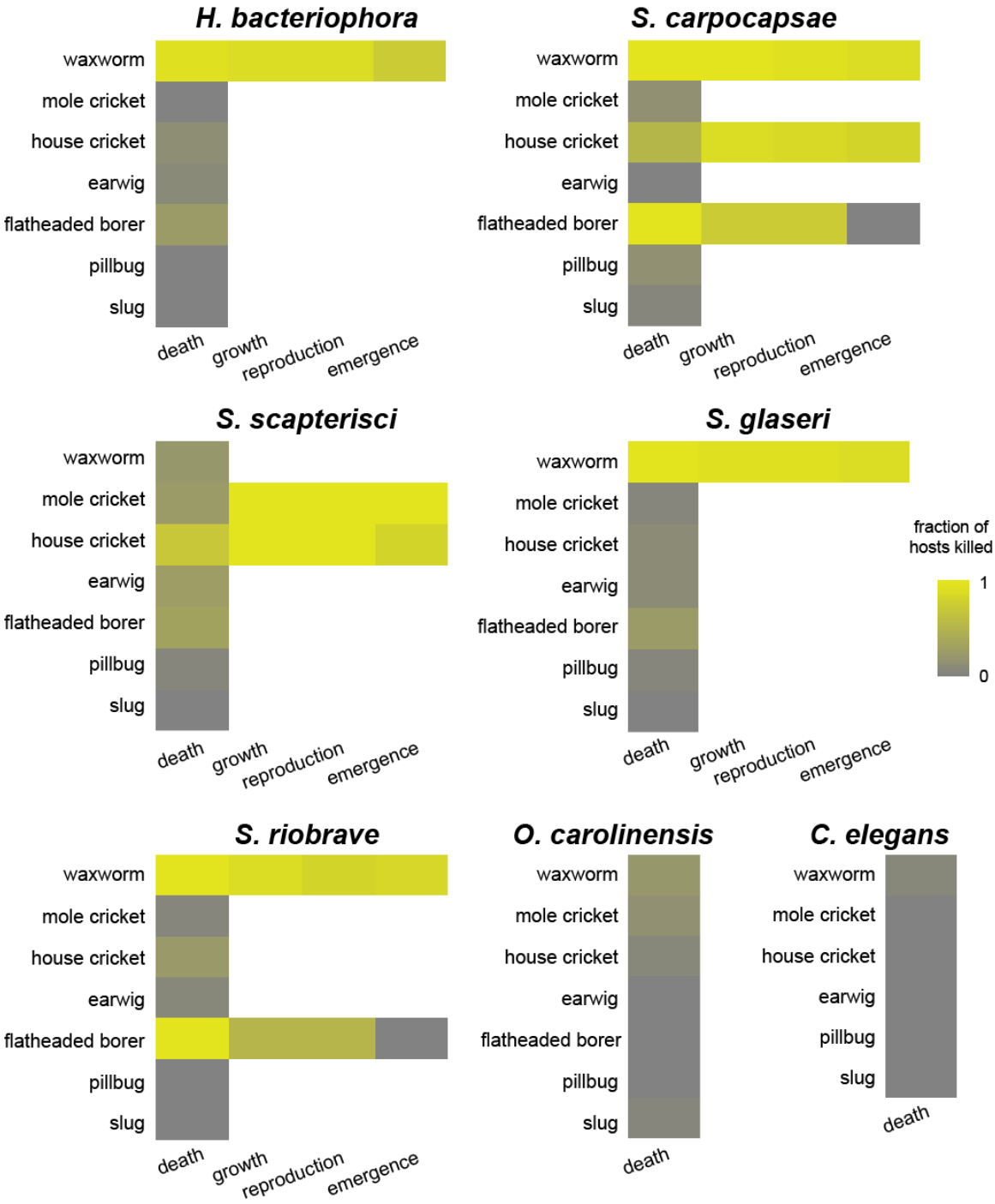


Figure 4.2 | EPNs differ in their virulence toward potential hosts. Graphs show the virulence of each nematode toward the panel of potential hosts. Values for "death" represent the

fraction of hosts that died within 48 hours following exposure to nematodes. Values for "growth," "reproduction," and "emergence" represent the fraction of dead hosts that supported nematode growth, reproduction, and emergence, respectively. The frequency of death following exposure to nematodes was scored for all potential hosts; growth, reproduction, and emergence were scored only when host killing was observed at statistically significant levels. Each virulence assay consisted of a single potential host and 200 IJs. n = 20–50 assays for all invertebrates except flatheaded borers; n = 8–12 assays for flatheaded borers due to limited availability of these insects. For each EPN-host combination, statistical significance was determined relative to an uninfected control using a chi-squared test. Mean values for death, growth, reproduction, and emergence are given in Appendix B.

EPNs vary in their virulence toward potential hosts

We then tested the virulence—i.e., the disease-producing power [60]—of the six different EPNs toward the seven potential hosts. EPN virulence is usually tested by exposing potential hosts to a defined number of IJs (typically between 1 and 1000 per potential host) [58, 61, 62]. Previous work suggests that using high doses of IJs in mortality experiments allows poor host suitability to be overcome by high number of parasites [35]. Therefore, in our virulence assays, individual host animals were exposed to 100 IJs and host survival was scored after 48 hours. In cases where the EPNs successfully killed the host, we subsequently scored EPN growth, reproduction, and emergence from host cadavers. We found that EPN virulence varied greatly among species (Figure 4.2, Appendix B). For example, *S. carpocapsae* was virulent toward three of the seven species tested, while *O. carolinensis* was not virulent toward any of these species at the concentration of IJs tested. Overall, we found that waxworms are

very efficient hosts for most EPNs: all species except *S. scapterisci* and *O. carolinensis* were highly successful at parasitizing waxworms. This could reflect the proclivity of these species to infect lepidopteran hosts, or the isolated environment of larval waxworms; as pests of beehives, they are unlikely to have evolved behavioral and immune defenses against soil-dwelling EPNs. It could also reflect unintentional laboratory selection toward virulence in waxworms, since most of these species have been maintained in waxworms since being collected from the wild. As expected, we found that *S. scapterisci* was most virulent toward crickets. In our assay, *S. scapterisci* was not as efficient at killing its natural host, the mole cricket, as it was at killing the house cricket: only 25% of mole crickets were killed compared to 71% of house crickets. However, mole crickets that were successfully killed were the most effective hosts: 100% of the mole cricket cadavers supported *S. scapterisci* growth, reproduction, and emergence (Figure 4.2, Appendix B). We note that *S. scapterisci* has been shown to be extremely effective at killing both house crickets and mole crickets at higher IJ densities than we tested here [40]. Flatheaded borers proved to be dead-end hosts for both *S. carpocapsae* and *S. riobrave*: although the EPNs could infect borers and in some cases grow and reproduce inside borer cadavers, emergence of IJs from borer cadavers was never observed (Figure 4.2, Appendix B). None of the EPNs were able to successfully kill earwigs, pillbugs, or slugs in our assay (Figure 4.2, Appendix B). Thus, at this inoculum (100 IJs per host), EPNs differ in their host ranges.

CO_2 is a host-seeking cue for both generalist and specialist EPNs

We then examined the host-derived odorants that stimulate host-seeking behavior. We first examined responses to CO_2, which is emitted by all animals as a byproduct of respiration and is a host cue for a wide range of parasites, including many types of parasitic nematodes [1, 12, 63]. To examine the chemotactic response to CO_2, we used a CO_2 chemotaxis assay in which worms were allowed to distribute on a plate in a CO_2 concentration gradient [3]. We found that all of the tested EPNs are attracted to CO_2 (Figure 4.3A, Appendix B) and all three of the jumping species jumped in response to CO_2 (Figure 4.3B, Appendix B). However, CO_2 attractiveness varied among EPNs, with *S. scapterisci* and *O. carolinensis* showing less attraction to low concentrations of CO_2 than the other species (Figure 4.3A, Appendix B). Responses to low CO_2 concentrations were highly correlated with overall host attraction, suggesting that differences in overall host attraction may be attributable to differences in CO_2 sensitivity among EPNs (Figure 4.3C). Thus, CO_2 is an important host-seeking cue for both specialist and generalist EPNs.

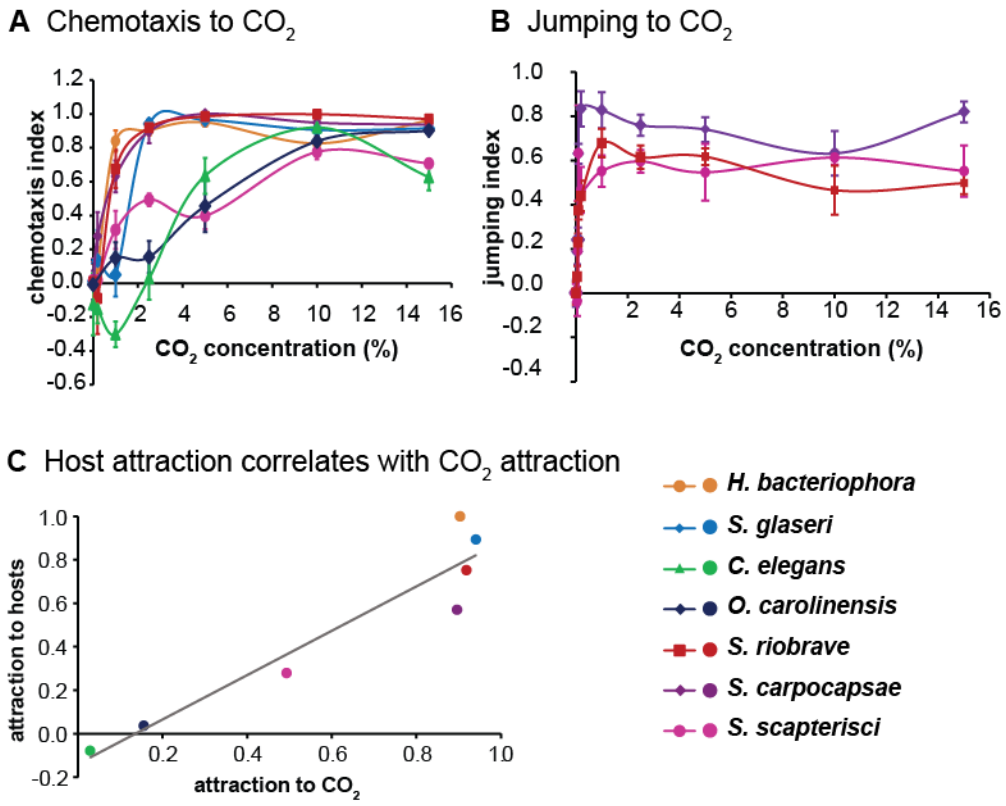


Figure 4.3 | CO_2 stimulates host-seeking behavior of EPNs. A. Chemotaxis of EPN IJs and *C. elegans* dauers to CO_2. n = 5–23 trials. Data for *H. bacteriophora* and *S. carpocapsae* are from Hallem *et al.,* 2011 [3]. **B.** Jumping of EPNs to CO_2. n = 43–192 animals. **C.** Host attraction correlates with CO_2 attraction. The x-axis indicates the chemotaxis index in response to 2.5% CO_2; the y-axis indicates the normalized sum of the chemotaxis indices toward all hosts. The best-fit linear trendline is shown. R^2 = 0.90. Mean, n, and SEM values for each assay are given in Appendix B.

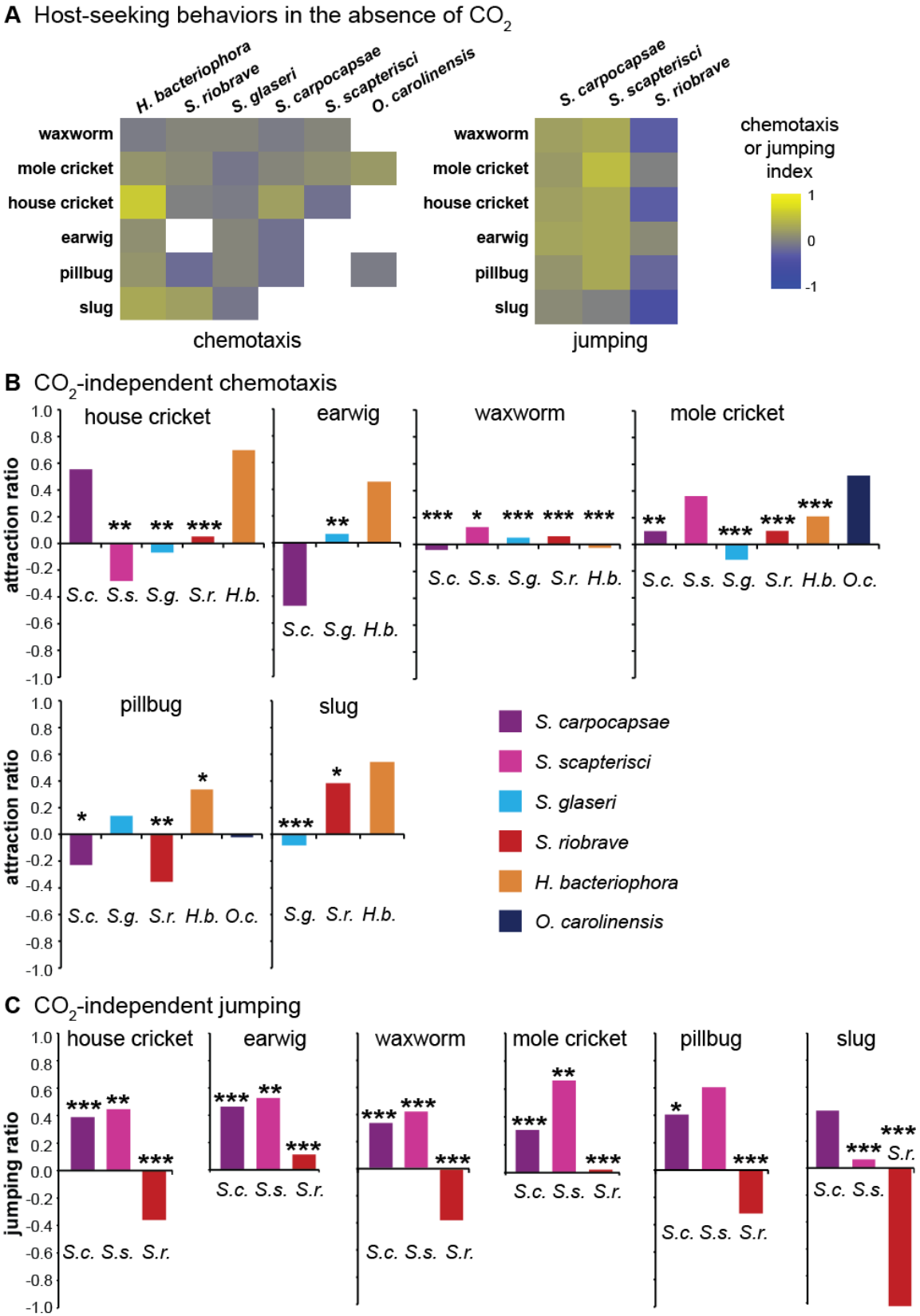

A Host-seeking behaviors in the absence of CO_2
H. bacteriophora
S. riobrave
S. glaseri
S. carpocapsae
S. scapterisci
O. carolinensis
waxworm
mole cricket
house cricket
earwig
pillbug
slug
chemotaxis
S. carpocapsae
S. scapterisci
S. riobrave
jumping
chemotaxis or jumping index
1
0
-1
B CO_2-independent chemotaxis
attraction ratio
house cricket
earwig
waxworm
mole cricket
pillbug
slug
S.c. S.s. S.g. S.r. H.b.
S.c. S.g. H.b.
S.c. S.s. S.g. S.r. H.b.
S.c. S.s. S.g. S.r. H.b. O.c.
S.c. S.g. S.r. H.b. O.c.
S.g. S.r. H.b.
S. carpocapsae
S. scapterisci
S. glaseri
S. riobrave
H. bacteriophora
O. carolinensis
C CO_2-independent jumping
jumping ratio
house cricket
earwig
waxworm
mole cricket
pillbug
slug
S.c. S.s. S.r.

Figure 4.4 | Host-seeking behavior is reduced in the absence of CO_2. A. Chemotaxis to live hosts is significantly reduced when CO_2 is removed from the host airstream using soda lime (left graph) ($P < 0.0001$ for all species except *O. carolinensis* and $P < 0.05$ for *O. carolinensis*, two- factor ANOVA with replication). Chemotaxis with CO_2 removed was tested only for EPN-host combinations where host attraction was initially observed. Jumping to live hosts is also reduced when CO_2 is removed from the host airstream using soda lime (right graph) ($P < 0.001$, two-factor ANOVA with replication). n = 6–22 trials for chemotaxis and 2–7 trials for jumping for each EPN- host combination. **B.** Levels of CO_2-independent attraction to potential hosts. Attraction ratios indicate the chemotaxis index for host attraction with CO_2 removed divided by the chemotaxis index for host attraction with CO_2. **C.** Levels of CO_2-independent jumping to potential hosts. Jumping ratios indicate the jumping index for host-evoked jumping with CO_2 removed divided by the jumping index for host-evoked jumping with CO_2. For **B** and **C**, asterisks indicate cases where the response to host with CO_2 removed was significantly different from the response to host with CO_2 present. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$, two-factor ANOVA with replication with a Bonferroni post-test. Mean, n, and SEM values for each assay in **A** are given in Appendix B; *P* values for each post-test are given in Appendix B.

The requirement for CO_2 varies for different EPN-host combinations

To test whether CO_2 is required for host attraction, we assayed the response to live hosts in the presence of soda lime, which removes CO_2 [3]. We found that for all EPN-host combinations, chemotaxis was reduced in the absence of CO_2 (Figure 4.4A, Appendix B). However, the extent of the reduction varied greatly for different EPNs

and different hosts. For example, none of the EPNs were attracted to waxworms in the absence of CO_2, whereas mole crickets, house crickets, and earwigs were still attractive to some EPNs but not others (Figure 4.4B, Appendix B). Removal of CO_2 did not render any hosts significantly repulsive (C.I. ≤ -0.2) (Figure 4A). Host-evoked jumping was also reduced in the absence of CO_2, and as for chemotaxis, the requirement for CO_2 differed for different EPN-host combinations (Figures 4.4A and 4.4C, Appendix B). Thus, while CO_2 is sufficient for eliciting host-seeking behavior from all EPNs, it is both necessary and sufficient for some EPN-host combinations but not others. To further test the role of CO_2 versus host-specific odors in host seeking, we performed a chemotaxis competition experiment with *S. carpocapsae* in which CO_2 was introduced into one side of the chemotaxis plate and odor from a single mole cricket was introduced into the other side (Figure 4.S3). We found that *S. carpocapsae* prefers live mole crickets to 1% CO_2 (Figure 4.S3), despite the fact that 1% CO_2 is highly attractive to *S. carpocapsae* and that attraction of *S. carpocapsae* to mole crickets is greatly reduced in the absence of CO_2 (Figure 4.4A). However, higher concentrations of CO_2 are more attractive than mole crickets (Figure 4.S3). These results demonstrate that EPNs use both CO_2 and host-specific odorants for host location.

A diverse array of host-derived odorants stimulate host-seeking behaviors

We next identified host-derived odorants that elicit host-seeking behavior. We previously used thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS) to identify odorants emitted by waxworms and house crickets [3]. We have now extended this analysis to all seven potential invertebrate hosts using TD-GC-MS and solid-phase

microextraction-gas chromatography-mass spectrometry (SPME-GC-MS) [64]. Overall, we identified 21 odorants emitted consistently and at relatively high abundance by the potential hosts (Figures 4.5 and 4.S4). (One of these odorants, p-dichlorobenzene, is a common pesticide that is unlikely to be insect-derived.) The number of odorants we identified from each invertebrate ranged from nine for house crickets to two for waxworms to zero for slugs (Figure 4.5). The fact that we identified more odorants from crickets than waxworms is consistent with our finding that crickets evoke higher levels of CO_2-independent attraction than waxworms (Figure 4.4B) and suggests that the relative contribution to host seeking of CO_2 versus host-specific odorants may be partly dependent on the number of odorants the host emits. We then examined the behavioral responses to these odorants, and found that many strongly stimulated host-seeking behaviors (Figure 4.6, Appendix B). Overall, we observed strong responses to at least one odorant identified from each of the tested invertebrates (with the exception of slugs, for which we did not successfully identify any odorants), suggesting that a wide variety of chemically diverse olfactory cues contribute to host-seeking behavior. The odorants that stimulated the strongest host-seeking responses differed for the different species—for example, 2-propanone, 4-methylphenol, and tetradecane were strongly attractive for *S. carpocapsae* but repulsive or neutral for the other species (Figure 4.6, Appendix B). In addition, all EPNs displayed unique chemotaxis and jumping odor response profiles to host-derived odorants with the exception of *S. riobrave* and *O. carolinensis*, whose chemotaxis odor response profiles did not differ significantly (Figure 4.6, Appendix B). Thus, most EPNs display species-specific responses to host-derived odorants.

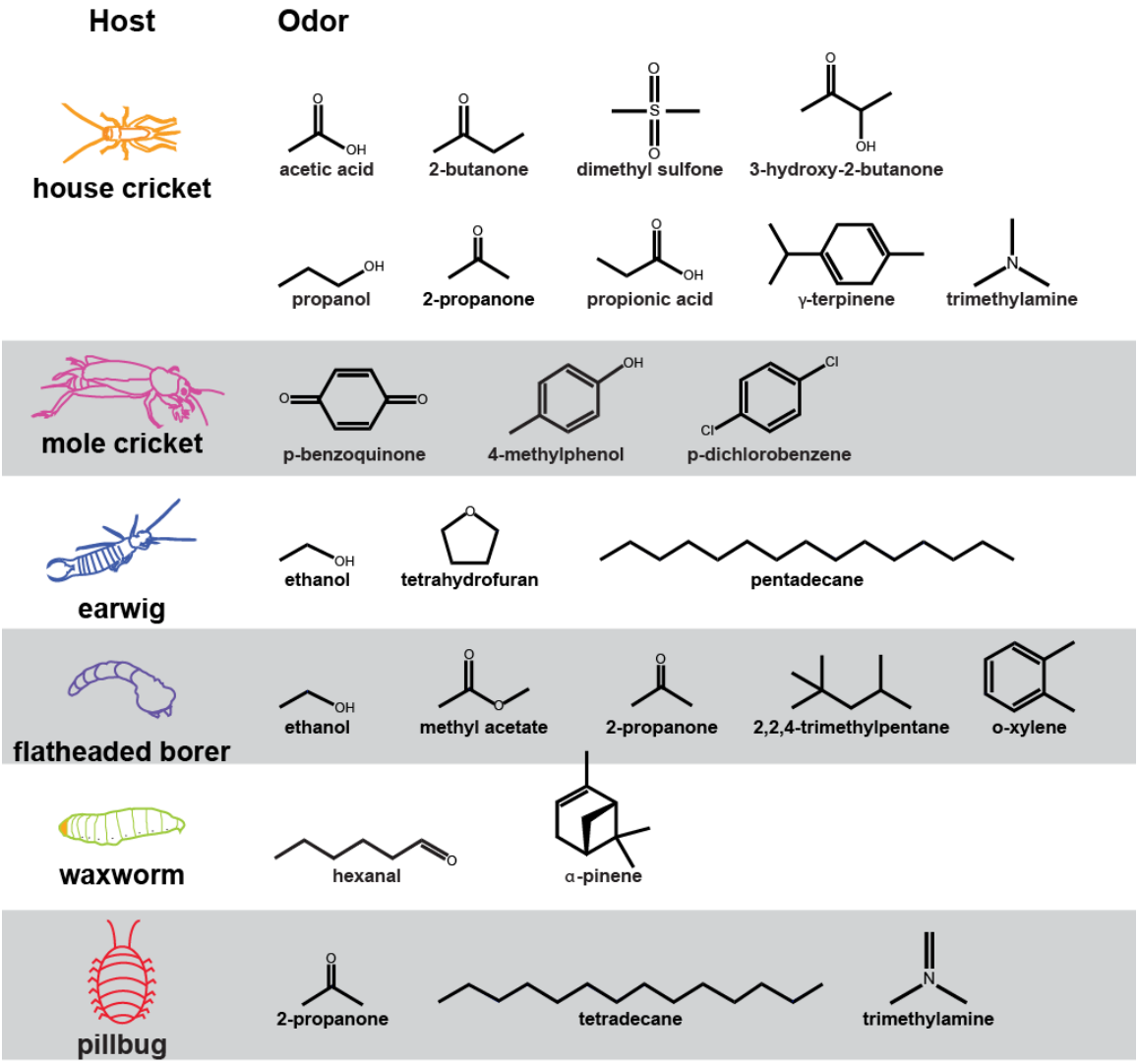


Figure 5. Host-derived odorants identified by TD-GC-MS and SPME-GC-MS. Each listed odorant was identified in at least two different experimental replicates at a relative abundance of ≥ 20,000 and with library matches of at least 95% confidence. Odorants identified from earwigs, flatheaded borers, and pillbugs, as well as 2-propanone identified from house crickets, were identified by SPME-GC-MS; all other odorants were identified by TD-GC-MS.

In the case of the cricket specialist *S. scapterisci*, we found that all of the odorants that elicited a strong response (as defined by a chemotaxis or jumping index of ± 0.5 or stronger) were cricket-derived, and seven of the ten cricket-derived odorants elicited a

positive chemotactic or jumping response (as defined by a chemotaxis or jumping index of ± 0.2 or stronger). Thus, the odor response profile of *S. scapterisci* appears to reflect its specialized host range.

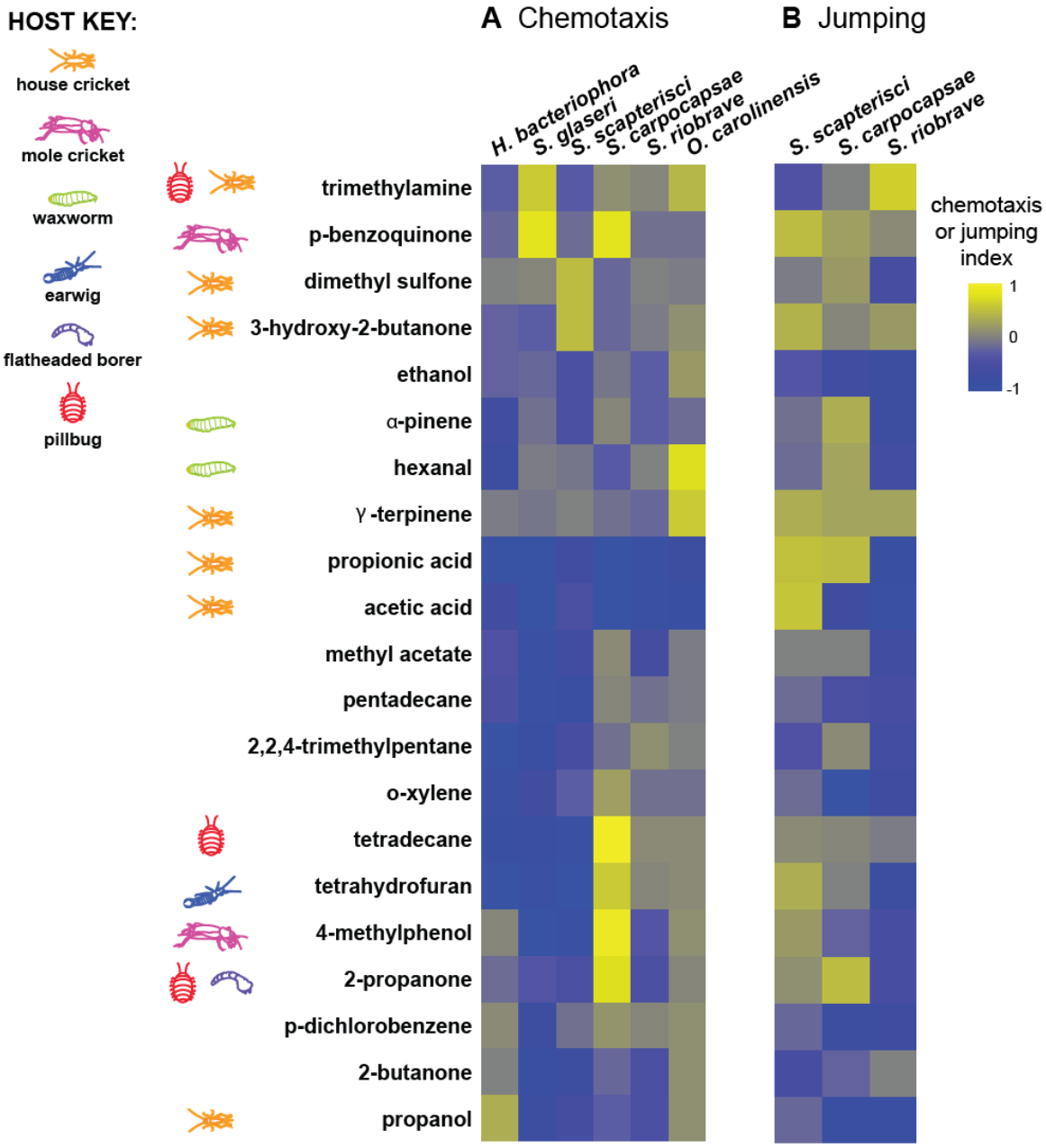


Figure 4.6 | A wide variety of host-derived odorants stimulate host-seeking behavior by EPNs. A. Chemotaxis of EPNs to host-derived odorants. The order of both the nematodes and odorants in the heat map was determined by hierarchical cluster analysis

(Ward's method). EPNs respond differently to different host-derived odorants ($P < 0.001$, two-factor ANOVA with replication). EPNs also displayed unique odor response profiles ($P < 0.05$, two-factor ANOVA with replication, with a Bonferroni post-test), with the exception of *S. riobrave* and *O. carolinensis*, which were not significantly different from each other. n = 4–10 trials for each EPN-odorant combination. Data for *H. bacteriophora* and *S. carpocapsae* responses to acetic acid, 2-butanone, dimethyl sulfone, ethanol, hexanal, 3-hydroxy-2-butanone, methyl acetate, α-pinene, propanol, propionic acid, γ-terpinene, and trimethylamine are from Hallem *et al.*, 2011 [3]. Mean, n, and SEM values for each assay are given in Appendix B; *P* values for each post-test are given in Appendix B. **B.** Jumping of EPNs to host-derived odorants. The order of nematodes in the heat map was determined by hierarchical cluster analysis (Ward's method); the order of the odorants is as in **A**. EPNs respond differently to different host-derived odorants ($P < 0.0001$, two-factor ANOVA with replication), and all three species display unique jumping odor response profiles ($P < 0.001$). n = 2–11 trials for each EPN-odorant combination. Mean, n, SEM, and *P* values for each post-test are given in Appendix B.

Dose-response analysis indicated that for chemotaxis behavior, most odorants were consistent attractants or repellants across concentrations (Figure 4.S5A, Appendix B). The one exception was acetic acid, which was repulsive for *S. carpocapsae* at high concentrations but attractive at lower concentrations (Figure 4.S5A, Appendix B). Jumping behavior was more dynamic across concentrations. One odorant, trimethylamine, inhibited *S. scapterisci* jumping at high concentrations but stimulated it at low concentrations; other odorants such as p-benzoquinone stimulated *S. carpocapsae* and *S. scapterisci* jumping at high concentrations but inhibited it at low concentrations (Figure 4.S5B, Appendix B). These results suggest that EPNs may use olfactory cues to encode information about host proximity as well as host identity.

To further explore the role of host-specific odors in EPN host-seeking behavior, we examined the responses to attractive host-derived odorants in the presence of either a neutral mixture of host-derived odorants (i.e., odorants we identified from hosts but that did not elicit a response when tested individually) (Figure 4.6), or soil odor. We found that host-derived odorants that attracted EPNs when tested individually were still attractive in the presence of both the neutral odorant mixture and the soil odor (Figure 4.7). Thus, EPNs can detect and respond to host-derived odorants even in the presence of other unrelated olfactory cues. These results suggest that EPNs are likely to use olfactory cues for host seeking even in complex soil environments.

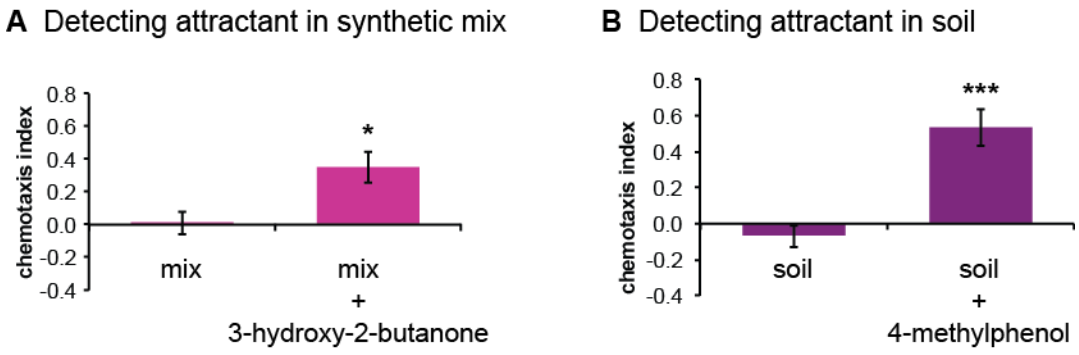


Figure 4.7 | EPNs detect and respond to host-derived odorants in the presence of complex odor mixtures. A. Response of *S. scapterisci* IJs to a 10^{-1} dilution of the cricket-derived odorant 3-hydroxy-2-butanone in the presence of a synthetic mix containing 10^{-1} dilutions of hexanal, γ-terpinene, and p-dichlorobenzene. Left bar, response to the synthetic mix vs. a paraffin oil control. Right bar, response to the synthetic mix vs. the synthetic mix with 3-hydroxy-2-butanone added. n = 6–9 trials for each condition. The response to the synthetic mix with 3-hydroxy-2- butanone added was significantly different from the response to the synthetic mix alone ($P < 0.05$, unpaired t test). **B.** Response of *S. carpocapsae* IJs to 4-

methylphenol in the presence of soil odor. Right bar, response to soil odor vs. an air control. Left bar, response to 4-methylphenol + soil odor vs. soil odor alone. n = 6 trials for each condition. The response to 4-methylphenol + soil odor was significantly different from the response to soil odor alone ($P < 0.001$, unpaired t test). In addition, the response to 4-methylphenol in the presence of soil odor was not significantly different from the response to 4-methylphenol in the absence of soil odor (unpaired t test). Mean, n, and SEM values for each assay are given in Appendix B.

Discussion

Heterorhabditis, Steinernema, and *Oscheius* are phylogenetically distant genera of EPNs that have convergently evolved similar entomopathogenic lifestyles. The entomopathogenic lifestyle is highly specialized: EPNs locate and infect insect larval hosts, deposit their bacterial symbiont into the host, rapidly kill the host, and then resume normal development [14]. The convergence of three separate genera in the EPN guild is therefore a striking example of adaptive plasticity among nematodes. Our results demonstrate that even closely related EPNs display different odor response profiles, raising the possibility that olfaction contributes to this adaptive plasticity.

Overall, we found that chemotaxis behaviors exhibit more species specificity than jumping behaviors. For example, the relative attractiveness of different potential hosts in a chemotaxis assay varied for different EPN species (Figure 4.1B). By contrast, all of the jumping species tested displayed the same relative host preferences; i.e., hosts that evoked higher levels of jumping for one species also evoked higher levels of jumping for the other species, and vice versa (Figure 4.1D). We also observed that odorants did not always stimulate equivalent responses for jumping and chemotaxis,

indicating that these behaviors are controlled by different chemosensory cues and may therefore serve different functions in the host-seeking process. The evolution of jumping behavior likely played a major role in niche partitioning among EPNs, since jumping ambushers are found primarily in epigeal (soil-air interface) habitats while cruisers are often found deeper in the soil column [65]. However, our results suggest that among jumping species, odor-driven chemotaxis behavior may have played a more important role in further partitioning of the epigeal niche than odor-driven jumping behavior. This is consistent with the possibility that jumping is a less specific short-range host-seeking strategy that facilitates rapid attachment to nearby hosts at the expense of specificity, while chemotaxis prior to jumping and tactile or other cues subsequent to jumping are used for host discrimination. However, it is possible that jumping can also be used as a long-range strategy for rapid movement toward potential hosts.

S. scapterisci is the only tested species known to have a narrow host range and for which a natural host, the mole cricket, has been convincingly demonstrated [38–40]. We found that the olfactory responses of *S. scapterisci* reflect its host range: *S. scapterisci* IJs showed the highest virulence to orthopteran hosts and appear to respond primarily to crickets and cricket-derived odorants (Figures 4.1 and 4.6). In addition, we found that *S. scapterisci* showed a reduced response to low concentrations of CO_2 (≤ 1%) compared to most EPNs in a chemotaxis assay but not a jumping assay (Figure 3), and the response of *S. scapterisci* to mole crickets in a chemotaxis assay was not significantly different when CO_2 was removed from the host airstream (Figure 4.4A and Appendix B). Thus, *S. scapterisci* may rely more on host-specific cues and less on CO_2 for long-range host seeking than generalist EPN species. In addition, we found that

S. scapterisci was attracted to the cricket-derived odorant 3-hydroxy-2-butanone even in the presence of a mixture of other odorants (Figure 4.7A), suggesting that *S. scapterisci* is capable of responding to cricket-derived odorants even in complex odor environments. Taken together, our results suggest an important role for olfaction in the evolution of host specificity for *S. scapterisci*.

The lack of overlap in the odorants identified from the two cricket species (Figure 4.5) suggests that either *S. scapterisci* uses different olfactory cues to locate the different species, or that *S. scapterisci* relies on low abundance odorants common to multiple cricket species that were not included in this study. However, we note that the odorant dimethyl sulfone, which we identified as a house cricket-derived odorant, was also identified from mole crickets but did not meet our stringent criteria for inclusion in our analysis (Figure 4.S4). Dimethyl sulfone elicited behavioral responses from *S. scapterisci* even at low concentrations (Figure 4.S5A), suggesting it may be an important orthopteran host-seeking cue.

O. carolinensis showed the lowest levels of host attraction in our assays, and like *S. scapterisci*, attraction of *O carolinensis* to CO_2 declined around 1% (Figures 4.1B and 4.3A). *O. carolinensis* is one of two recently described EPNs in the genus *Oscheius*; these species are thought to have evolved an entomopathogenic lifestyle more recently than *Heterorhabditis* and *Steinernema* species [14, 25, 66]. Thus, the olfactory system of *O. carolinensis* may be less highly specialized for insect parasitism than those of the more anciently evolved EPNs. It is also possible that none of the seven hosts tested are natural or preferred hosts for *O. carolinensis*. In support of this possibility, the closely

related species *O. necromenus* is associated with millipedes, which are non-insect arthropods in the class Diplopoda [66, 67].

Our virulence assays revealed that all EPNs, even those with very broad host ranges such as *S. carpocapsae,* are better able to infect some insects than others (Figure 2). Thus, virulence varies greatly for different EPN-host combinations. However, we note that the number of IJs to which hosts are exposed is positively correlated with both the number of nematodes entering the host and the number of resultant infections [68]. Many EPNs are capable of infecting a wide variety of insect larvae and even some non-insect invertebrates at high doses [61, 69–71]. Thus, it is likely that at least some of the potential hosts we tested that appeared resistant to EPN infection can serve as hosts if exposed to a high enough concentration of IJs. We also note that host efficiency is determined not only by the rate of host killing but also by the level of reproduction supported by the host [35], and reproduction levels are not tested here.

A comparison of host virulence with host-evoked chemotaxis and jumping behaviors revealed that some EPNs are attracted to invertebrate species that are not effective hosts (Figures 4.1 and 4.2). This finding is consistent with the observation that EPNs can engage in phoresy—a relationship in which nematodes use an organism for transportation to new environmental niches—with both non-host insects and non-insect invertebrates such as isopods and earthworms [72–74]. Attraction to non-hosts in the absence of hosts may offer a survival advantage to EPNs by facilitating dispersal to more favorable environmental niches. It is also possible that olfactory preferences can in some cases lead EPNs to pursue non-hosts or dead- end hosts. Host selection is a complex process that can be broken down into multiple steps, including host location,

host attachment, host recognition, and host penetration [21, 55]. Host attraction is only one component of this process, and other behaviors such as those that mediate host recognition and penetration may prevent the fatal decision to infect an inappropriate host. We note that the gastropod-parasitic nematode *Phasmarhabditis hermaphrodita,* which is in the Rhabditid family and is closely related to *C. elegans, H. bacteriophora,* and *O. carolinensis,* also displays host-seeking behavior toward various species of gastropods [75–77].

In addition to examining responses to live hosts, we also examined responses to CO_2 and other host-derived odorants. We found that all EPNs tested are attracted to CO_2 and that CO_2 sensitivity is positively correlated with overall host attraction (Figure 3). Thus, CO_2 is a critical host-seeking cue for EPNs regardless of host-seeking strategy or host range. However, the importance of CO_2 as a host-seeking cue varies for different hosts. For example, CO_2 appears to be more important for attraction to waxworms than crickets: waxworms were no longer attractive to any of the EPNs in the absence of CO_2, while crickets were still attractive to some but not all EPNs (Figure 4.4). In addition, *S. carpocapsae* preferred mole cricket odor to 1% CO_2 in a competition chemotaxis assay, demonstrating that at least some live hosts are more attractive than low concentrations of CO_2 alone (Figure 4.S3). The importance of CO_2 also varies for different EPNs. For example, *S. riobrave* responded only to slugs in the absence of CO_2, and in fact host-evoked chemotaxis and jumping were in many cases suppressed in the absence of CO_2 (Figure 4.4). Consistent with the reliance of *S. riobrave* on CO_2, we did not identify any host-derived odorants that were strong attractants for *S. riobrave* and we identified only one host-derived odorant that strongly stimulated jumping (Figure 4.6). These

results suggest that EPNs differ in the extent to which their olfactory systems have evolved to mediate specific host-parasite interactions: some EPNs rely primarily on CO_2 for host location, while others use CO_2 in combination with host-specific odorants for host location. We also found that at least some EPNs are attracted to host-specific odorants even in the presence of complex mixtures (Figure 4.7), further confirming an important role for host-specific odorants in host location.

EPNs inhabit all continents except Antarctica and have been isolated from diverse soil ecosystems ranging from forests in Germany to coastlands in Kenya to the Arctic regions of Russia [78–80]. As a result of their strikingly diverse biogeography, EPNs are promising biocontrol agents for nearly all climates and locales, and have been successfully used throughout the world for the control of a wide variety of insect pests [81]. However, the commercial success of EPNs as biocontrol agents is often unpredictable. For example, *S. scapterisci* has proven to be as effective as chemical pesticides for the control of mole crickets, and it is now widely used on golf courses, pastures, and other grassy terrains subject to mole cricket infestation [38, 81]. By contrast, EPNs have been much less successful against Colorado potato beetles, chafers, and armyworms [81]. A better understanding of how EPNs locate hosts and discriminate among potential hosts may be useful for enhancing the efficacy of EPNs as biocontrol agents.

The ability to find and infect hosts using host-emitted chemosensory cues is essential for many endoparasites such as parasitic nematodes and schistosomes, as well as many ectoparasites such as blood-feeding insects, ticks, and lice [82–86]. We show that EPNs respond differently to the odors of different potential hosts, and we identify a

number of host-derived odorants that stimulate strong attractive and repulsive behavioral responses. Our results provide a foundation for future investigations into the mechanisms of these responses.

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Chapter 5:

Incorporating Genomics Into the Toolkit of Nematology*

*This chapter first published in *Journal of Nematology* in 2012 and was written by Adler R. Dillman, Ali Mortazavi, and Paul W. Sternberg. This chapter includes discussion of *Steinernema* genomes for which a separate manuscript is being prepared but was not yet ready to include in this thesis.

Abstract

The study of nematode genomes over the last three decades has relied heavily on the model organism *Caenorhabditis elegans*, which remains the best-assembled and annotated metazoan genome. This is now changing as a rapidly expanding number of nematodes of medical and economic importance have been sequenced in recent years. The advent of sequencing technologies to achieve the equivalent of the $1000 human genome promises that every nematode genome of interest will eventually be sequenced at a reasonable cost. As the sequencing of species spanning the nematode phylum becomes a routine part of characterizing nematodes, the comparative approach and the increasing use of ecological context will help us to further understand the evolution and functional specializations of any given species by comparing its genome to that of other closely and more distantly related nematodes. We review the current state of nematode genomics and discuss some of the highlights that these genomes have revealed and the trend and benefits of ecological genomics, emphasizing the potential for new genomes and the exciting opportunities this provides for nematological studies.

Introduction

Nematoda is one of the most expansive phyla documented with free-living and parasitic species found in nearly every ecological niche [1]. Traditionally, nematode phylogeny was based on classical and often incomplete understanding of morphological traits, but traditional systems have been revised and supplemented by a growing body of insight from molecular phylogenetics that is primarily based on ribosomal DNA for higher level taxonomic studies [2–4]. The study of the evolutionary relationships between

species in vertebrates and in arthropods is transitioning to the comparative analysis of entire genomes due to the exponentially decreasing cost of sequencing and the study of nematodes is now following the same path [5–7]. While the model organism *Caenorhabditis elegans* was the first metazoan sequenced [8], there have been only a few additional nematodes sequenced until recently and many representative clades and ecological niches remain unexplored. There are several advantages to whole genome sequencing for nematology. The simplest and most obvious is that the complete genome harbors the full repertoire of genes that are the inherited common core of any given species. Furthermore, the genome contains the structural and regulatory elements that lie in and between genes, even if we cannot yet identify them all. The genome also provides the foundation for future experimentation such as transformation and RNA interference (RNAi). The genome is the natural framework for indexing and organizing the massive genetic content of species within a phylum. The genetic 'blueprint' represented by a genome may prove to be the most valuable and enduring piece of knowledge we can currently obtain for any particular life form [8].

As in many other fields of biology, the nematode *C. elegans* has proven invaluable as a model for genomic analysis, and thousands of investigators have contributed to our understanding of its 20,431 protein-coding genes [8, 9]. This is likely for the same reasons that make this hermaphrodite so powerful and useful in genetics: 1) its ease of culture, 2) its simple, rapid, invariant development, 3) many biological principles are universal, even if specific details are not, and 4) the more detailed our understanding of any biological phenomenon, the more interesting it tends to become [10]. While sequencing efforts have expanded exponentially as technology improves and

the cost continues to diminish, the finished *C. elegans* genome remains unrivaled in completeness compared to other metazoans. This is not likely to change, due partly to differences in technology but primarily because closing the remaining gaps in genomic sequence is a prolonged and expensive process with diminishing biological return [8]. The top-down approach of completing genome sequences by breaking the genome down into large, known fragments, which provide a physical map, and the subsequent sequencing of those fragments in their entirety, will probably not be common until new technologies sharply reduce the costs of finishing genomes.

Over the last two decades, sequencing technology has advanced from relying on the hierarchical sequencing and assembly of cloned fragments of DNA (i.e., automated Sanger sequencing as used in the *C. elegans* project), to the shotgun, high-throughput ~ 500 bp reads produced by 454 Roche sequencing and the even cheaper ≤ 150 bp reads produced by Illumina sequencing [11–13]. Due to the rapid pace of sequencing technology development and turnover, we will refer to the newer technologies as 'next-generation' (next-gen) technologies throughout rather than focus on any specific platform. These next-gen technologies are driven with the eventual goal to achieve a ≤ $1000 human genome to enable health applications. Given that the typical nematode genome is less than 1/15 of the size of the 3.2 Gb human genome (see Table 5.1 for nematode genome sizes), sequencing nematode genomes is already affordable and, as technology improves, could become monetarily negligible. Current next-gen technologies use DNA fragments of various size to generate sequence, which range from less than 500 bp up to ≤ 20 kb, and can produce either single or paired end reads (either one or both ends of prepared fragments can be sequenced (Figure 5.1)). Next-gen sequencing technologies

generate many more sequencing reads that have a higher error rate than traditional Sanger sequencing, but this is balanced by higher overall coverage (whereas 2 Gb of generated sequence would provide 20-fold coverage of a 100 Mb genome, 10 Gb of generated sequence would provide 100-fold coverage). When considering these sequencing technologies it is important to distinguish fragment size and read length as distinct variables that will affect the resulting assembly, because it is easy to sometimes conflate or combine these separate aspects. Fragment size refers to the length of the DNA insert, from which sequence will be generated either from one or both sides, while read length refers to how many base pairs are actually being sequenced from one or both sides of the fragment (Figure 5.1).

The hierarchical application of Sanger sequencing to the assembly of the *C. elegans* genome helped to facilitate completeness and circumvented the potential problems of long repeats, homopolymeric regions, and low G+C content, along with the community effort of researchers, which was crucial and is ongoing [8, 14]. Next-gen technologies are more affordable and allow for much higher fold coverage of genomes, leading to hundreds of millions of genomic reads. In contrast to the hierarchical approach previously used, the shotgun strategies in favor today are based on breaking the entire genome into many more small fragments. These require more computational effort to assemble into multigenic sized contigs, let alone chromosomes (Figure 5.1) [11]. The contiguity of the resulting draft genomes can be dramatically improved by library construction with inserts of larger but approximately known sizes as well as ‘jumping libraries’ (Figure 5.1C) [15, 16].

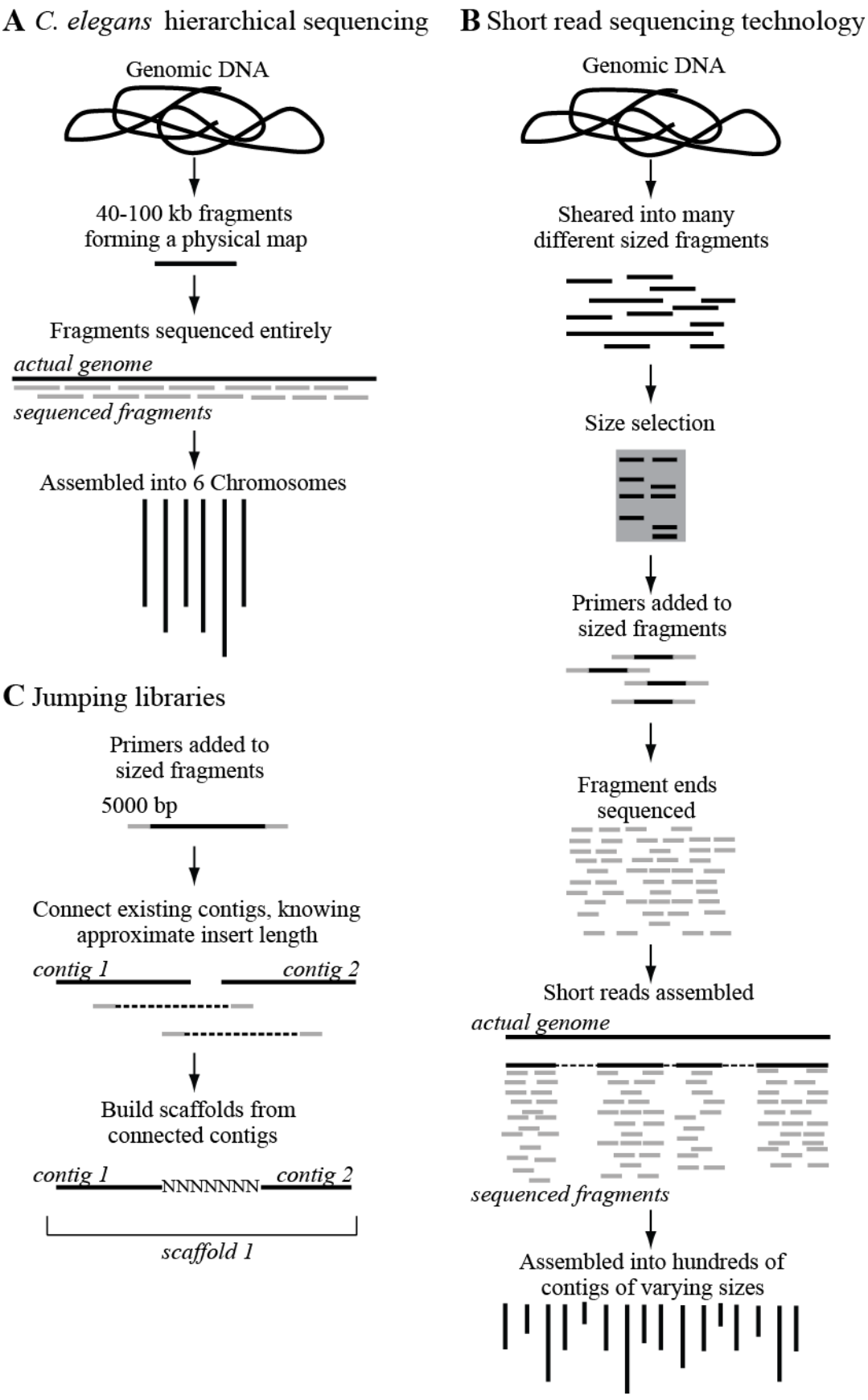
A C. elegans hierarchical sequencing
Genomic DNA
40-100 kb fragments
forming a physical map
Fragments sequenced entirely
actual genome
sequenced fragments
Assembled into 6 Chromosomes
B Short read sequencing technology
Genomic DNA
Sheared into many
different sized fragments
Size selection
Primers added to
sized fragments
Fragment ends
sequenced
Short reads assembled
actual genome
sequenced fragments
Assembled into hundreds of
contigs of varying sizes
C Jumping libraries
Primers added to
sized fragments
5000 bp
Connect existing contigs, knowing
approximate insert length
contig 1
contig 2
Build scaffolds from
connected contigs
contig 1
NNNNNNN
contig 2
scaffold 1

Figure 5.1 | Hierarchical and shotgun sequencing. A) A shortened diagram of the hierarchical (top-down) sequencing technique used for the *C. elegans* genome. The genomic DNA was broken into large fragments (40–100 kb) that formed a physical map. The order of the fragments was known before they were actually sequenced. The fragments were then fully sequenced and assembled, resulting in six chromosomal contigs. B) A diagram of the shotgun-sequencing techniques used to prepare genomic DNA for sequencing using 454 Roche or Illumina short-read technology. The genomic DNA is sheared into approximately sized fragments of 0.5 to 1 kb. These fragments then have primers attached to one or both ends, depending on whether they will be run on a paired-end sequencer. The fragments are not sequenced in their entirety, but 50–500 bp of one or both ends of each fragment are sequenced. The resulting short reads are then assembled, with some gaps remaining as shown (gaps are represented by the dotted lines). These reads are then assembled into hundreds up to thousands of larger contigs (contiguous sequence). C) Jumping libraries are used in short-read sequencing to improve assembly quality. During the size selection, larger fragment sizes are selected and sequenced. Only one size is selected per library, but the sizes range from 2 kb to 20 kb. Read assembly is facilitated by knowing the approximate distance between the paired end reads, helping to overcome issues of repeats and homopolymeric regions, jumping large regions (as large as the insert length). Assembly quality is improved as multiple previously unconnected contigs are now known to connect, just as contig 1 and contig 2 are joined to form a larger contig called ‘scaffold 1’ in the figure.

Since the first nematode genome was first published in 1998, twelve more whole nematode genomes have been sequenced and made publicly available [8, 12, 17–25]. There are at least 13 more nematode genomes scheduled for release in 2012, and several others in preparation (Table 5.1) [26]. Because Nematoda is so ecologically diverse and

species-rich (1 to 10 million species [27, 28]), phylogenetic relationships along with human health and agricultural considerations should inform sequencing efforts.

Nematode Species	Date Published	Size (Mb)	# Protein-coding genes	G+C Content
Ascaris suum	Oct. 2011	273	18,542	38%
Brugia malayi	Sept. 2007	96	21,252	31%
Bursaphelenchus xylophilus	Sept. 2011	75	18,074	40%
Caenorhabditis angaria	Oct. 2010	80	22,851	36%
*Caenorhabditis brenneri**	Feb. 2009	190	30,670	39%
Caenorhabditis briggsae	Nov. 2003	106	21,963	37%
Caenorhabditis elegans	Dec. 1998	100	20,431	35%
*Caenorhabditis japonica**	Feb. 2009	83	25,879	40%
*Caenorhabditis remanei**	Feb. 2009	145	31,471	38%
Caenorhabditis sp. 5†	~2012	132	35,000	39%
Caenorhabditis sp. 11†	~2012	79	22,330	38%
Dictyocaulus viviparus§	-	230	-	-
Dirofilaria immitis§	-	90	-	-
Haemonchus contortus†	~2012	315	-	42%
Heterorhabditis bacteriophora†	~2012	74	-	33%
Heterorhabditis indica†	~2012	64	-	34%
Heterorhabditis megidis†	~2012	71	-	34%
Heterorhabditis sonorensis†	~2012	64	-	34%
Litmosoides sigmodontis§	-	90	-	35%
Loa loa§	-	90	-	-
Meloidogyne hapla	Sept. 2008	53	13,072	27%
Meloidogyne incognita	Jul. 2008	86	19,212	31%
Nippostrongylus brasiliensis§	-	80	-	-
Onchocera ochengi§	-	90	-	-
Onchocera volvulus§	-	90	-	-
Oscheius tipulae§	-	120	-	-
Panagrellus redivivus†	~2012	62	27,266	44%
Pristionchus pacificus	Sept. 2008	173	24,216	43%
Steinernema carpocapsae†	~2012	86	-	46%
Steinernema feltiae†	~2012	101	-	47%
Steinernema glaseri†	~2012	94	-	48%
Steinernema monticolum†	~2012	114	-	45%
Steinernema scapterisci†	~2012	86	-	48%
Strongyloides ratti§	-	50	-	-
Trichinella spiralis	Mar. 2011	64	15,808	34%
Wuchereria bancrofti§	-	90	-	-

Table 5.1 | Genome statistics for published and selected forthcoming whole nematode genomes. Additional genomes planned and in progress can be viewed at www.nematodes.org/nematodegenomes/index.php/959_Nematode_Genomes.

* Genomes that were not explicitly published in genome papers but discussed in Barrière *et al*. [22].

† Forthcoming genome statistics were provided by investigators working on those projects (P.W. Sternberg, E.M. Schwarz, and H.T. Schwartz).

§ Genomes in production with data available from the 959 genome project website.

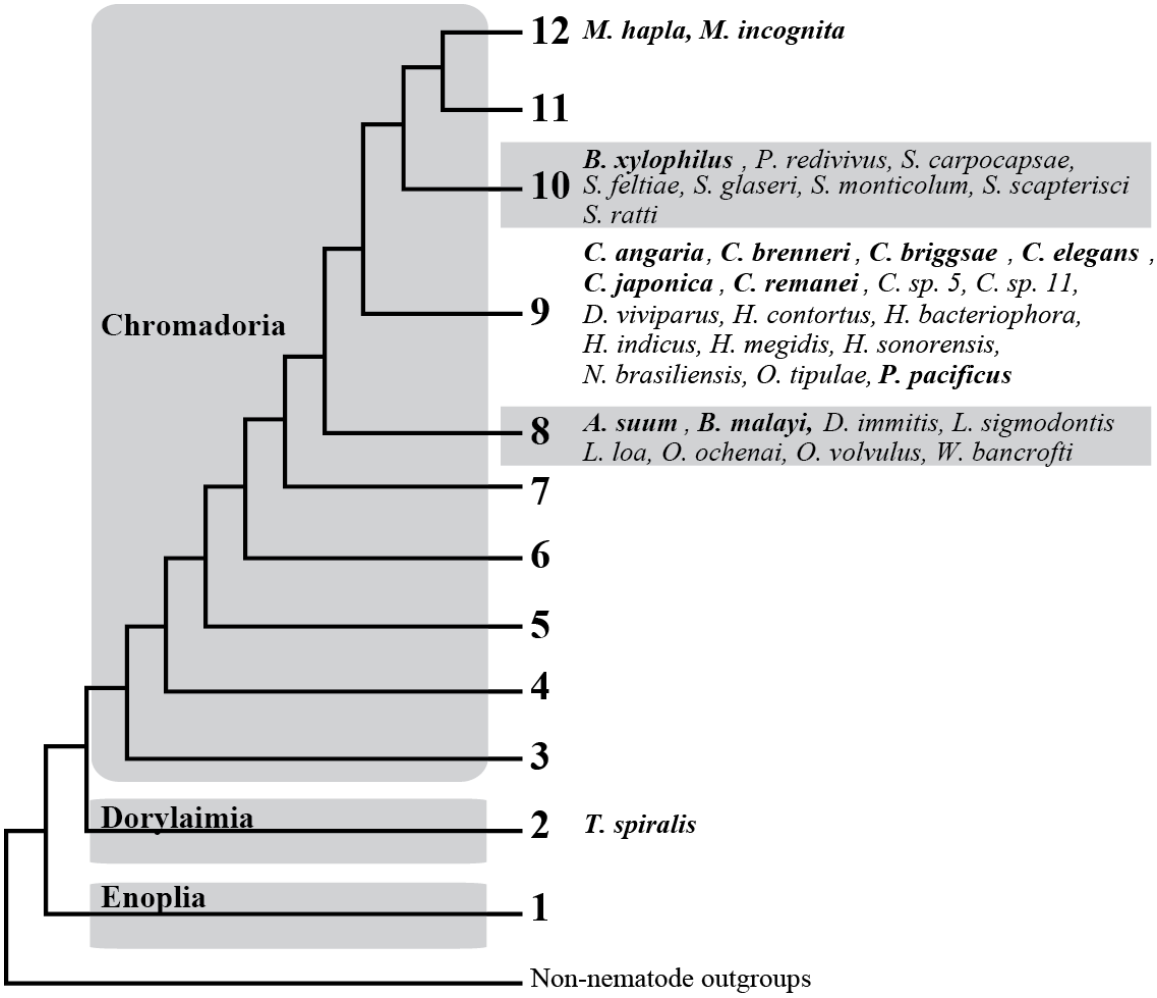

Figure 5.2 | A phylogenetic representation of sequenced and selected forthcoming nematode genomes. This diagram depicts twelve monophyletic clades representing the phylum Nematoda. Sequencing efforts have focused on a few select crown clades of Chromadoria. The clade designations are after Holterman *et al.* [4]. Taxa with published genomes have bolded names while taxa for which genomes are underway and scheduled for release in 2012 are regular typeface. Full genus names can be found in Table 1. Forthcoming genomes were selected from genomes with data available from the 959 genome project [26] and from genome projects of which the authors had knowledge.

The current view of nematode genealogical relationships divides the phylum into 3 major clades: Enoplia, Dorylaimia, and Chromadoria [2, 3, 29]. Chromadoria is further broken down into 10 clades, which together with Enoplia and Dorylaimia form a total of 12 major monophyletic branches within the phylum (Figure 5.2) [4, 6, 30]. Sequencing efforts so far have focused on nematodes in the crown clades of Chromadoria, which include *C. elegans* as well as most medically and agriculturally relevant species (Figure 5.2). A systematic genomic survey of the phylum would facilitate a better understanding of the evolution of Nematoda, enhance comparative studies, and could illuminate striking differences across the phylum such as differences in parasitic lifestyle (e.g., endoparasitic vs. ectoparasitic) or mode of reproduction (e.g., amphimictic vs. parthenogenetic) as well as developmental differences (e.g., asymmetric vs. symmetric cleavages; presence vs. absence of a prominent coeloblastula [31]), among others.

What are the benefits of genomics for nematologists? Herein we briefly review the basic information provided by most nematode genome analyses. We discuss the highlights of the 13 available nematode genomes, how their utility increases as the number of possible comparisons increases, and how the focus of nematode genomics is changing to emphasize the specific biology and ecology of each species. We finish by illustrating the potential benefit of sequencing additional nematode genomes, using as an example the prospects of entomopathogenic nematode genomes and discussing how they can contribute to our understanding of parasitism, mutualism, and nematode biology in general.

The steps in sequencing a genome

With such a diversity of nematodes to choose from, which nematodes should be sequenced first? In addition to the above-mentioned biological motivations of phylogenetic position and human health and agricultural concerns, there are practical considerations such as the availability and homogeneity of material. Culturability is also a consideration, especially if investigators are interested in the transcriptome and subsequent experimentation. Adding transcriptional data can dramatically improve gene predictions and assembly quality [23, 32]. Whole-genome amplification techniques may make it possible to analyze interesting-but-unculturable nematodes in a cost-effective way. However, such amplification techniques may introduce additional problems such as polymorphisms and amplification errors, while culturable worms escape these difficulties since they can provide large quantities of DNA (typically 5 micrograms are needed to construct robustly a representative DNA library, which corresponds to ~ 50,000 worms for *C. elegans*) and can be inbred to decrease heterozygosity. While the study of sequence variation within a species is of great importance, the same variation can make it difficult to assemble a genome *de novo* without producing assembly errors. Therefore every effort should be undertaken, if possible, to inbreed the strains used, to minimize polymorphisms. The genomic value of a culturable worm increases with complementary transcriptome data and the possibility of further experimentation. In fact, the implementation of some experimental techniques such as RNAi may depend on optimized culturing techniques that do not stress the nematodes being cultured [33]. We believe that there are plenty of interesting culturable nematodes that can shed light on the evolution of the phylum and thus should be prioritized to fill sequencing pipelines. While the bulk of our discussion

below focuses on genomic libraries, RNA-seq libraries for transcriptome sequencing can be built from as little as 100 ng of total RNA thus lowering the numbers of worms needed to collect data. As next-gen technologies mature, we can expect that the starting amounts of material necessary will decrease.

Once a suitable nematode is identified, the simplified, general pipeline for genomic sequencing is as follows: 1) extraction and purification of genomic DNA, 2) selection of a sequencing platform, 3) library construction, 4) sequencing, 5) assembly of the sequence into as long and as few contigs as possible, 6) gene predictions and subsequent annotation.

1) DNA extraction and purification. There are numerous DNA extraction and purification methods and proprietary kits that have been tested and are known to work well both for populations and individual nematodes [34–36].

2) Selection of a sequencing platform. Careful consideration should be given to selecting the appropriate sequencing technology and accompanying parameters, such as read length and fragment size. A common priority is to select the most cost-effective source of high-quality sequence while simultaneously collecting as many reads as possible to ensure good coverage. Good assemblies with short-read technologies typically require 100x average coverage to compensate for high error rates. Coverage takes into account the size of the genome and the length of sequenced reads; for a 100 Mb genome, 100 million 100 bp reads are needed to achieve 100x coverage. Matters are further complicated by the effect of GC-content (GC content of the genome is the percentage of guanines and cytosines) on the coverage in some next-gen technologies, which necessitate greater overall sequencing depth (i.e., more sequencing reads) to cover GC-

poor regions well [23]. Certain sequencing platforms may be advisable for particularly GC-poor genomes (e.g. < 35%), such as 454.

3) Library construction. Good library construction is often a critical step, depending on the sequencing technology used [37]. A genomic library is essentially genomic DNA that has been sheared into fragments, which are then size selected for an approximate distribution. These fragments then have sequencing primers ligated to one or both ends (Figure 5.1). Because of the massive number of reads, and increasingly longer read lengths, the construction of good libraries with a normally distributed fragment size can make the difference between good and poor quality assemblies. Libraries with average fragment sizes of 500 bp are sufficient to assemble most nematode-size gene loci onto a single contig [32]. Genomes that are rich in longer repeat sequences or gene clusters that are larger than the fragment lengths will benefit from additional jumping libraries, which are paired-end libraries that are typically 3–20 kb apart (Figure 5.1C) [12]. In addition to traditional genomic jumping libraries, transcriptome data can be used to scaffold expressed genes that are broken across multiple contigs [23].

4) Sequencing. After a library is constructed, it is then sequenced, which is typically handled by dedicated facilities. The sequencing run may take 1 to 10 days, but this may be prolonged depending on facility scheduling considerations. The resulting raw reads each consist of a DNA sequence and a corresponding quality score; these can be used to filter all but the highest-quality reads, which will improve the overall assembly.

5) Genome assembly. Reads are assembled into contigs using one of several available programs such as Velvet and SOAPdenovo [38, 39]. Genome assembly is a resource-intensive step that can require substantial memory, but the relatively small size

of nematode genomes makes assembly practical on servers with 128 to 256 gigabytes of RAM. Assembly programs work by finding overlap between reads into contigs and by connecting contigs using the paired information from paired-end (or jumping libraries) into scaffolds (connected contigs). In an ideal situation, one contig or even one scaffold per chromosome would be recovered, but this has only been achieved for *C. elegans* and *C. briggsae* (Figure 5.1A) [8, 17]. Assembly programs are often run multiple times with different parameters to maximize several of the assembly metrics described in the basic genome statistics section below.

6) Gene prediction and genome annotation. Once reads have been assembled, gene-finding programs that identify protein coding or non-protein coding genes such as Augustus and tRNAscan are used to annotate the genome (Figure 5.3) [40, 41]. Perhaps the most helpful additional dataset for this step is transcriptome data that is generated by high-throughput sequencing of mRNA (RNA-seq). This provides expression data and identifies *bona fide* transcripts (either full length or fragments) directly. These data can also be used to train prediction software, thus facilitating more reliable gene predictions [23, 32]. The transcriptome provides interesting biological data about global gene expression and can be applied to nematodes at specific stages such as infective juveniles or embryos. RNA-seq data for any biological sample, whether strain (e.g., drug-resistant mutant compared to the wild type) or stage-specific, can be used to identify genes with expression patterns of interest.

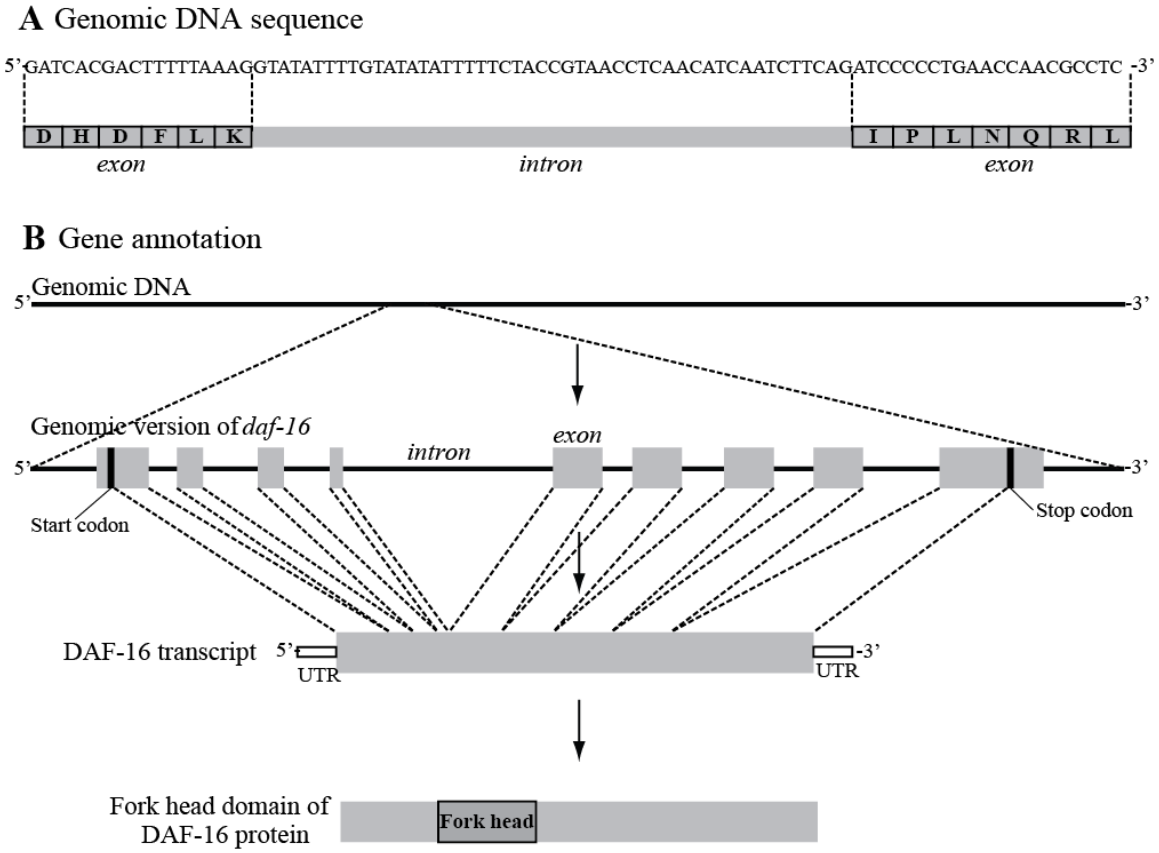

Figure 5.3 | Gene annotation. A) An overview of gene architecture, showing a stretch of DNA demarcated into protein-coding exons and non-coding introns. B) An example of gene annotation, proceeding from identification of genes from the genome either predicted *de novo* from gene-like patterns in the sequence or by comparison to known genes. In this case, the *daf-16* gene is shown with its introns and exons. Below that is a transcript from the *daf-16* gene, with the open reading frame (ORF) along with the upstream and downstream untranslated regions (UTR). The genome is also scanned for known protein domains; in this case, *daf-16* has a single protein domain, although other proteins may contain several domains.

Basic genomic metrics

The quality of a genome assembly can be assessed by metrics such as the total size of the genome relative to the fold coverage. This is estimated by dividing the total

number of assembled nucleotides by the genome size, which varies from 50–315 Mb for published and forthcoming nematode genomes (Table 5.1). For example, the *Ascaris suum* genome was sequenced with ~ 80–fold coverage, meaning that the 309 megabase genome was assembled from about 25 gigabases of sequence [12]. The GC content of the genome is usually reported, and varies between 27–48% among published and forthcoming nematode genomes (Table 5.1). Other commonly reported quality metrics of genomic assemblies address contiguity and completeness. One commonly used metric is the 'N50' value, which indicates that half of the genome is in contigs at least as large as that value. For instance, the N50 of the *A. suum* genome is 408 kb, meaning that half of the assembly is in contigs at least 408 kb in length [12]. Also important is the number of predicted protein coding genes, ranging from 13,000–45,000 among published and forthcoming genomes (Table 5.1). There are several other genomic statistics that have become potentially useful in comparisons such as gene density, number of transfer RNAs, and the percentage of high copy repeated sequences in the genome [8, 17, 18].

Quality assessments of genomic assembly provide confidence and a framework for interpreting subsequent analyses while other genomic metrics provide more information about the biological content of the genome. For instance, all known metazoan genomes require a certain number of tRNAs for codon recognition and for shuttling specific amino acids during translation, such that the number of tRNAs, tRNA pseudo-genes, and tRNA-derived repeats found in a genome assembly can serve as a rough estimate of completeness [42].

How protein sequences are analyzed and what they reveal about your nematode of choice

Annotation of nematode whole genomic sequence is complicated by several factors, including the structural complexity of introns, alternative RNA splicing, variable gene density, transplicing, and the presence of operons. Fortunately, annotation efforts on novel nematode species can leverage the excellent annotation of the *C. elegans* genome. These annotations are carefully curated and maintained in WormBase (www.wormbase.org), an expandable model for genome curation and annotation that already includes many available nematode genomes including *Ascaris suum, Brugia malayi, Bursaphelenchus xylophilus, Meloidogyne hapla, Meloidogyne incognita,* and many others. WormBase, with its established infrastructure and fulltime maintenance could serve as a repository for all nematode genomes and subsequent annotation [9]. As more genomes are sequenced and annotated, it has become clear that the availability of transcriptome data (e.g., RNA-seq; see above) is paramount for more accurate and comprehensive gene predictions, as well as elucidating biological function. While RNA requires more careful handling to avoid degradation, the reverse transcribed cDNA can be sequenced in the exact same manner as genomic DNA and for a similar cost.

While the specific details of annotation for each nematode genome differ, a general approach to protein analysis involves the following: identification of the protein-coding gene set, characterization by protein domain analysis and comparison to other protein databases, and comparative analysis with other nematodes and beyond. The identification of protein-coding genes is done using one or multiple gene prediction software packages, which generate *ab initio* predictions using machine-learning methods

such as hidden Markov models to identify open reading frames indicative of protein coding genes. The accuracy of these predictions can be improved by training the prediction software on experimental datasets such as ESTs, cDNA, protein similarity matches, and RNA-seq datasets. In particular, RNA-seq data can be used to partially or fully confirm gene-finder predictions [12, 17, 23, 43]. While computationally intensive, gene finding requires fewer resources than assembly.

As part of the annotation process, genes and proteins of the newly sequenced genome are evaluated by comparison to previously annotated genes and proteins from databases and genomes. Such evaluations identify putative homologous genes and proteins by sequence similarity. Homologous genes can be subdivided into orthologs and paralogs, depending on their history [44]. Orthologs are homologous sequences in different species that descended from a common ancestral gene during speciation, such that the ortholog of a gene in one species is the gene in the second species that shares decent from a common ancestral gene and is uniquely closely related to the gene in the first species. For example, the last common ancestor of *Pristionchus pacificus* and *C. elegans* may have possessed only one copy of the *daf-16* gene, which encodes a transcription factor in the insulin/IGF-1-mediated signaling pathway, and each of these extant species has one copy of *daf-16*, making these genes *daf-16* orthologs [8, 20, 45–47] (Figure 5.4A). We make this inference about *C. elegans* and *P. pacificus* knowing that both of these species as well as an outgroup taxon (in this case *A. suum*) all only have one copy of *daf-16*.

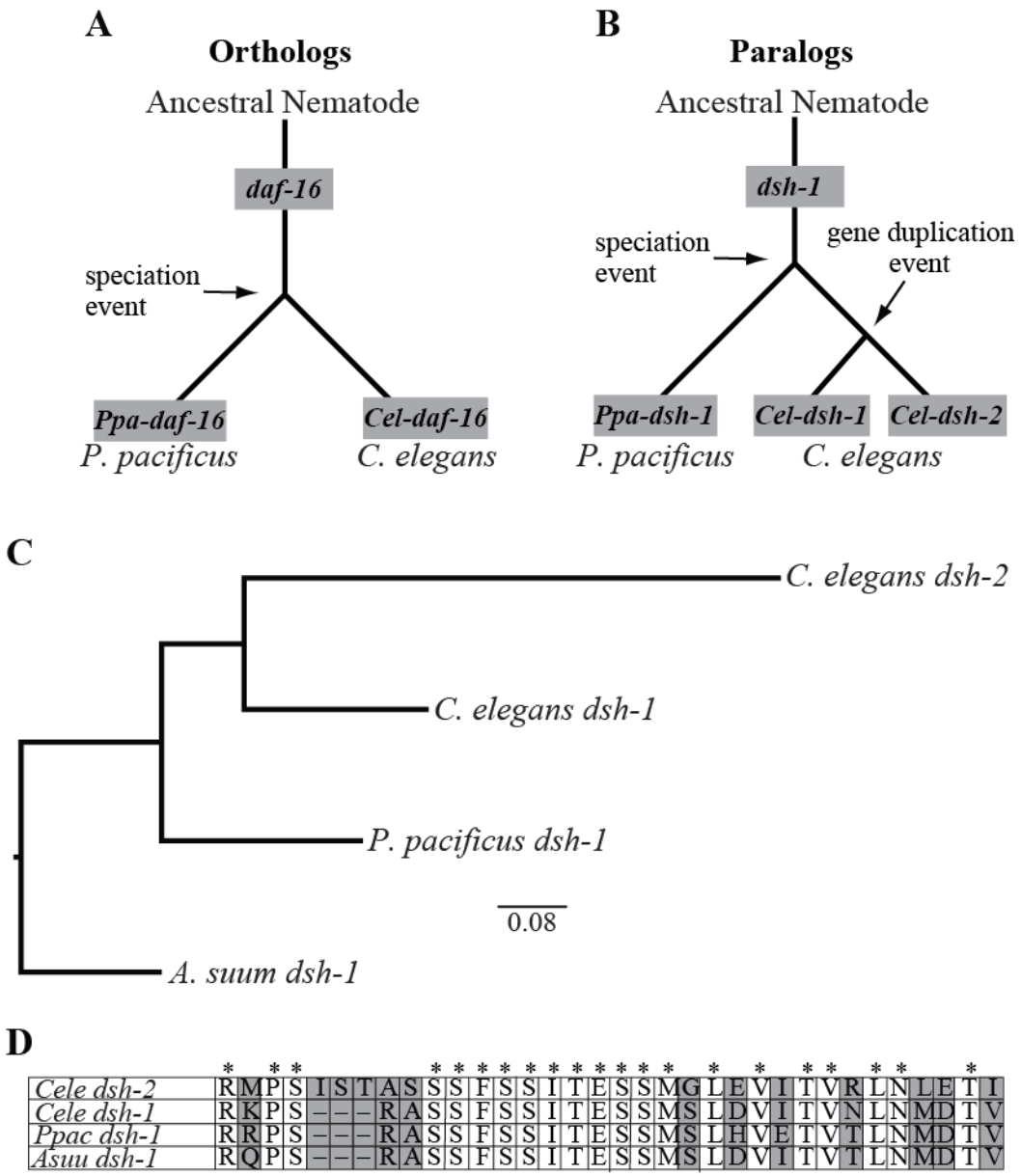

Figure 5.4 | Distinction between orthologous and paralogous genes. A) Orthologs are homologous sequences in different species that descend from a common ancestral gene during speciation. The *daf-16* gene in an ancestral nematode was conserved in both extant lineages resulting from a speciation event that lead to *P. pacificus* and *C. elegans*. *Ppa-daf-16* is the conserved *daf-16* gene in *P. pacificus* and *Cel-daf-16* is the conserved gene in *C. elegans*. B) Paralogs are homologous sequences within a species, having arisen by gene duplication or similar event. While *Ppa-dsh-1* and *Cel-dsh-1* are orthologs in this example, having both been conserved

from the same parental *dsh-1* copy, *Cel-dsh-1* and *Cel-dsh-2* are paralogs, having been duplicated within *C. elegans*. C) Neighbor-joining tree generated from gene comparisons of *dsh-1* homologs between *A. suum*, *P. pacificus*, and *C. elegans*, providing evidence that *Cel-dsh-1* is the ortholog of *Ppa-dsh-1* while *Cel-dsh-1* and *Cel-dsh-2* are paralogs. The small bar at the bottom center of the tree shows the approximate distance equal to 0.08 nucleotide changes. *A. suum* was used as the outgroup taxon (see Figure 2). The tree was made from an alignment of the full proteins in MUSCLE and subsequently analyzed using default parameters of the 'Dnadist' and 'Neighbor' programs from PHYLIP 3.68 software package [48, 49]. D) A 34 amino acid window of the protein alignment from which the tree in part C was generated. It shows the sequence conservation of the *dsh-1* orthologs and the subsequent divergence of *Cel-dsh-2* from the other sequences. Areas of sequence divergence are highlighted in grey while asterisks (*) indicate conserved amino acid identity across all four genes. Hyphens (-) indicate gaps in the alignment, likely the result of insertion/deletion events. *A. suum* serves as the outgroup taxon.

Paralogs are homologous sequences within a species, having arisen by gene duplication. Paralogs are thought *a priori* to share similar function, but this may not always be the case, as gene duplication and subsequent modification is thought to be the major way organisms evolve genes with novel functions [50]. For example, *P. pacificus* contains a single copy of the gene *dsh-1*, which encodes a signaling protein involved in embryogenesis, while *C. elegans* has two paralagous copies of the dishevelled gene, *dsh-1* and *dsh-2*. Relative to the outgroup *A. suum*, there appears to have been a duplication event in the *C. elegans* lineage since it diverged from *P. pacificus*; the last common ancestor of *P. pacificus* and *C. elegans* likely also possessed a single copy of this gene (Figure 5.4B) [47, 50, 51]. Based on higher sequence conservation with the sole *P. pacificus* protein, only *Cel-dsh-1* is considered to be a genuine ortholog of *Ppa-dsh-1*,

though experimental confirmation of conserved function would validate this inference (Figure 5.4C–D).

Once a gene set has been identified, putative functions are ascribed by database searching and similarity comparisons of the proteins from the new genome to those with known function. Commonly used databases include the NCBI BLAST database, the EMBL-EBI InterProScan, Pfam, and Gene Ontology databases [43, 52–55]. This initial assignment of protein function is based on the assumption of homology by sequence or domain similarity. In essence, the proteome (the full complement of protein coding genes) that results from whole genome sequencing and annotation has functions ascribed to its individual protein-coding sequences by comparing them to a number of different databases in search of sequence or domain similarity [20]. When a protein sequence from the genomic dataset has the highest degree of similarity to one sequence in another genome, it is *a priori* assumed to be homologous or to be derived from shared ancestry. The protein is further inferred to have similar function. In molecular phylogeny, homology infers shared ancestry. One important caveat of identifying homologs by sequence similarity is that it is not uncommon for two proteins to share functional similarity without shared ancestry, as a result of convergent evolution [47, 50, 56]. For example, *Heterorhabditis* and *Steinernema* nematodes utilize a specific type of insect parasitism and are known as entomopathogenic nematodes (EPNs), a characteristic they share not through ancestry but convergent evolution [34]. A notable molecular example is the convergent evolution of nearly identical antifreeze proteins in both Antarctic notothenioid fishes and Arctic cod, which show remarkable sequence and functional similarity that is due to evolutionary convergence rather than shared ancestry [57].

Another nematode example of convergence is the hermaphroditism of *C. elegans* and *C. briggsae*, which though outwardly similar as self-fertile hermaphrodites, have different molecular mechanisms for achieving this mode of reproduction [58]. The opposite caveat is also true; proteins of shared ancestry do not necessarily share similar function [59].

Orthologous gene associations across multiple genomes can provide powerful evolutionary insights into biological functions of individual genes as well as the evolution of species. They can be used to identify conserved genes, as in the case of pan-nematode genes or clade-specific genes. The identification of widely conserved or more specific genes serves as the basis for designing molecular diagnostic tools and elucidating the relationships between species. Multigene analyses from EST datasets have previously been successfully used to inform nematode phylogeny, and additional whole genome sequencing could identify new diagnostic markers to overcome sequencing identification difficulties and lack of phylogenetic resolution in some vexing taxa such as the tylenchids [60, 61]. Furthermore, such comparisons can be used in pursuit of non-conserved taxon-specific genes, which may reveal something about the particular biology and adaptations of individual species. For example, Kikuchi et al. (2011), in conjunction with publishing the *Bursaphelenchus xylophilus* genome included an orthology analysis across 10 nematode genomes. Although the genes shared across the 10 species did not fit an obvious phylogenetic pattern, the comparison revealed several gene families that are broadly conserved as well as small groups of genes shared between pairs or groups of nematodes that may be involved in the ecologies of those species. For example, 144 genes are shared exclusively between *P. pacificus* and *B. xylophilus* [25]. These nematodes occupy different ecological niches (one is necromenic and the other is a

migratory endoparasite of plants), but they both share a close association with insects during their lifecycle. Kikuchi *et al.* (2011) suggest that these genes are candidates for being involved in that association. The case for such a conclusion would be stronger if genome comparisons could show that the last common ancestor of both species also shared an association with insects.

Orthology analyses can also be used to explore the conservation of important biological pathways, such as sex determination, dauer formation, or the RNAi pathway. Because of the extent of detailed genetic exploration in *C. elegans*, a common starting place is to identify pathways of interest in *C. elegans* and search for their orthologs in another nematode of interest, though these results should be interpreted conservatively. For example, the RNAi pathway in *C. elegans* has been well-studied and found to be quite complex, with at least 77 genes known to be involved in core aspects of the process [33]. As a powerful reverse genetics technique, RNAi is a commonly examined pathway in newly sequenced genomes and has been developed as an experimental tool in both plant- and animal-parasitic nematodes including *Globodera pallida*, *Heterordera glycines*, *M. incognita*, and *B. malayi* [62–64]. It may even have practical utility in agriculture in controlling plant-parasitic nematodes or at least increasing plant resistance [65, 66]. How many of the 77 known RNAi effector genes are absolutely necessary for RNAi in general and how many are part of the specific mechanism of RNAi in *C. elegans*? For instance, *sid-1* is necessary for systemic RNAi in *C. elegans*, but systemic RNAi has been reported in several other species that do not seem to contain an identifiable homolog of *sid-1*, including *B. malayi*, *Globodera* and *Meloidogyne* spp., *Pristionchus pacificus*, and *Panagrolaimus superbus* [62, 63, 67–70]. The successful application of experimental

RNAi in species that are apparently missing some genes required for systemic RNAi in *C. elegans* implies that either these genes are rapidly evolving or have only become necessary in *C. elegans,* or that an alternate pathway exists [18, 33, 62]. Although RNAi has been shown to work in a number of both plant- and animal- parasitic nematodes, it is thought that culturability and the feasibility of maintaining non-stressful culturing conditions may better explain RNAi competencies than the disparity of RNAi effector genes across taxa [33]. As more species are added to these types of genomic analyses and genetic experimentation in non-model systems continues to grow, our understanding of these processes and which parts are conserved, derived, or rapidly evolving will become more clear.

Operons

One striking feature of nematode genomes studied thus far is the presence of operons. Though originally thought to be a genomic feature unique to prokaryotes, operons have been found in nematodes as well as some ascidians and fruit flies [71]. Bacterial operons comprise 2 or more genes that are transcribed to form a single mRNA transcript (Figure 5.5). In nematodes, multiple genes are transcribed into a single primary transcript, which is then processed into separate mRNAs; through RNA-splicing events, a spliced leader is added to the 5' end of each downstream transcript in operon (Figure 5.5). In *C. elegans*, about 70% of mRNAs include a spliced leader, the majority of which (~ 55%) are of the SL1 type. These SL1 spliced leaders are typically either from non-operonic transcripts or are from the first gene in an operon (Figure 5.5) [72]. Downstream transcripts from within an operon each have an SL2 leader [72]. Operons can be inferred

from the genome by the presence of very closely spaced genes in the same orientation in the genome and from the presence of SL2 spliced leaders. Apparent operons have been identified in all published nematode genomes with the exception of *Trichinella spiralis*, a highly unusual nematode, quite distantly related to all other sequenced nematodes and one of the world's largest intracellular parasites (Figure 5.2) [24, 73]. Although *T. spiralis* is missing both canonical nematode *trans*-spliced leaders, SL1 and SL2, the presence of a number of other distinct spliced leader sequences leaves open the possibility that this species does contain operons. Additional nematode genomic data, especially from taxa in Enoplia, Dorylaimia, and basal clades of Chromadoria, may reveal the untold story of operon evolution among nematodes (Figure 5.2). Operons are thought to have evolved in nematodes to facilitate transitions from arrested development to rapid growth [74].

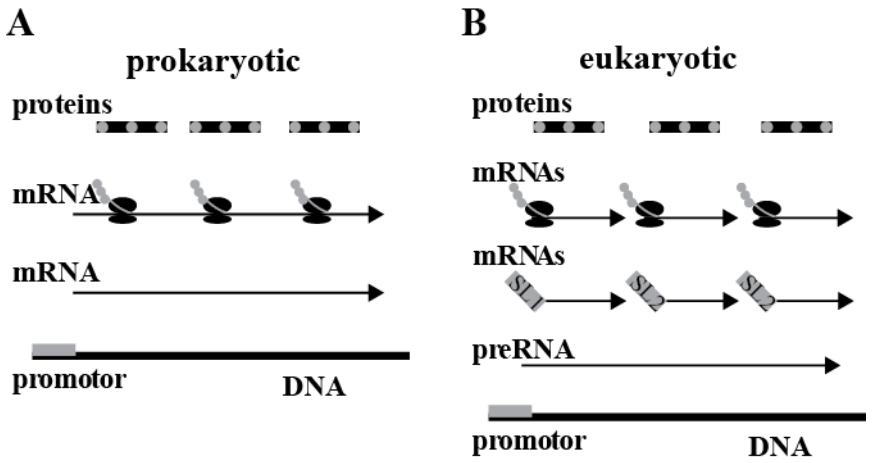

Figure 5.5 | Prokaryotic and eukaryotic operons. A) The prokaryotic operon model is that polycistronic mRNA is produced from a single promoter, containing several genes in the same transcript. Each protein is translated from a different location of a single mRNA. B) Eukaryotic operons also produce a single preRNA transcript with multiple genes, but are then processed to

form mRNAs for each individual protein. As part of the processing each mRNA has a splice leader added to the 5' end of the transcript. These mRNAs are then translated. Usually the first gene from the preRNA in a nematode operon transcript is spliced with the SL1 splice leader attached while all other downstream genes have the SL2 splice leader attached.

Genomes and ecology

The first report of a nematode genome focused on the sequencing methodology, the development of physical and genetic maps, assembly, and annotation, as well as a comparison of the genome to prokaryotes and yeast [8, 14]. This comparison revealed that *C. elegans* has an unusually high number of nuclear hormone receptor proteins (NHRs), prompting researchers to propose that NHRs were perhaps important in the evolution of multicellularity [8]. Though originally thought to be normal among nematodes, it is now known that even among close relatives, *C. elegans* is an outlier in terms of its number of NHRs and G protein-coupled receptors (GPCRs) and in these respects is not an archetypical nematode [6, 75]. The anomalously high number of NHRs and GPCRs in the *C. elegans* genome was found by examining the top 20 most prevalent protein domains in the genome. Such comparisons of gene and domain prevalence among species may reveal important differences in the genome that ultimately underlie differences in the evolution, ecology, and lifestyles of nematodes. In this way, comparative genome analyses will serve as a tool for testing hypotheses about the ecology and evolution of related species; and the resolving power of such comparisons will increase with the addition of more sequenced taxa.

The sequencing of *C. briggsae* greatly enhanced our understanding of the *C. elegans* genome by providing strong evidence for 1,300 previously unidentified genes,

thus demonstrating how sequencing closely related species can enhance the annotation of genomes [17]. Analysis of repeat regions revealed that *C. elegans* and *C. briggsae* have undergone rapid evolutionary turnover at the sequence level, providing evidence for a more recent divergence of these two nematodes compared to the evolutionary split between human and mouse lineages (~ 40 million years ago for the nematodes and ~ 75 million years ago for mouse/human). Similarly, the amino acid identity revealed between putative orthologs (~ 80% for *C. briggsae*/*C. elegans* and ~ 78.5% for mouse/human) supports this conclusion [17, 76].

As sequencing technology has advanced and costs have dropped, additional nematode genomes have been sequenced, including close relatives of *C. elegans* (*C. angaria, C. brenneri*, *C. japonica*, *and C. remanei*) and a handful of economically important parasites such as *Bursaphelenchus xylophilus*, *Meloidogyne incognita*, and *M. hapla* [12, 18, 19, 21–25]. One of the rationales for vertebrate-parasitic nematode sequencing projects (*B. malayi, A. suum*, and *T. spiralis*) was the identification of candidate genes to target pharmacologically [12, 18, 24]. This is a particularly important avenue of research given the large number of humans affected by nematode diseases and our current reliance on a small pool of drugs, whose effectiveness is at risk due to increasing resistance [77]. In addition to identifying new drug targets, these genomic analyses identified genes likely to be involved in the vertebrate-parasitic lifestyle, or perhaps parasitism in general. The abundance and diversity of secreted proteases and protease inhibitors in these genomes was an interesting result and has produced a long list of genes that are candidates to be involved in invasion of host tissues and degradation or evasion of host immune responses. The *B. malayi* genome's lack of key metabolic

enzymes provided evidence for this nematode's reliance on host- or *Wolbachia*-supplied molecules for purine, riboflavin, and heme biosynthesis [18]. Due to the basal position of *T. spiralis* in Dorylaimia (Figure 5.2), its genome was compared to all other available nematode genomes to identify pan-Nematoda-specific conservation. The resulting list of genes and proteins may have fundamental importance in all nematodes and points to potential targets for control of parasitic nematodes throughout the phylum [24]. Because of the highly specific and derived lifestyle of *T. spiralis*, which is an intracellular parasite, it is likely that examination of additional basal taxa will improve and solidify a pan-Nematoda candidate gene list, which, in addition to providing potential pharmacological targets could be used to inform deeper level phylogenetic studies.

Root-knot nematodes are among the most agriculturally devastating plant pathogens known in any phylum [19, 78]. This motivated the sequencing of *Meloidogyne incognita*, closely followed by *Meloidogyne hapla* [19, 21]. These genomes have provided intriguing insights into the adaptive strategies used by metazoans to circumvent immunity and successfully parasitize plants [19, 21]. They also provided evidence to support the long-suspected role of horizontal gene transfer (HGT) in the evolution of plant parasitism [79, 80]. Both of these parasites seem to have benefitted from the acquisition of plant cell wall-degrading enzymes that appear bacterial in origin. The idea that nematodes can acquire and utilize such enzymes in a cross-kingdom way was further bolstered by similar findings from genomic analyses of the mycophagous plant parasite *B. xylophilus* and the necromenic species *P. pacificus* [20, 25]. Recent follow-up work on HGT in multiple *Pristionchus* and related species utilized genome, transcriptome, and EST data sets, and revealed functional laterally acquired cellulase genes in several

diplogastrid species, notable turnover of cellulase genes inferred from elevated gene birth and death rates, and showed evidence for selective forces working on individual cellulase genes with a high degree of specificity [81]. Moreover, some cellulases found in *B. xylophilus* have not been found in any other nematode and appear fungal in origin, providing evidence that, if these genes are the result of HGT and not the independently arising result of convergent evolution, nematodes may not be limited to bacteria as sources of adaptational armament [25]. The evidence for HGT in multiple distantly related nematodes (*Bursaphelenchus*, Koerneria, *Meloidogyne*, and *Pristionchus*) suggests that this mode of gene acquisition may play a broadly significant role in nematode adaptation and evolution (Figure 5.2).

One clear theme that has emerged from genomic comparisons is that there may not be an archetypal nematode [6, 75]. For example, the massive expansions in GPCRs and NHRs reported in *C. elegans* are thus far not replicated in the genomes of any other sequenced nematodes, and likely play a significant role in *C. elegans*' natural ecology, which has only recently been explored through modern investigation [82–84]. As more nematode species are fully sequenced, it is becoming clear that the ecology and specific biology of each species will become increasingly valuable in the interpretation and use of these genomes. While earlier reports of nematode genomes focused heavily on sequencing methodologies and the technical details of gene prediction and annotation, more recent studies have highlighted genomes in the context of nematode ecology and evolution; this trend is likely to continue. For instance, *P. pacificus* is an omnivorous feeder, necromenic but not parasitic. It associates with arthropods and waits for them to die, feasting on the microbial and fungal bloom resulting from the arthropod host's death

[82, 85]. A broad view of the *P. pacificus* genome reveals expansions in protein families playing key roles in stress tolerance and the metabolism of xenobiotics (foreign chemical compounds; e.g., host defense molecules) [20]. Tolerance to low oxygen concentrations and toxic host enzymes as well as complex metabolic pathways and other morphological adaptations were predicted to assist this nematode in its lifestyle, but prior to its genome being sequenced the molecular architecture of these adaptations could only be speculative [20]. The genetic underpinnings of necromeny in *P. pacificus* and its adaptation to this particular niche have been revealed through its genome. These findings lead to additional genomically generated hypotheses and sow fertile ground for future experimentation.

Ecological genomics is a burgeoning field aimed at understanding the genetic mechanisms that underlie organismal responses and adaptations to their natural environments [86]. Model organisms, often chosen for ease of culture and a host of other traits that favor laboratory growth and experimentation, usually lack the extensive ecological context and framework that has been painstakingly built for many non-model systems. In contrast, many organisms used in ecological studies do not have the extensive experimental tool development (e.g., transformation and RNAi) or genetic pathways and interactions mapped out as in model systems. The time is ripe for dramatic expansion of ecological studies using model systems and genomic/transcriptomic sequencing and accompanying tool development to be done in favored ecological systems [87]. Nematodes are in a superb position to see progress in both areas, with several well-developed model systems being explored from an ecological context [82–84, 88, 89] and for nematode species for which archives of ecological data have been accumulated to be scrutinized from a genomic context [90, 91].

Entomopathogenic nematodes as an example of question-driven genomics

Nematode genomics, now highlighting specific aspects of organismal biology, life history traits, and ecology and evolution, provides opportunity for researchers to utilize the powerful broad view of sequencing to learn more about their nematode of choice. As an illustrative example of ecological genomics and what could be accomplished for every niche occupied by nematodes, we conclude by discussing some of the interesting genomic insights that can be gleaned from examining the forthcoming entomopathogenic nematode genomes.

EPNs occupy an interesting niche somewhere between parasitoids and pathogens, utilizing insect-pathogenic bacteria to facilitate their form of parasitism, acting as a vector for the bacteria and, working together as a complex, the nematode and bacteria rapidly kill their host [92, 93]. This very specific form of parasitism seems to have arisen at least twice among nematodes, in *Heterorhabditidae* and *Steinernematidae*, which are not closely related. The genomic sequencing of heterorhabditid and steinernematid nematodes will provide the framework for a genetic comparison of the evolution of entomopathogeny in these lineages [87]. In contrast to the vertebrate- and plant-parasitic nematode genome studies, which compare organisms that obtain resources by different means, the intra-guild comparisons of EPN genomes will focus on species that exploit the same kind of environmental resources in similar ways [94, 95]. A genomic comparison of EPNs from multiple genera has the advantage of decades of ecological research and will

increase our understanding of adaptation and convergent evolution in addition to revealing just how similar or different this niche exploitation is at the genetic level.

EPNs have rapidly become models for studying parasitism and mutualism. The genetic components of their association with symbiotic bacteria have been heavily studied from the bacterial side, but largely neglected in terms of the nematode's contribution [90, 96]. Genome-wide expression analysis against the backdrop of the genomic sequence could shed light on what, if any, contribution is made by the nematodes to symbiosis. Within *Steinernema*, there are more than 60 described species [97–105]. Though only a handful of these have been tested, the host-range and specificity of insects they can infect is diverse and varied. A striking example is *S. carpocapsae*, which is the most heavily studied steinernematid. With an extremely broad host range, *S. carpocapsae* is capable of infecting more than 250 species of insects across 10 orders, although some infections were only demonstrated under laboratory conditions [106]. Closely related to *S. carpocapsae* is *S. scapterisci*, which is known to have a much narrower host range and seems to be a cricket specialist [107, 108]. The wide view afforded by protein family abundances revealed by genomes will provide testable hypotheses about the breadth of specific of EPNs' host-range and the specificity of some EPNs for certain insect hosts, beyond what is currently known.

EPN research has also seen recent developments in the neuronal basis of behavior and the molecular mechanisms underlying host tissue invasion and death [91, 109]. Understanding protein domain abundance against this backdrop will likely hone existing hypotheses and direct future experimentation, leading to a deepening of our knowledge in both of these areas of research. Along with the broad overview on the architecture of

parasitism, it is anticipated that EPN genomes will provide insights to the above mentioned and other aspects of EPN ecology. A hopeful expectation of most new nematode genomes is that they will pave the way for techniques such as transformation and RNAi to be used in experimentally testing the genomically generated hypotheses, as exemplified with *P. pacificus* [20, 68].

Conclusion

Many new nematode genomic sequencing projects are underway, and improving technologies means still more will become feasible and affordable. These widening horizons are generating a need for more nematodes to be cultured and have their DNA harvested. More importantly, it opens the door for collaborations between genomicists and nematologists. We expect that fruitful collaborations will entail far more than merely providing material and could include various aspects such as (a) knowledge of the ecological background and candidate pathways or biological phenomena to explore within the sequence, (b) phylogenetic knowledge of sister taxa or associated nematodes for comparison or particularly informative developmental stages for transcript analysis, and (c) interesting morphological features that remain to be genetically explored. We urge the members of the Society of Nematologists to utilize their expertise and the wealth of their collective ecological knowledge to contribute to sequencing efforts and to adopt genomics into the toolkit of nematology. As nematology stands at the precipice of genomic grandeur, with 959 nematode genomes planned (a number chosen to reference the 959 somatic cells of *C. elegans* [26]), we will soon be suffused with genomic data,

offering the potential to discover long-sought answers to the biology, ecology, and evolution of genomes, and promising in turn to raise many more new questions.

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Chapter 6:

Genomic Sequencing of 5 *Steinernema* Nematodes (Rhabditida: Steinernematidae): Insights Into the Evolution of Parasitism*

*This chapter is not yet published but is currently in preparation and in its current form is written solely by Adler Dillman. It will ultimately include additional authors when published, including Ali Mortazavi, Marissa Macchietto, Byron J. Adams, Paul W. Sternberg, and possibly other others as well. Some of the data presented herein will change prior to publication, as additional sequencing data is acquired.

Abstract

Nematodes are amazing animals, both old and diverse. Among their diversity are many plant and animal parasites, many of which negatively affect humans. However, not all parasitic nematodes are bad and some are currently being used as organic alternatives to chemical pesticides for controlling damaging insect pests. Although there are many insect-parasitic nematodes, the entomopathogenic nematodes are the best studied of these and are remarkably different in their lifestyle and in their particular parasitism. Herein I discuss the difference between entomopathogenic nematodes and other insect parasites and what makes them so interesting and useful.

Introduction

When first looking for projects to propose for my graduate thesis, I was intrigued by the jumping abilities of some species of *Steinernema* and had hoped to explore this behavior in the context of foraging and host seeking. In addition to host seeking, I am interested in understanding the architecture of parasitism within the genome and how the genome of a free-living nematode might differ from that of a parasite. If we were to look at overviews of 2 genomes, could we tell just by the genomic content that one belonged to a parasite? I also thought it would be neat to understand how some species of *Steinernema* are capable of jumping but not others. Is this due to differences in physical structure or musculature, or does the difference lie deeper and hidden at the molecular level? Though I knew it was an impossibly risky proposal, it seemed that by sequencing the genomes of jumpers and non-jumpers, we might learn something about what

facilitates this amazing behavior. Admittedly this was a naïve supposition, but the EPNs are potentially well-suited to answering the question of parasitic architecture in the genome, since they are easily cultured within a short generation time and can be synchronized as IJs in, in addition to the abundance of ecological and behavioral data, as previously presented. However, there are currently no EPN genomes publicly available. There are over 70 species in the genus *Steinernema*, making it somewhat difficult to decide which species to sequence [1, 2]. Ultimately this decision was made based on availability of material, usefulness in biological control, and their phylogenetic position within the genus. I have sequenced and begun annotation on 5 steinernematids: *S. carpocapsae*, *S. scapterisci*, *S. monticolum*, *S. feltiae*, and *S. glaseri*. Several of these taxa were also included in behavioral studies detailed in Chapter 4 of this thesis. Among these 5, *S. carpocapsae* and *S. scapterisci* are known to be capable of jumping and are considered ambush foragers [3–5]. *S. monticolum* is reported as being capable of jumping but is thought to employ an intermediate foraging strategy [3, 5–7]. Originally I had wanted to include this species in my behavioral assays described in Chapter 4, but this nematode is not a very good jumper and it was not practical to use it in jumping assays. *S. feltiae* is not capable of jumping but is commonly used in biological control and is thought to use an intermediate foraging strategy, leaning toward the cruising side of the continuum [3, 5]. *S. glaseri* is not capable of jumping and is a classic example of a cruise forager among steinernematids [3, 5]. Sequencing these species also meant we were using taxa from clades II, III, and V of the five clades identified within the genus [8, 9].

Materials and Methods

Strain culturing and maintenance of *Steinernema* sp. *S. carpocapsae* were from the inbred strain ALL [10–12]. *S. glaseri* were from the inbred NC strain [13]. *S. scapterisci* were inbred from the FL strain [14]. *S. feltiae* were from the inbred SN strain [15]. *S. monticolum* were inbred from the originally isolated strain from Korea [7]. All nematodes were cultured as previously described [12]. Briefly, 5 last instar *Galleria mellonella* larvae (American Cricket Ranch, Lakeside, CA) were placed in a 5 cm Petri dish with a 55 mm Whatman 1 filter paper acting as a pseudo-soil substrate in the bottom of the dish. ≤ 250 ml containing 500–1000 IJs suspended in water was evenly distributed on the filter paper. After 7–10 days the insect cadavers were placed on White traps [16]. *Steinernema glaseri* was placed onto a modified White trap containing plaster of Paris as previously described [17]. Emerging IJs were harvested and rinsed 3 times with water. *S. scapterisci* was also cultured by infecting house crickets and mole crickets using similar techniques. IJs were stored harvested and used to isolate either total genomic DNA or stage specific RNA. To obtain *S. carpocapsae* stage-specific RNA for embryo, L1, and adult stages, nematodes were grown on lipid agar plates inoculated overnight with *Xenorhabdus nematophila* cultures [18]. Fresh bacterial lawns were inoculated with IJs and given three days to develop and reproduce. After 3 days, all nematodes were harvested and bleached for synchronization, then harvested at the appropriate times for stage-specific material.

Isolation of DNA and RNA. Once harvested, nematodes were frozen at −80°C until used. To extract nucleic acid, the nematodes were thawed and refrozen two to three times

to facilitate breaking the tough cuticle before extracting either genomic DNA or bulk RNA. Genomic DNA was extracted using a Promega Wizard® genomic DNA purification kit and following the protocol described in that kit. The genomic DNA was then treated with RNase A for digestion of any RNAs present in the sample. Bulk RNA was extracted using a Trizol® extraction as previously described [19].

Genomic and RNA-Seq library construction. Genomic library was constructed using Illumina Paired End DNA Sample Preparation Kit according to the manufacturer's instructions. Briefly, 3 μg of genomic DNA were fragmented using nebulization. The fragments were end repaired, 3' adenylated and ligated to Illumina's paired end adaptors. The ligation products were size selected on an agarose gel to yield fragments of approximate length of 350 bp and PCR amplified to produce the finished library. For *S. carpocapsae*, we also made a jumping library with in insert fragment length of 2kb to help facilitate a better assembly [20]. RNA-Seq library was created from 10 μg of total RNA. mRNA was purified using Dynal magnetic oligo(dT) beads (Invitrogen) and fragmented with 40mM Tris-acetate, pH 8.1, 100 mM KOAc, 30 mM MgOAc buffer for 4 min at 94°C. First and second cDNA strands were synthesized using random primers and SuperScript II RT (Invitrogen), and RNaseH and DNA Pol I, respectively. The rest of the procedure was identical to that used for the genomic library preparation, except that the gel cut for the RNA-seq library was ~ 300 bp. Libraries were quantified using Qubit fluorometer (Invitrogen) and size distributions were verified using Agilent Bioanalyzer and the High Sensitivity DNA Kit. Libraries were sequenced on Illumina Genome Analyzer IIx sequencer in paired-end mode with the read length of 100 nt.

Genome assembly and annotation. Both the genomic and the mixed-stage transcriptome libraries were built, sequenced, assembled, filtered, and repeat-masked as previously described [21] using Velvet 1.0.9. Genome and RNA-seq reads will be submitted to the public database once the assembly is complete. Assembled cDNA was used to train Augustus 2.5 [22] for protein-coding gene finding. Separately, RNA-seq reads were mapped onto the genome using TopHat 1.3.1 [23], assembled intro transcripts using Cufflinks 1.2.0 [24] and merged with the Augustus annotations using the RABT method [25]. Candidate SNVs in the genome and transcriptome mapped reads were called using the SAMtools [26] pileup and varFilter options. Candidate SNVs in the transcriptome that fell within 5 bp of exon junctions were filtered out as likely splicing artifacts.

Orthology analyses. To study the evolution of gene families across nematodes, we used the available predicted protein datasets from WormBase release WS225 (www.wormbase.org)—*Brugia malayi*, *Caenorhabditis elegans*, *Meloidogyne hapla*, *Pristionchus pacificus*, and *Trichinella spiralis*. We also included the *Ascaris suum* and *Bursaphelenchus xylophilus* predicted proteome data sets from WormBase release WS229. For outgroup and comparative analysis we used the predicted protein datasets of the *Arabidopsis thaliana* (vGNOMON 7/9/07), *Drosophila melanogaster* (v10/30/11), *Homo sapiens* (v9/7/11), *Mus musculus* (v3/4/11), *Nasonia vitripennis* (v1.2), *Saccharomyces cerevisiae* (v2/3/11), and *Tribolium castaneum* (vTcas 3.0) genome projects, obtained from the NCBI/NIH repository (ftp://ftp.ncbi.nih.gov/genomes).

Version 1.4 of the OrthoMCL pipeline was used to cluster proteins into families of orthologous genes, with default settings and the BLAST parameters recommended in the OrthoMCL documentation [27].

Protein domain analyses. To evaluate the prevalence of protein domains in the proteome of *S. carpocapsae*, *S. scapterisci*, *S. monticolum*, *S. feltiae*, and *S. glaseri* and other species, we used the HMMscan program from the latest version of HMMER (3.0) software package, which implements probabilistic profile hidden Markov models [28]. We set our threshold *E*-value criterion at 10^{-6}, so that no known false-positive matches would be detected in assigning Pfam domain identities. We ran this analysis on the proteomes mentioned above and filtered out splice isoforms from the *C. elegans* proteome.

Gene tree analyses. Some protein families were further explored by evaluating gene trees, either with whole protein sequences or by protein domain sequences. To do these analyses we aligned protein sequences using MUSCLE [29]. Aligned protein sequences were then evaluated by distance analysis using the JTT matrix and a subsequent neighbor-joining tree was created using the PHYLIP software package version 3.68 [30].

Results and Discussion

The *Steinernema* species selected proved to be amenable to short-read sequencing technology, resulting in assembled genomes between 82 and 114 Mb in size with variable contig sizes (Table 1). With the quality expectation of newly sequenced genomes on the

rise, we have decided to do additional sequencing prior to publishing these genomes, but Table 6.1 clearly indicates the usability of these genomes. The quality should dramatically improve with additional sequencing. In addition to high N50 values for several of these genomes, an analysis of 458 core eukaryotic genes reveals these genomes are largely complete: 98.67% for *S. carpocapsae*, 97.13% for *S. scapterisci*, 96.68% *S. monticolum*, 97.57% for *S. feltiae*, and 97.13% for *S. glaseri* [31].

Genome	Size (Mb)	# scaffolds	Max scaffold	N50	# genes predicted
S. carpocapsae	85.66	8,470	890.8 kb	93.15 kb	27,706
S. scapterisci	82.54	16,412	479.7 kb	46.0 kb	31,939
S. monticolum	114.25	82,427	347.9 kb	8.0 kb	41,294
S. glaseri	93.83	28,194	261.2 kb	27.6 kb	34,109
S. feltiae	101.04	66,553	566.99 kb	18.2 kb	36,178

Table 6.1 | *Steinernema* Genome Statistics. This table lists the 5 *Steinernema* genome species that were sequenced and provides several statistics. The size of the assemblies are given in Mb. The number of pieces or scaffolds that are in the assembly are reported, the fewer the pieces the better. Ideally there would be one scaffold per chromosome; obviously these genomes are not in the same state as the *C. elegans* genome. The max scaffold gives the size of the largest scaffold of the assembly. The N50 statistic provides the size of at least half of the scaffolds in that assembly. For example, at least half of the *S. carpocapsae* scaffolds are 93.15kb or larger in size, meaning that each scaffold is likely a multigenic-sized piece, making this a good quality genome. Also listed is the number of predicted genes, which is artificially high due to splice isoforms but will get better as annotation continues.

In order to evaluate the potential differences and similarities in genome architecture between a parasite and a 'free-living' nematode, a comparison was made between *S. carpocapsae* and *C. elegans*. The Pfam database was used to assign protein domain families to each of the full proteomes of these species. It is possible for some

proteins to have no recognizable protein domains while others can have several. I compared the prevalence of protein domains between these two species to see if anything stands out as being more abundant in the parasite or more abundant in the non-parasite, thus giving me a starting place to identify underlying differences in life strategy (Figure 6.1). I find that the *C. elegans* genome has an abundance of G-protein-coupled receptor (GPCR) domains, including members of the Srh, Sri, Srd, Str, and Srj families. Many olfactory receptors in nematodes are known to be GPCRs, which sparked my interest, especially considering the host-seeking studies I had performed previously, described in Chapters 3 and 4 [12, 32–34]. It is striking that Srh and Sri are much more abundant in *C. elegans*, given what is known about their expression. A particular promoter sequence called the E-box has been shown to be enriched in Srh and Sri GPCRs, and it is thought that this promoter drives expression in the chemosensory ADL neuron, making these GPCRs likely olfactory receptors [35]. Other Pfam domains that are much more abundant in *C. elegans* include F-box and F-box associated domains, which are involved in protein-protein interactions (Figure 6.1). On the other hand, I find trypsin inhibitor, aspartyl protease, and trypsin domains to be much more prevalent in the *S. carpocapsae* genome. This is not particularly surprising, given the potential utility of proteases and protease inhibitors in affecting insect immunity and tissue digestion. One surprising finding is the abundance of Srt family GPCRs in *S. carpocapsae*. Evolutionary analysis of this family has been limited and almost nothing is known about their function, but their expression pattern seems consistent with a role in chemosensation. This is a promising and unexpected expansion that could prove interesting regarding host seeking among steinernematids.

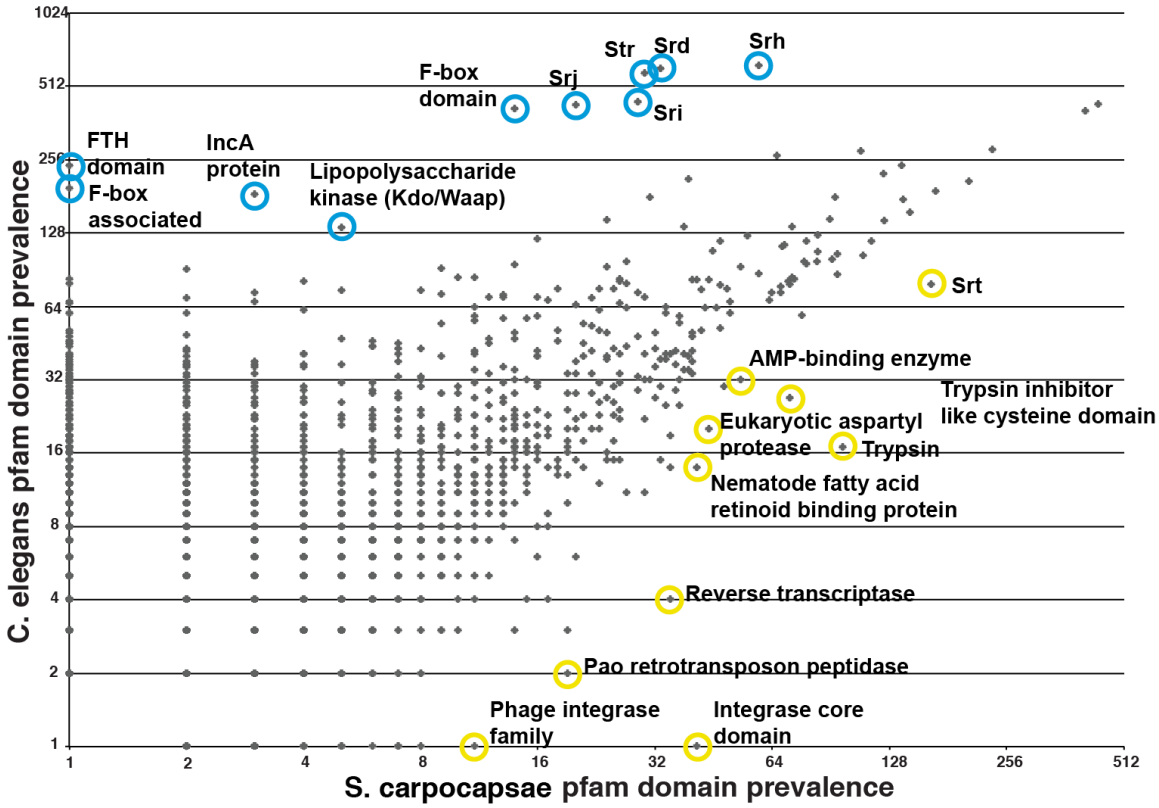

Figure 6.1 | Comparison of Pfam protein domain prevalence between *C. elegans* and *S. carpocapsae*. Protein domains that are in equal abundance in both species will show up on the diagonal axis, while those more abundant in *C. elegans* will cluster in the upper left and those more abundant in *S. carpocapsae* will appear toward the lower right. Several of the most divergently abundant protein domains have been highlighted in blue and yellow for those more abundant in *C. elegans* and *S. carpocapsae*, respectively.

These two findings, the abundance of GPCRs in *C. elegans* and the abundance of proteases and protease inhibitors, have shaped much of the rest of my genomic research, and I will discuss each in further detail below.

The abundance of GPCRs in *C. elegans* is interesting, since it is known that *C. elegans* is a fruit-dwelling nematode, not normally found in soil but in rotting fruit or

plant material. It spends its entire life cycle in a very complex environment avoiding predators while seeking resources and mates. It is unsurprising that an abundance of potential olfactory receptors would be useful for its lifestyle, but what of other nematodes? I searched for the abundance of potential olfactory receptors across available nematode genomes and found there is a common trend for GPCR abundance (Table 6.2).

	C. elegans	P. redivivus	B. xylophilus	P. pacificus	M. hapla	A. suum	B. malayi	T. spiralis	S. carpocapsae	S. scapterisci	S. monticolum	S. feltiae	S. glaseri
Number of proteins with multiple GPCR domains	1026	295	296	229	34	24	3	3	152	178	185	219	277
Number of proteins with only one GPCR domain	558	289	198	307	115	78	20	25	452	553	505	664	527
Total GPCR domain-containing proteins	**1654**	**584**	**494**	**536**	**149**	**102**	**23**	**28**	**604**	**731**	**690**	**883**	**804**

Table 6.2 | Total GPCRs Identified by Pfam Across Nematodes. This table lists total number of potential GPCRs as identified by a Pfam analysis.

	C. elegans	P. redivivus	B. xylophilus	P. pacificus	M. hapla	A. suum	B. malayi	T. spiralis	S. carpocapsae	S. scapterisci	S. monticolum	S. feltiae	S. glaseri
Serpentine type 7TM GPCR chemoreceptor Srh	625	208	221	154	2	9	0	0	58	90	90	200	220
Serpentine type 7TM GPCR chemoreceptor Srd	606	143	206	146	28	16	0	0	32	40	43	65	90
Serpentine type 7TM GPCR chemoreceptor Str	578	155	238	176	20	13	0	0	29	39	40	59	96
Serpentine type 7TM GPCR chemoreceptor Sri	439	127	90	61	0	1	0	0	28	27	26	74	84
Serpentine type 7TM GPCR chemoreceptor Srj	427	64	143	131	2	6	0	0	19	18	18	31	55
Serpentine type 7TM GPCR chemoreceptor Srx	241	33	23	50	9	20	9	2	136	154	172	201	172
Serpentine type 7TM GPCR chemoreceptor Srw	179	24	27	15	5	23	7	4	30	27	29	31	26
Serpentine type 7TM GPCR chemoreceptor Srsx	154	67	62	41	60	35	2	18	143	171	167	177	137
Srg family chemoreceptor	144	93	13	39	8	1	0	0	23	23	23	22	32
Serpentine type 7TM GPCR chemoreceptor Srbc	120	10	4	4	0	4	0	0	15	21	6	13	12
Serpentine type 7TM GPCR receptor class ab chemoreceptor	112	45	51	25	13	5	5	1	67	74	77	69	80
Sre G protein-coupled chemoreceptor	81	55	30	38	9	5	2	0	40	43	44	38	50
Serpentine type 7TM GPCR chemoreceptor Srv	80	49	10	36	1	2	0	4	25	38	31	31	45
Serpentine type 7TM GPCR chemoreceptor Srz	79	0	0	0	0	0	0	1	0	1	0	0	0
Serpentine type 7TM GPCR chemoreceptor Srt	78	39	33	35	21	3	0	1	163	192	153	191	138
Serpentine type 7TM GPCR chemoreceptor Sra	69	4	6	5	2	1	3	0	12	15	19	11	15
Serpentine type 7TM GPCR chemoreceptor Sru	68	1	0	1	1	1	0	0	1	3	2	1	12
Serpentine type 7TM GPCR chemoreceptor Srb	46	0	0	4	0	1	0	0	11	11	15	6	11
Serpentine receptor-like protein, class xa	41	1	0	0	0	0	0	0	1	1	2	1	1
Total Serpentine GPCR Domains	**4167**	**1128**	**1157**	**961**	**191**	**146**	**28**	**31**	**833**	**989**	**957**	**1221**	**1276**

Table 6.3 | All GPCR Families Identified by Pfam Across Nematodes. This table lists the number of all potential GPCRs as identified by a Pfam analysis and categorizes them by family [36, 37].

Nematode species that spend little to none of their foraging time in complex soil environments, such as the passively ingested vertebrate parasites *Ascaris suum*, *Brugia malayi*, and *Trichinella spiralis* have very few potential olfactory receptors (as GPCRs). While species that have free-living stages or forage in complex environments, including plant parasites, insect parasites, and free-living nematodes, have an abundance of potential olfactory receptors (Table 6.2).

In addition to looking at the total number of potential olfactory receptors, breaking these down into their respective families provides additional details about which GPCRs are highly conserved across all nematodes and which families have been expanded for particular use among the different lineages (Table 6.3). This analysis reveals that Srx, Srw, and Srsx GPCRs are the most highly conserved numerically across nematodes. Finding that Srsx GPCRs are conserved across nematodes is not a surprising finding and agrees with previous research indicating that the Srsx family of GPCRs seems evolutionarily stable [37]. This analysis also reveals that an abundance of Srt GPCRs is common among all of the steinernematids we sequenced and potentially all steinernematids. I suggest that it is the Srt GPCRs among *Steinernema* that have led to host preferences and specializations within the genus and that they merit further investigation. This analysis also reveals that many of the GPCRs in *C. elegans* are unique to it or the *Caenorhabditis* lineage and are not shared among other nematodes, such as the abundance of Sri, Srj, Srx, and Srw GPCRs (Table 6.3). The general trend that GPCR abundance seems to correlate with environmental foraging can also be observed within the steinernematids, with intermediate and cruise foraging seeming to have more potential olfactory receptors than ambush foragers, a trend that should be explored in

further detail (Tables 6.2 and 6.3). I was able to explore this trend in somewhat more detail, at least informatically. The Srh, Srd, Str, Sri, and Srj families of GPCRs are at least two-fold more abundant in intermediate and cruise foragers *S. feltiae* and *S. glaseri* than they are in the well-known ambushers *S. carpocapsae* and *S. scapterisci* (Table 6.3). I constructed a gene tree including all identified Srd GPCRs to examine the evolutionary dynamics of this particular gene family (Figure 6.2). Although you won't be able to read the names of individual Srd genes, by looking at the colors a trend is easily observed. There are regions where all five colors seem well represented, likely indicating conserved GPCRs across the species and other regions of abundant red or purple, indicating expansions in *S. glaseri* and *S. feltiae*, respectively. It appears that evolution is driving the expansion of this GPCR family among the cruise foragers, indicating that this GPCR family may be involved in the different odor preferences of these nematodes.

It is known that EPNs can affect their host's immune response, and several researchers have already implicated a handful of *Steinernema* proteases and protease inhibitors as influencing the insect immune system. However, the full complement of these proteins and their modes of action still remains unknown [13, 38–43]. Using the agnostic approach described above to identify differences in protein abundances in the steinernematids, I was led to explore in more detail the abundance of proteases among these species and which families seem to be expanded. I find that the metalloproteases and serine proteases are expanded in *Steinernema*, above what is seen in other non-parasitic species, as well as the abundance of protease inhibitors (Table 6.4). It is likely that these types of proteases are involved in insect parasitism and could play a role in host specificity.

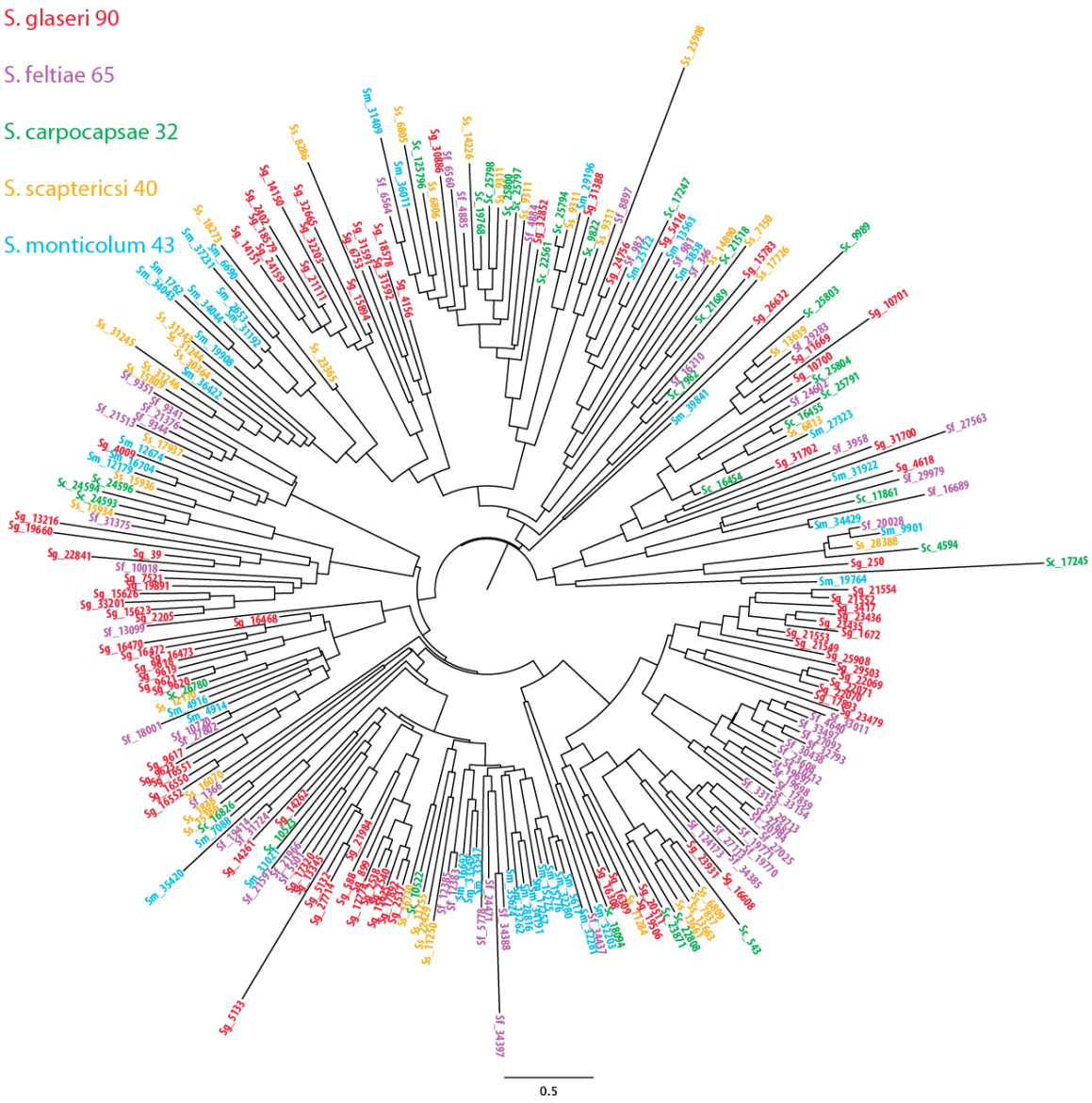

Figure 6.2 | Srd Family GPCRs Among Sequenced Steinernematids. This is a gene tree including all Srd GPCR family genes among the five steinernematids I sequenced. Although the gene names are too small to read, the pattern of conservation or expansion of genes is visible in the colors, with several apparent expansions in *S. glaseri* and *S. feltiae*.

These data lead me to believe that it could be the abundance and diversity of proteases that determines what kinds of insects an EPN is capable of infecting, while it is the abundance and diversity of GPCRs that determine which insects they are attracted to.

Clearly proteases, protease inhibitors, and GPCRs have played a significant role in the evolution of parasitism among steinernematids as well as niche partitioning among these species.

Proteases

Type of Protease	S. carpo	S. scapt	S. felti	S. glase	S. monti	B. xylop	P. rediv	C. japon	C. elega	C. brenn	C. reman	C. brigg	P. pacif
Aspartic	51	77	48	56	83	78	24	36	27	36	36	33	38
Cysteine	141	155	152	130	171	148	112	108	141	171	151	126	100
Metallo	232	306	365	360	439	209	232	209	203	275	233	185	215
Serine	264	370	333	288	531	174	227	134	156	169	168	156	237
Threonine	28	37	36	38	54	21	21	102	23	42	74	26	22
Unassigned	2	1	5	3	17	1	1	2	1	1	1	2	1
Total	718	946	939	875	1295	631	617	591	551	694	663	528	613
Inhibitors	189	225	231	158	191	60	91	80	94	132	119	102	64

Table 6.4 | Protease and Protease Inhibitors Across Selected Nematodes. This table displays the number of proteases in each of the subtypes. These data were assembled using the MEROPS protease database [44].

As mentioned earlier, these data are unpublished and remain unrefined. Additional sequencing is currently taking place and more analyses including the conservation of certain important biological pathways such as sex determination, RNA interference, dauer, and cell death pathways will be analyzed. I plan to do a more detailed analysis of the proteases, including those with signal peptides, to narrow down a list of potentially secreted proteases.

As a collected work this thesis contributes to our understanding of parasitism, host-seeking behavior, and the architecture of parasitism among nematodes. I have shown a conserved role for the BAG neurons in detected CO_2 in *C. elegans*, *H. bacteriophora*, and *S. carpocapsae*. This conservation spans considerable phylogenetic distance. I have

shown clear differences in olfactory preferences and virulence among EPNs and have demonstrated that this preference correlates with host suitability. I have placed these findings in the broader context of what it means to be an EPN and how these differ from other insect parasites.

I have discussed the role of genomics in nematology and exerted considerable effort to encourage genomic sequencing and analysis among nematologists and have been a driving force in steering the sequencing that is currently being done at Caltech. Though no genome papers have yet been published based on my work, I anticipate several significant contributions coming out in the next two years. It is clear that much of the information in my thesis builds on the work of others, but I have still conducted new research and contributed new knowledge of appreciable application across many fields.

It has been a pleasure to be involved in this work, and I have particularly enjoyed the conditions and working environment I experienced at Caltech. I close with my favorite Charles Darwin quote: “Doing what little one can to increase the general stock of knowledge is as respectable an object of life, as one can in any likelihood pursue.”

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Appendix A: Supplementary Materials for Chapter 3*

*This appendix is available as supplementary material for the published manuscript in *Current Biology* in 2011.

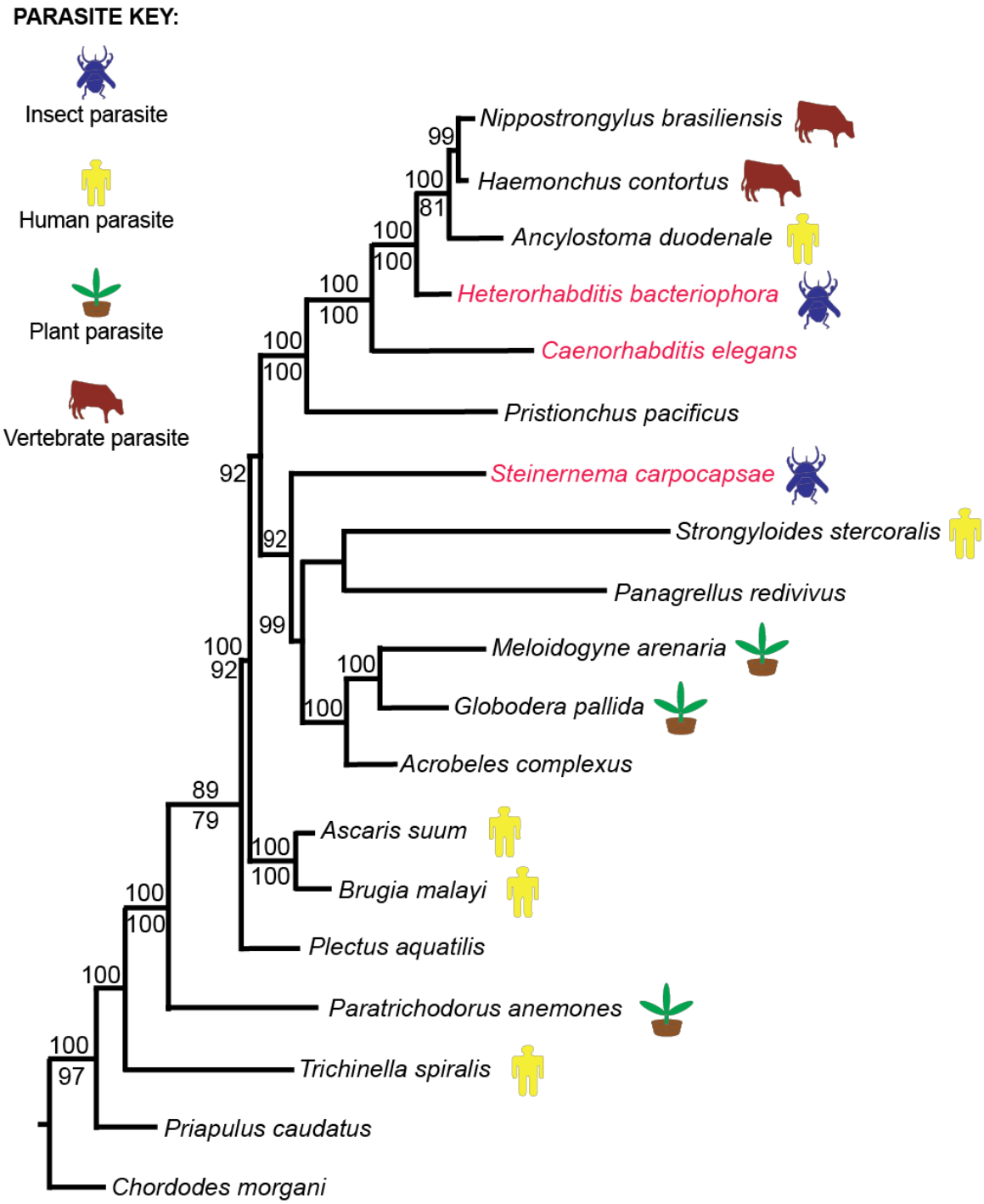


Figure 3.S1 | Phylogenetic relationships of 17 well-studied or representative species within Nematoda.

Relationships are based on ML and Bayesian analyses of nearly complete SSU sequences. Values above each branch represent Bayesian posterior probabilities; ML bootstrap indices (1000 replicates) appear below each branch. Values lower than 75 are not reported. Both analyses

produced concordant tree topologies. The ecologies of parasitic taxa are represented by colored icons. *Priapulus* (a priapulid) and *Chordodes* (a nematomorph) were defined as outgroups.

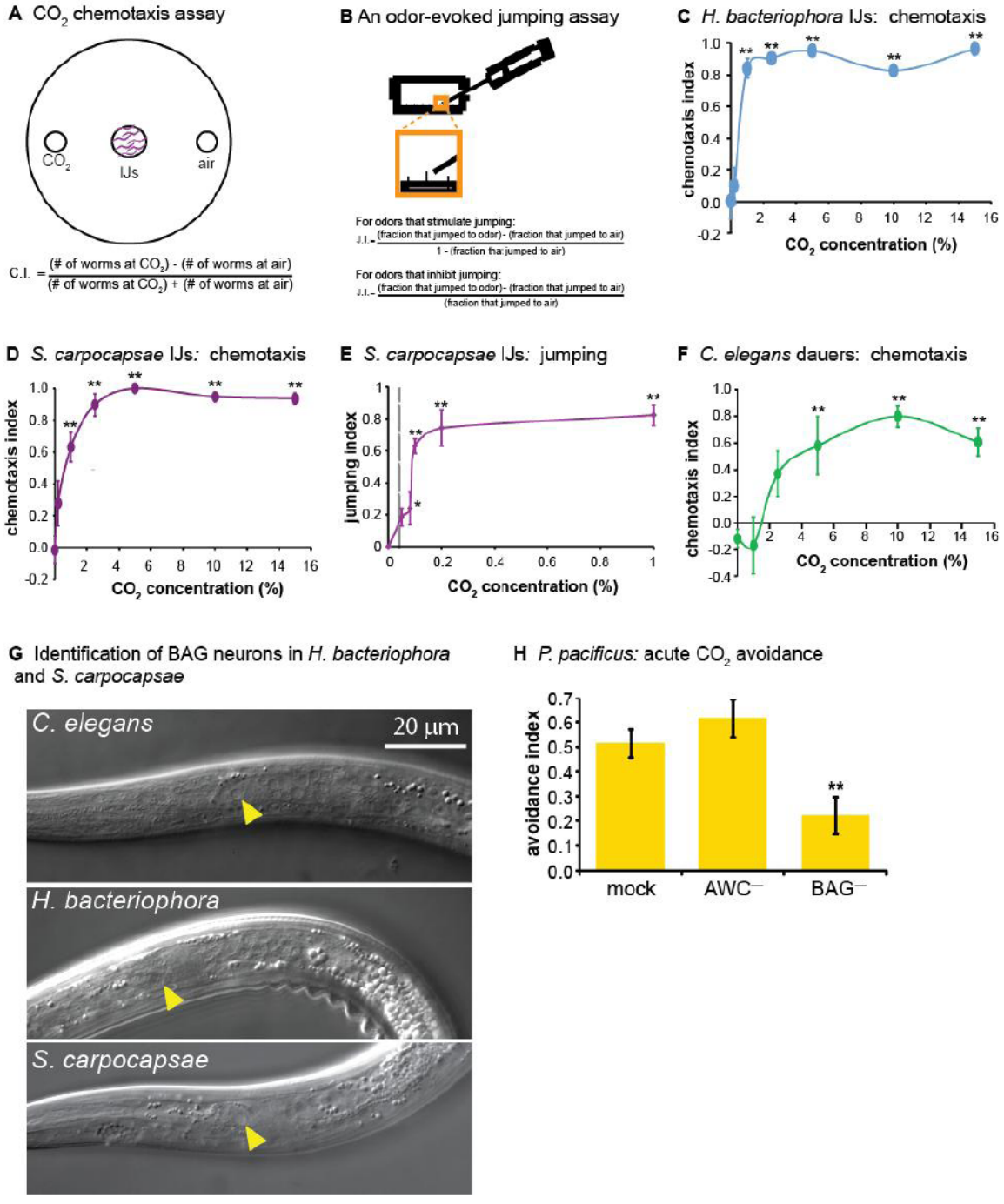
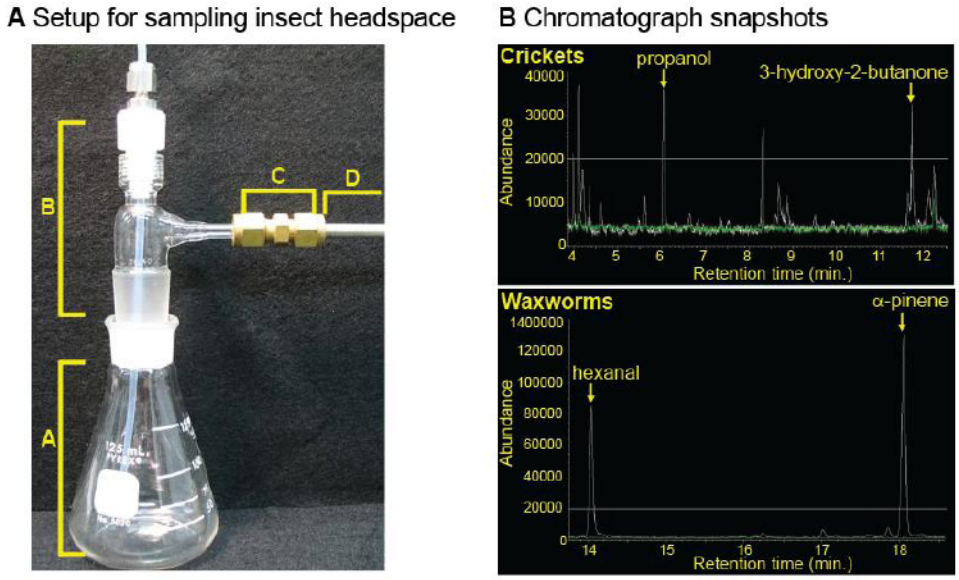
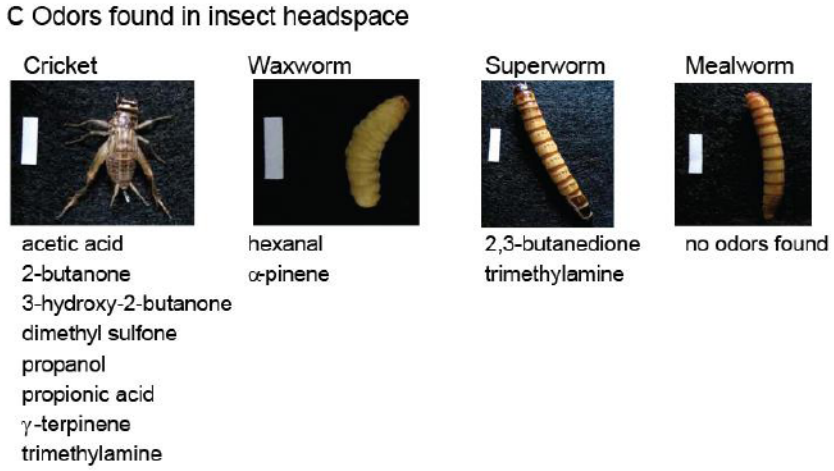


Figure 3.S2 | CO_2 response across species. A. The CO_2 chemotaxis assay. Nematodes are

placed in the center of the plate, and allowed to distribute in the CO_2 gradient. The number of worms in each scoring region is then counted. Inner boundaries of scoring regions are indicated by the horizontal lines. The chemotaxis index (C.I.) is calculated as indicated (bottom). **B.** The odor-evoked jumping assay. Individual or populations of nematodes are placed on a piece of filter paper inside a Petri dish. A non-beveled syringe with an attached needle is brought to within 2 mm of an individual nematode that is standing, and a small puff of stimulus is delivered. The percentage of animals that jump within 8 sec. is scored. A jumping index (J.I.) is then calculated as indicated (right) such that the J.I. is normalized to a scale of -1 to +1. The orange box shows an enlarged view of the standing IJ and stimulus syringe. **C–D.** *H. bacteriophora* and *S. carpocapsae* IJs are attracted to CO_2 across concentrations. n = 6–12 trials. **E.** CO_2 also stimulates jumping in *S. carpocapsae* IJs at concentrations as low as 0.08%. The atmospheric concentration of CO_2 (0.04%) is indicated by the dashed line. Saturation was achieved at 0.1% CO_2; CO_2 concentrations of 0.1% to 15% evoked similar levels of jumping (data not shown). The jumping index was calculated as described in Figure S2B. n = 3–7 trials; for each trial, ~ 60 individual IJs were tested. **F.** *C. elegans* dauers are attracted to CO_2. n = 5–10 trials. For **C–F**, *, $P < 0.05$; **, $P < 0.01$, one-way ANOVA with Dunnett's post-test. **G.** Identification of BAG neurons in *H. bacteriophora* and *S. carpocapsae.* Nomarski images of the left side of a *C. elegans* larva and parasitic IJs. Arrowheads indicate left BAG neurons. Anterior is to the left; dorsal is up. In *C. elegans* as well as the parasites, BAG neuron cell bodies are located laterally within the body just anterior to the nerve ring. **H.** BAG neurons are required for acute CO_2 avoidance in the necromenic nematode *Pristionchus pacificus*. The acute assay for CO_2 avoidance was performed as previously described [1]. The avoidance index was calculated as a.i. = (fraction of worms that reversed in response to CO_2)—(fraction of worms that reversed in response to air control). n = 17–19 worms for each treatment. **, $P < 0.01$, Fisher's exact test. For all graphs, error bars represent SEM. We note that for all experiments, assay chambers were open to the external

environment; thus the same ambient level of CO_2 (~ 0.04%) was present in all experiments.





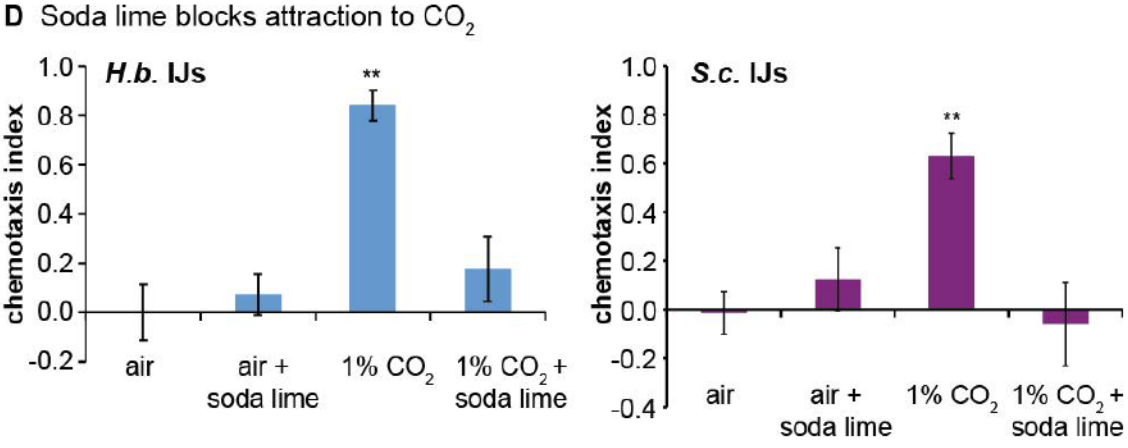
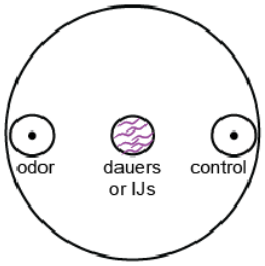


Figure 3.S3 | Identification of insect volatiles by TD-GC-MS. A. Little is known about

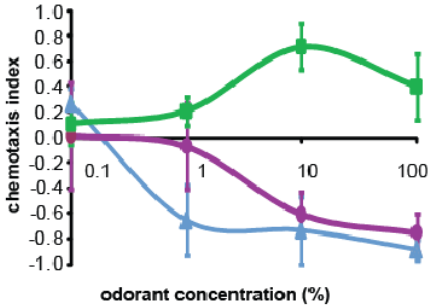
the odorants emitted by insect larvae. We therefore performed TD-GC-MS to identify odorants emitted by three species of insect larvae (*Galleria mellonella*, *Zophobas morio*, and *Tenebrio molitor*), as well as young adult crickets (*Acheta domesticus*). **A.** The unit used to sample insect headspace. (A) is a 125 ml glass Erlenmeyer flask. (B) is a hand-blown glass adaptor with a ground glass attachment fit into the flask, a Teflon top piece fit to accommodate a 1/8" O.D. Teflon tube for air flow, and a small side neck tapered to 1/4" O.D. (C) is a 1/4" female/female Swagelok compression fitting for the attachment of the thermal desorption tube to the flask, where air and any volatiles flow out of the set-up. (D) is the thermal desorption tube. For each species, six experimental replicates and three control replicates were obtained. The number of insects sampled in each run, and the average population weights (± SEM), are as follows: *A domesticus:* 50 insects, 8.64 g (± 0.15); *G. mellonella:* 100 insects, 28.89 g (± 2.79); *Z. morio:* 40 insects, 27.48 g (± 0.65); *T. molitor:* 50 insects, 17.0 g (± 0.61). Control samples did not contain insects. **B.** Representative snapshots of the ion chromatograph data acquired from cricket (upper trace) and waxworm (lower trace) headspace. White traces represent insect headspace samples and green traces represent controls. Compounds identified in multiple traces at relative abundances of $\geq$ 20,000, and that were not present in the controls at detectable levels, were then positively identified. Compounds meeting these criteria are indicated with yellow arrows. **C.** Compounds identified from the four insect species tested. Scale bars in insect photographs are 1 cm x 2.5 mm. **D.** A soda lime assay for examining the responses to host volatiles besides CO_2. The assay is a modified version of the host chemotaxis assay in which the airstream containing host volatiles is passed through a column of soda lime before entering the assay plate. As a control, here we show that for both *H. bacteriophora* IJs (left graph) and *S. carpocapsae* IJs (right graph), soda lime alone does not elicit a behavioral response and passing an airstream containing 1% CO_2 through a column of soda lime eliminates the attractive response to CO_2. Thus, a soda lime column can be used to chemically remove CO_2 from an airstream. n = 8–16 trials. **, $P <$ 0.01, one-way ANOVA with Dunnett's post-test.

A Chemotaxis assay

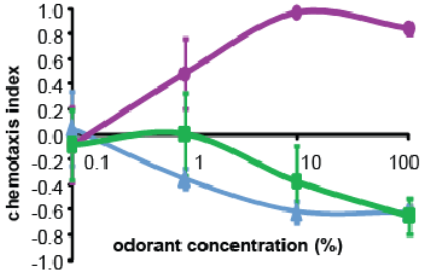


B Chemotaxis across species

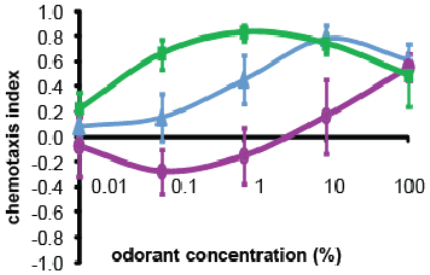
Responses to 2,3-butanedione



Responses to 1-heptanol



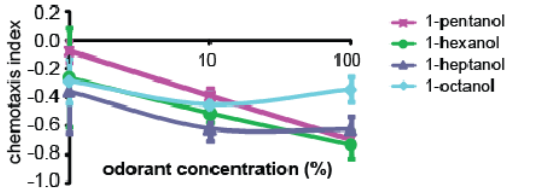
Responses to 4,5-dimethylthiazole



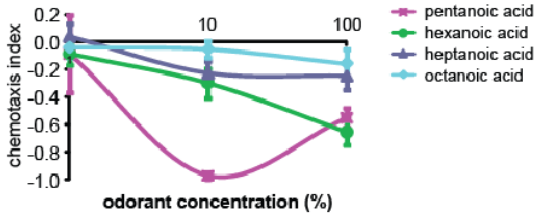
C. elegans dauers
H. bacteriophora IJs
S. carpocapsae IJs

C Chemotaxis by *H. bacteriophora* IJs

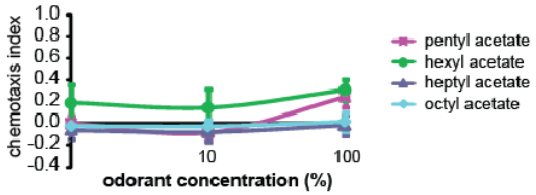
Response to alcohols



Response to acids

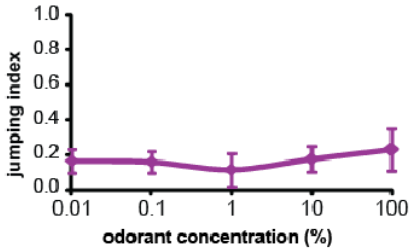


Response to acetates

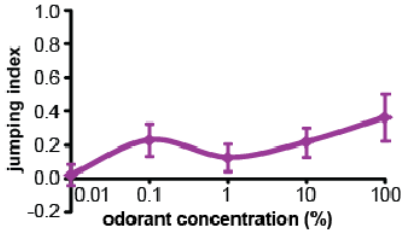


D Jumping by *S. carpocapsae* IJs

Response to 1-heptanol



Response to α-pinene



Response to 2,3-butanedione

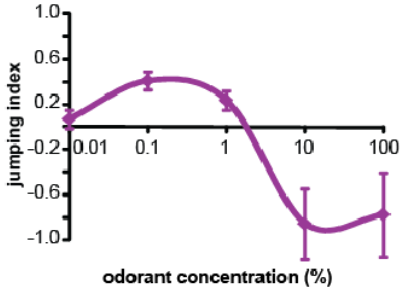


Figure 3.S4 | Dose-response analysis across species. A. A schematic of the chemotaxis assay. Nematodes are placed in the center of the plate, and allowed to distribute in the odor gradient. After three hours, the number of worms in each scoring region is counted. Scoring regions are indicated by the circles at either side of the plate. The chemotaxis index (C.I.) is then calculated as indicated (bottom). **B**. Responses to increasing concentrations of 2,3-butanedione, 1-heptanol, and 4,5-dimethylthiazole in a chemotaxis assay. n = 5–33 trials. **C.** Responses of *H. bacteriophora* to alcohols, acids, and acids. n = 5–33 trials. Error bars represent SEM. Responses to 1-heptanol are from **B**. **D.** Jumping responses of *S. carpocapsae* to increasing concentrations of 1-heptanol, a-pinene, and 2,3-butanedione in a jumping assay. n = 3–8 trials; for each trial, ~ 60 individual IJs were tested. For all graphs, error bars represent SEM.

Table 3.S1 | Odorants tested in chemotaxis and jumping assays. Odorants were selected based on their chemical diversity and ecological relevance. All of the odorants tested are present either in plants or bacteria [2–5], and many have been shown to elicit responses from free-living adult nematodes [6–9]. Eleven of the odorants were identified in insect headspace (Figure 2.S7). In addition, hexadecanoic and octadecanoic acid have been identified in larval cuticular extracts from insect larvae [10]; acetic, propionic, and pentanoic acids have been identified in cricket excreta [11]; benzaldehyde and linalool have been identified in butterfly wing extracts [12]; and nonanal, undecanal, and nonanol are emitted by adult wax moths [13, 14]. Product numbers are from Sigma-Aldrich, except for ethanol (Pharmco-AAPER), ethyl acetate (Mallinckrodt chemicals), and acetic acid (J.T. Baker).

	C. elegans			*H. bacteriophora*			*S. carpocapsae*					
	C.I.	**n**	**SEM**	**C.I.**	**n**	**SEM**	**C.I.**	**n**	**SEM**	**J.I.**	**n**	**SEM**
ethanol	0.33	5	0.21	-0.22	5	0.12	-0.06	5	0.09	-1.00	3	0.30
1-propanol	0.51	5	0.22	0.36	5	0.13	-0.28	5	0.21	-1.00	3	0.30
1-pentanol	-0.24	7	0.27	-0.66	15	0.06	0.39	5	0.10	-0.92	4	0.31
1-hexanol	-0.61	7	0.27	-0.73	22	0.04	0.83	6	0.08	-0.50	4	0.42
1-heptanol	-0.65	10	0.14	-0.62	33	0.05	0.84	8	0.04	0.16	8	0.10
1-octanol	-0.88	5	0.08	-0.34	15	0.07	0.48	5	0.14	0.24	9	0.12
1-nonanol	-0.79	5	0.11	-0.62	5	0.11	0.53	6	0.08	0.24	8	0.61
isopropyl alcohol	0.18	5	0.29	-0.39	5	0.22	-0.03	5	0.22	-0.68	3	0.43
isoamyl alcohol	0.29	7	0.24	-0.21	5	0.15	-0.05	5	0.05	0.06	6	0.09
linalool	0.38	5	0.14	-0.05	6	0.23	-0.09	5	0.28	0.12	6	0.10
methyl acetate	0.64	5	0.15	-0.38	5	0.09	0.11	5	0.11	-0.71	3	0.34
ethyl acetate	0.36	5	0.23	-0.16	15	0.10	0.26	5	0.13	-0.89	3	0.31
pentyl acetate	-1.00	5	0.00	0.26	13	0.09	-0.09	6	0.10	-1.00	3	0.30
hexyl acetate	-0.70	5	0.29	0.31	5	0.10	0.08	6	0.09	-0.01	8	0.64
heptyl acetate	-0.80	5	0.20	-0.02	14	0.09	0.33	5	0.17	0.08	8	0.10
octyl acetate	-0.37	6	0.31	0.01	14	0.09	0.49	5	0.14	0.37	8	0.14
nonyl acetate	-0.01	5	0.16	-0.25	5	0.15	0.15	5	0.04	0.24	10	0.12
decyl acetate	-0.40	5	0.19	0.10	5	0.20	0.08	5	0.09	-0.89	3	0.31
dodecyl acetate	0.19	5	0.17	0.45	5	0.10	-0.02	6	0.09	0.22	8	0.11
2-butanone	0.83	5	0.11	0.02	6	0.13	-0.18	5	0.24	-0.66	3	2.39
2-pentanone	0.67	5	0.13	-0.29	5	0.13	-0.39	5	0.07	-0.46	5	0.46
2-hexanone	-0.50	5	0.22	-0.36	7	0.15	0.11	5	0.09	-0.89	3	0.31
2-heptanone	-0.99	5	0.01	0.19	5	0.23	0.18	5	0.24	-0.68	3	0.35
2-octanone	-1.00	5	0.00	0.11	5	0.15	0.30	5	0.24	-0.68	3	0.43
2-nonanone	-0.95	5	0.04	0.05	5	0.19	0.52	5	0.12	-0.89	3	0.31
2,3-butanedione	0.40	5	0.26	-0.89	5	0.08	-0.75	5	0.15	-0.89	6	0.31
3-hydroxy-2-butanone	0.25	5	0.12	-0.23	5	0.06	-0.17	5	0.05	-0.74	3	0.33
dimethyl sulfone	0.02	5	0.25	0.00	6	0.19	-0.18	5	0.06	0.16	3	0.09
acetic acid	-1.00	6	0.00	-0.66	8	0.23	-0.98	5	0.02	-0.73	3	0.33
propionic acid	-1.00	5	0.00	-1.00	5	0.00	-1.00	5	0.00	0.43	3	0.06
pentanoic acid	-1.00	5	0.00	-0.55	7	0.07	-0.80	5	0.20	0.46	9	0.10
hexanoic acid	-1.00	5	0.00	-0.66	14	0.09	-0.43	7	0.18	0.42	8	0.11
heptanoic acid	-0.90	5	0.10	-0.25	17	0.10	-0.46	5	0.13	0.07	8	0.09
octanoic acid	-0.78	5	0.13	-0.16	14	0.10	-0.53	5	0.19	-0.47	6	0.34
nonanoic acid	-0.68	5	0.19	-0.33	5	0.20	-0.17	5	0.11	0.21	8	0.10
hexadecanoic acid	0.12	5	0.14	-0.06	7	0.24	-0.15	5	0.24	-1.00	3	0.30
octadecanoic acid	0.36	5	0.15	-0.16	5	0.11	-0.02	5	0.20	-1.00	3	0.30
hexanal	-0.70	5	0.14	-0.75	7	0.20	-0.31	7	0.20	0.30	10	0.10
nonanal	-0.45	6	0.06	-0.01	5	0.24	0.09	5	0.10	0.37	9	0.11
undecanal	-0.71	5	0.19	0.24	5	0.16	-0.09	5	0.17	0.01	4	0.06
2-acetylthiazole	0.03	5	0.34	-0.73	8	0.04	-0.15	5	0.25	-0.79	3	0.31
benzothiazole	0.71	5	0.12	-0.06	8	0.05	-0.49	5	0.18	-0.40	6	0.37
2-isobutylthiazole	-0.76	5	0.12	-0.04	8	0.09	-0.04	5	0.29	0.07	8	0.09
4,5-dimethylthiazole	0.49	7	0.25	0.61	6	0.12	0.55	5	0.11	-1.00	6	0.30
benzaldehyde	-0.75	5	0.14	-0.83	5	0.08	0.07	5	0.11	-0.53	5	0.43
methyl salicylate	-0.93	5	0.03	0.86	5	0.02	0.07	5	0.17	-0.79	3	0.36
m-xylene	-0.04	5	0.22	-0.45	5	0.21	-0.05	5	0.03	0.07	6	0.11
m-cymene	-0.04	5	0.17	0.19	7	0.23	-0.10	5	0.17	0.03	5	0.06
p-cymene	-0.30	5	0.20	0.41	5	0.22	-0.10	5	0.07	0.02	8	0.09
β-caryophyllene	0.27	5	0.24	0.06	10	0.07	-0.40	5	0.13	-0.89	3	0.31
α-humulene	0.45	7	0.27	0.00	5	0.25	0.30	5	0.20	-0.04	3	0.30
3-carene	0.48	5	0.25	-0.70	5	0.07	-0.13	6	0.05	-0.89	3	0.31
α-pinene	0.57	5	0.17	-0.63	5	0.12	0.05	5	0.21	0.36	8	0.14
β-pinene	0.30	5	0.17	-0.17	5	0.11	-0.17	5	0.25	0.23	6	0.10
limonene	0.16	5	0.14	-0.64	5	0.12	-0.35	5	0.07	-0.50	5	0.30
γ-terpinene	0.13	7	0.24	-0.02	5	0.14	-0.12	5	0.12	0.26	8	0.10
trimethylamine	0.75	5	0.08	-0.28	5	0.14	0.12	7	0.25	-0.03	3	0.36

Table 3.S1 | Mean values for the chemotaxis index (C.I.) and jumping index (J.I.) of each species in response to each tested stimulus. The number of trials (n) is shown. SEM, standard error of the mean. Odorants were selected based on their chemical diversity and ecological relevance. All of the odorants tested are present either in plants or bacteria [2–5], and many have been shown to elicit responses from free-living adult nematodes [6–9]. Eleven of the odorants were identified in insect headspace (Figure 5). In addition, hexadecanoic and octadecanoic acid have been identified in larval cuticular extracts from insect larvae [10]; acetic, propionic, and pentanoic acids have been identified in cricket excreta [11]; benzaldehyde and linalool have been identified in butterfly wing extracts [12]; and nonanal, undecanal, and nonanol are emitted by adult wax moths [13, 14]. Product numbers are from Sigma-Aldrich, except for ethanol (Pharmco-AAPER), ethyl acetate (Mallinckrodt chemicals), and acetic acid (J.T. Baker).

Supplemental Methods

Nematodes. *H. bacteriophora* were from the inbred strain M31e [15, 16], *S. carpocapsae* were from the inbred strain ALL [17], and *C. elegans* were from the standard N2 ("Bristol") strain, unless otherwise indicated. Other *H. bacteriophora* strains tested were HP88 [18], GPS11 [19], NC1 [20], and a strain we designate as "BU" that was derived from commercially available nematodes that were originally obtained from Becker-Underwood (http://www.beckerunderwood.com). The other *S. carpocapsae* strain tested was an inbred wild isolate that we designate as Base [17]. Other *C. elegans* strains tested were the wild isolate CB4856 ("Hawaii") and CX11697 [*kyIs536[flp-17::p17::s12GFP, elt-2::mCherry]; kyIs538[glb-5::p12::s12GFP, elt-2::mCherry]*], which contains a genetic ablation of the BAG neurons. *P. pacificus* were from the PS312 ("California") strain.

Nematode culture. *H. bacteriophora* was cultured at 27°C on either nutrient agar + cholesterol plates (23 g nutrient agar + 1 ml of 5 mg/ml cholesterol in 1 L) or lipid agar +

cholesterol plates [21] seeded with either TT01 or RET16 bacteria. RET16 is a GFP-labeled derivative of *P. temperata* strain NC1 mutated with HiMarGM (a hyperactive mariner transposon with gentamicin resistance) [15]. *Photorhabdus* was grown in PP3 broth (20 g proteose peptone #3 (Difco) in 1 L dH_20) and on either nutrient agar + cholesterol plates or lipid agar + cholesterol plates. IJs were stored in 0.85% NaCl (w/v) or dH_20 at room temperature or 15°C prior to use. Prior to behavioral testing, IJs were washed in dH_20. For Figure 1A, *H. bacteriophora* was cultured on plates seeded with GFP-labeled *P. luminescens,* as previously described [16].

S. carpocapsae was cultured as previously described [22]. Briefly, 5 last-instar *Galleria mellonella* larvae (American Cricket Ranch, Lakeside, CA) were placed in a 5 cm Petri dish with a 55 mm Whatman 1 filter paper acting as a pseudo-soil substrate in the bottom of the dish. ≤ 250 μl containing 500–1000 IJs suspended in water was evenly distributed on the filter paper. After 7–10 days the insect cadavers were placed on White traps [23]. Emerging IJs were harvested, washed for 10 minutes in 0.4% Hyamine 1622 solution (Fluka), and rinsed 3 times with water. To prevent differences in inbreeding between batches of IJs, the same stock population of IJs was used to generate all test batches of IJs. Stock populations were stored at 15°C and propagated in *G. mellonella* every 10 days to produce fresh test batches of IJs. Test batches were stored at room temperature and used in behavioral assays within 12 days of emergence. In some cases, *S. carpocapsae* used for chemotaxis assays were cultured at 27°C on nutrient agar + cholesterol or lipid agar + cholesterol plates as described above, except that plates were seeded with *X. nematophila* strain HGB081 [24]. For Figure 1B, *S. carpocapsae* were cultured on plates seeded with GFP-labeled *X. nematophila,* as previously described [25]. *X. nematophila* was grown in LB broth containing 0.1% sodium pyruvate, and on either nutrient agar + cholesterol or lipid agar + cholesterol plates.

C. elegans was cultured on NGM plates seeded with *E. coli* OP50 according to standard methods [26]. *C. elegans* dauers were grown primarily in liquid culture, although in some cases dauers were collected from the lids of starved plates. For dauers grown in liquid culture, embryos were collected as previously described and diluted to 10 eggs/ml in

S complete media [27]. *E. coli* HB101 bacteria was added at a final concentration of 0.5 mg/ml and worms were grown on a carousel at 20°C for 6 days to generate dauers (L.R. Baugh and P.W. Sternberg, unpublished). If necessary, the bacterial concentration was adjusted to generate populations of nearly 100% dauers. Dauers were stored in dH_2O at 15°C prior to use. For CO_2 assays, dauers were SDS-treated [28]; SDS treatment did not affect CO_2 response. For all other assays, dauers were not treated with SDS; in these cases, a small population sample was treated with SDS, and worms were only used for behavioral assays if nearly all of the sample population survived SDS treatment.

P. pacificus was grown on NGM plates seeded with *E. coli* OP50 bacteria at room temperature.

Phylogenetic analysis. Small subunit ribosomal DNA (SSU rDNA) sequences for all analyses were obtained from GenBank for all taxa included in the present study (accession numbers: AJ920356, AJ920348, AJ417024, EU086375, AF036593, AY268117, U81584, AF083007, AF279916, AF036604, AY284620, AY284621, AY284671, U94367, AF036588, U61761, AF036600, U60231, EU344798, X87984, and AF036639). Most of these sequences have been used in previous phylogenetic analyses [29, 30]. The SSU sequences for *C. elegans*, *S. carpocapsae*, *H. bacteriophora*, and *C. morgani* (a nematomorph) were used for the neighbor-joining (NJ) tree in Figure 4C. The sequences were first trimmed to 1783 characters and then aligned using MUSCLE [31]. The subsequent NJ analysis was done using the 'Dnadist' and 'Neighbor' programs from the PHYLIP 3.68 package [32] using default settings with *C. morgani* defined as the outgroup. A total of 19 nematode species and 2 outgroup taxa (a priapulid and a nematomorph) were used in the analyses for Figure S1. In order to facilitate comparison of the SSU sequences of varying lengths, the ends were trimmed by hand, prior to alignment, in MacClade 4 [33] to a maximum length of 1152 characters, which is the length of the taxon with the shortest sequence, *Parastrongyloides trichosuri*. Sequences were then aligned using MUSCLE [31], resulting in 1313 characters (including gaps). The TIM2+I+G model was selected as the best-fit model of substitution for all analyses using the AIC and AICc model selection criteria in the program jModelTest [34, 35].

Maximum likelihood and bootstrap (1000 replicates) analyses were carried out in PhyML 3.0 [35] using the parameters for base frequencies, substitution rate matrix, proportion of invariable sites, number of substitution categories, and shape distribution parameter determined as the best-fit by jModelTest (freqA = 0.2684, freqC = 0.1835, freqG = 0.2501, freqT = 0.2981, Ra(AC) = 1.6751, Rb(AG) = 2.5642, Rc(AT) = 1.6751, Rd(CG) = 1.0000, Re(CT) = 4.5613, Rf(GT) = 1.0000, p-inv = 0.1710, and gamma shape = 0.5840). Bayesian analysis was carried out using MrBayes 3.1.2 [36]. The number of substitution categories, substitution rate matrix, shape and proportion of invariant sites were based on the parameters determined by jModelTest (as above). The parameters for base frequencies and relative rates were allowed to vary throughout the analysis. The parameters were unlinked to allow for more flexibility in searching tree space. Trees were sampled every 1000 generations. The burn-in value was set to 2000 trees. The total number of generations was set to 8 million. Four parallel chains (one cold and three heated) were used. A majority-rule consensus tree was reconstructed after discarding the burn-in.

Thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS). Appropriately staged insects (adult *Acheta domesticus* and last-instar larvae of *Zophobas morio, Galleria mellonella,* and *Tenebrio molitor*) were placed in a 125 ml glass beaker and sampled for 30 minutes with a stream of air (10% oxygen, 90% nitrogen) flowing into the flask and out through a thermal desorption tube (Sigma-Aldrich 20913-U) at a flow rate of approximately 104 ml/min. Experiments were done in pairs and replicated 3 times, with an empty control flask being run each time. To prevent carry-over of odors between experiments, all tubing used was Nalgene Teflon tubing, connected with Swagelok compression fittings, and flasks were cleaned and sterilized after each use.

The contents of the thermal desorption tubes were transferred to a HP 6890 GC–5973 MS system (Agilent Technologies, US) with an Eclipse 4660 purge and trap sampler equipped with an airtube desorber accessory (OI Analytical, College Station, TX, US.). Tubes were desorbed at 200°C for 15 minutes and transferred via a flow of helium to an internal trap held at room temperature. After desorption, the internal trap was heated

to 200°C. This trap was brought in line with the GC carrier gas flow as the trap reached 180°C. The trap was then taken offline and subjected to a bake-out procedure. The sample flowed to a GC via a transfer line held at 120°C where it entered a split-splitless injector held at 200°C. The injector was operated in split mode with a split ratio of 30:1, and a 1 mm liner was installed to optimize chromatographic resolution. Separation was achieved with a HP-624 capillary column (30 m x 0.320 mm) where a volumetric flow of 1 ml/min was maintained with electronic pressure control. The transfer line to the mass spectrometer was held at 200°C, the ion source at 250°C and the quadrupole at 100°C. The mass spectrometer is equipped with an electron impact source. Electron energy was set to 70 eV to obtain the best possible library spectrum matches. The quadrupole mass spectrometer was operated with a full width at half maximum of 0.65 m/z. Mass calibration was verified weekly. The GC oven was ramped from 30°C to 260°C and run for 42 minutes. Data was analyzed with both Chemstation and Masshunter software. Mass spectra were searched against the Wiley library (275,000 spectra) of electron impact mass spectra. Only compounds that were found in multiple traces (≥ 2), with a relative abundance ≥ 20,000, and not present in the control traces were considered in this study. Compounds identified in this way were then positively confirmed by running the pure compound (Table S1) and comparing the retention time and mass spectra of the assay-identified compound to the known compound. In cases where the retention time was off by ≥ 0.5 minutes or the mass spectra did not match, the assay-identified compound was considered uncertain and not used in behavioral assays. All insects tested were obtained from commercial sources (American Cricket Ranch, Lakeside CA).

Data Analysis

Statistical analysis was performed using GraphPad InStat. Heat maps and dendrograms were generated using PAST [37].

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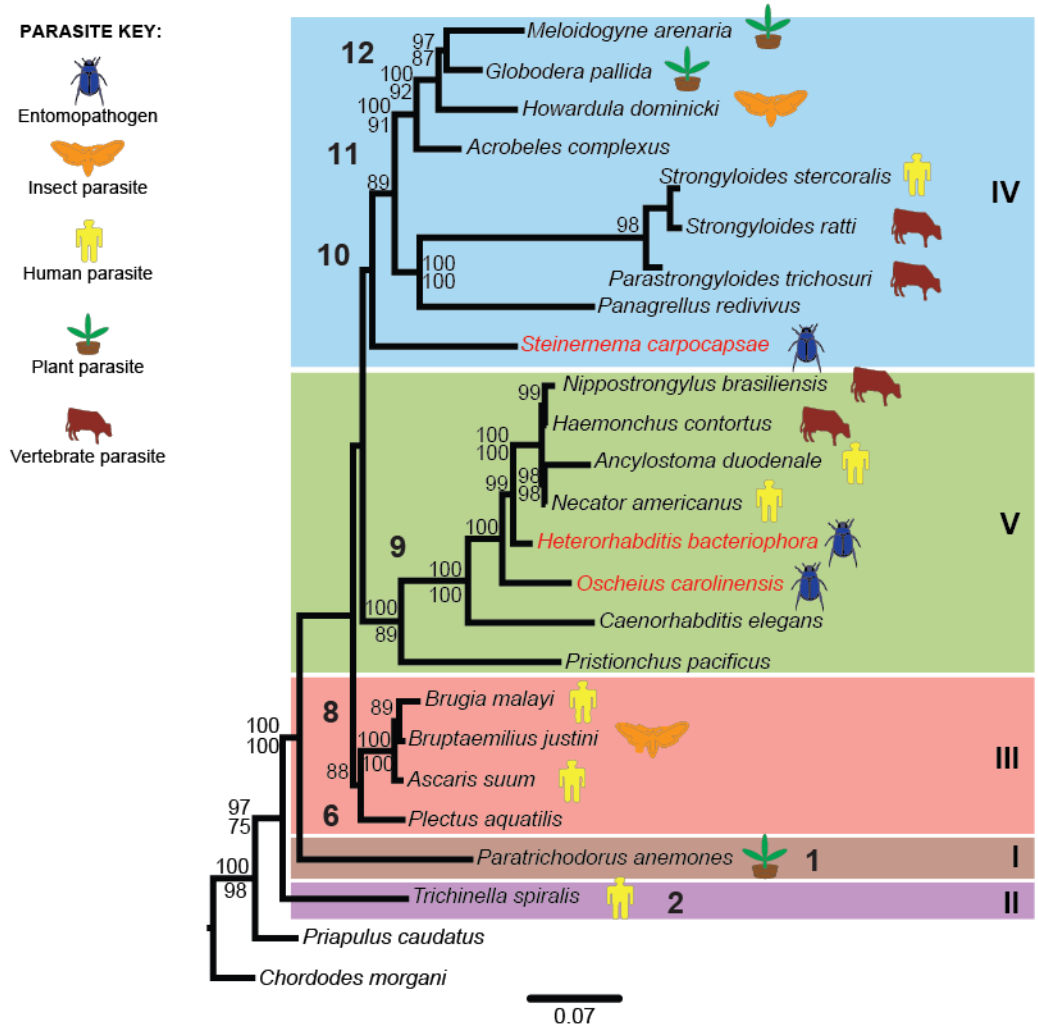
Appendix B: Supplementary Materials for Chapter 4*

*This appendix is available as supplementary material for the published manuscript in *PNAS* in 2012.

A Phylogeny of selected nematode species



B Phylogeny of selected *Steinernema* species

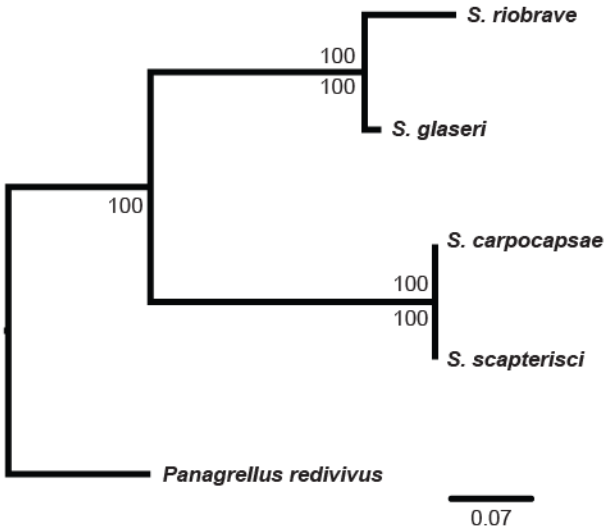


Figure 4.S1 | Phylogeny of selected nematodes. A. Phylogenetic relationships among free-living and parasitic nematodes. Relationships are based on maximum likelihood and Bayesian analyses of nearly complete SSU sequences. Values above each branch represent Bayesian posterior probabilities; ML bootstrap indices appear below each branch. Values lower than 75 are not reported. Both analyses produced concordant tree topologies. Nematode clades (1–12) are after Holterman *et al.,* 2006 [1] while clades after Blaxter *et al.,* 1998 [2] are indicated with roman numerals and colored boxes. For parasitic species, host ranges are indicated by colored icons. *Priapulus* (a priapulid) and *Chordodes* (a nematomorph) were defined as outgroups. **B.** Phylogeny of selected *Steinernema* species. Relationships are based on ML and Bayesian analysis of the large subunit ribosomal DNA. Values above each branch represent Bayesian posterior probabilities; ML bootstrap indices appear below each branch. Values lower than 75 are not reported. Both analyses produced concordant tree topologies. The tree was rooted with the free-living nematode *Panagrellus redivivus* as the outgroup species. **C.** EPNs tested. Photomicrographs of the different EPN infective juveniles (IJs), with their host-seeking strategies and host ranges.

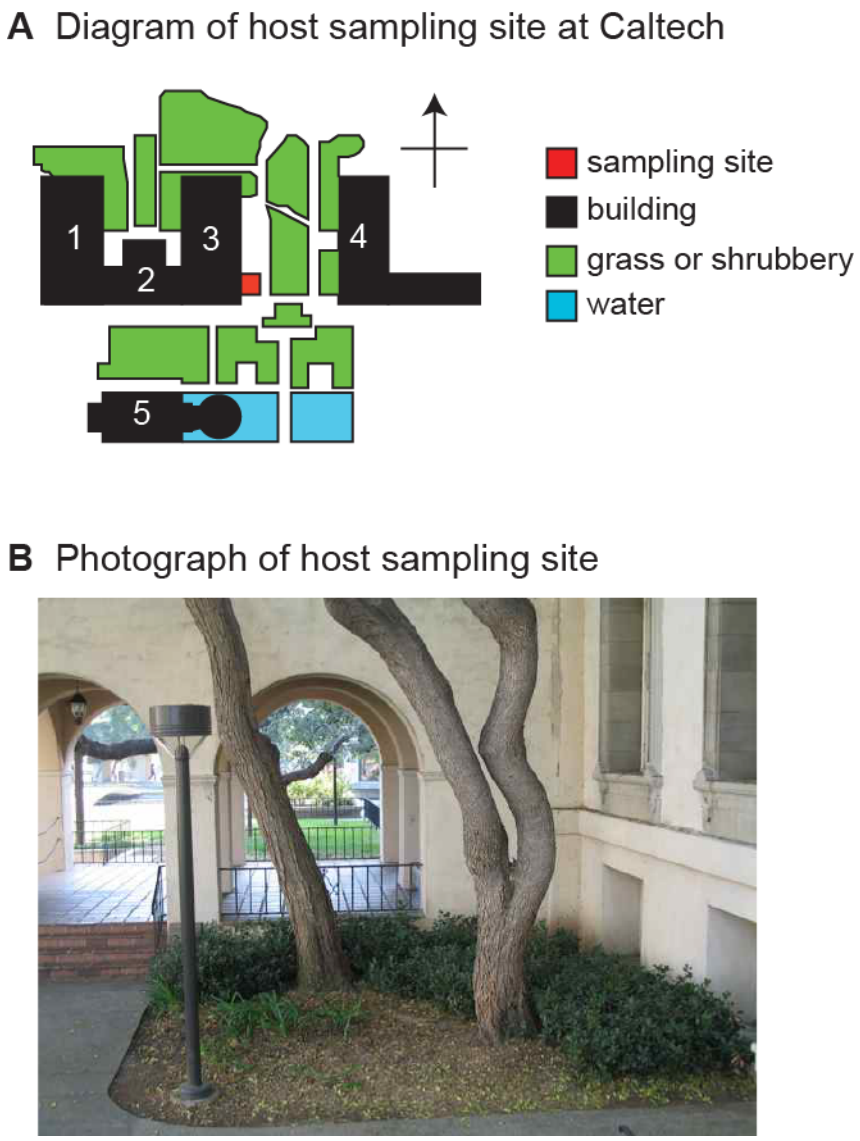


Figure 4.S2 | Sampling site from which the majority of potential hosts were collected. A. Diagram of the sampling site at Caltech. **B.** Photograph of the same sampling site. The sampling site is the small, shady grass plot visible in the foreground. Earwigs, pillbugs, and slugs were collected from the upper layers of moist soil in the vicinity of a leaky sprinkler. Flatheaded borers were collected from inside the wood of nearby rose bushes.

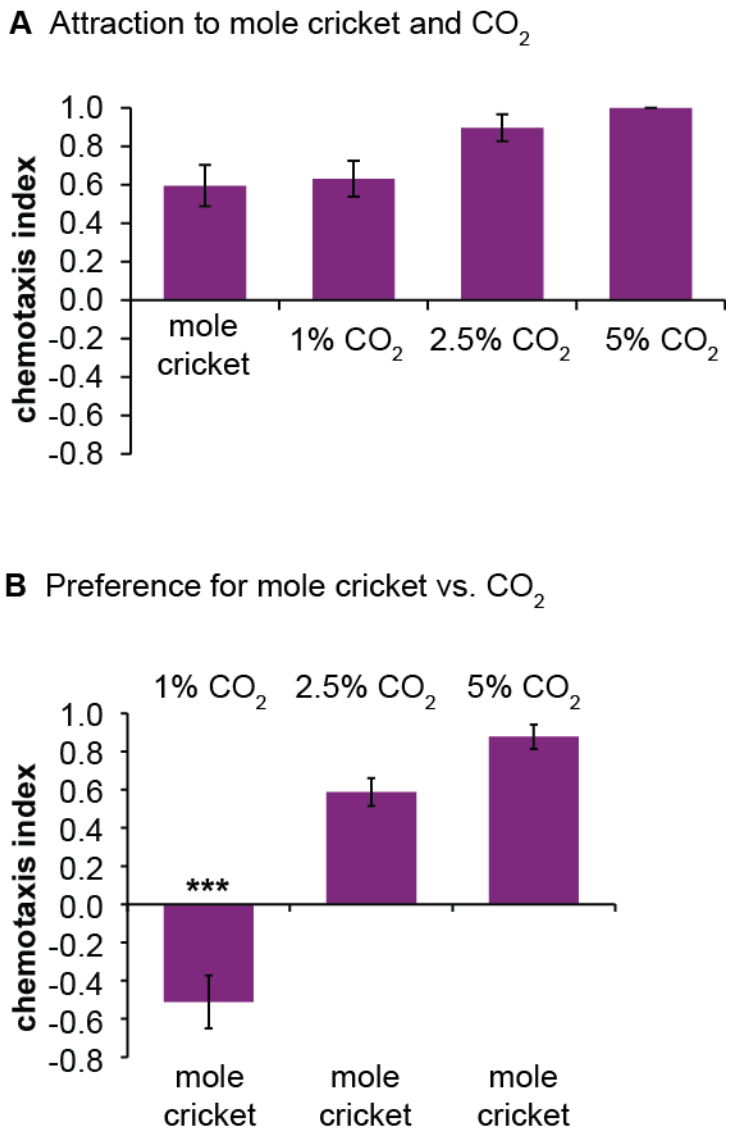


Figure 4.S3 | Preference of *S. carpocapsae* IJs for mole cricket odor vs. CO_2. A. Responses of S. carpocapsae IJs to volatiles from an individual mole cricket and to different concentrations of CO_2 in a chemotaxis assay. Data are from Figs. 4.1B and 4.3A. **B.** Responses of S. carpocapsae IJs to volatiles from an individual mole cricket vs. different concentrations of CO_2 in a competition chemotaxis assay. A positive C.I. indicates attraction to CO_2; a negative C.I. indicates attraction to mole cricket odor. n = 3–9 trials for each condition. The response to mole cricket odor when tested against an air control was not significantly different from the response to mole cricket odor when tested against 1% CO_2 (unpaired t test). The response to 1% CO_2 when tested against an air control was significantly different from the response to 1% CO_2 when tested against mole cricket odor (***, $P < 0.001$); however, responses to 2.5% CO_2 and 5%

CO_2 when tested against an air control vs. mole cricket odor were not significantly different (two-factor repeated measures ANOVA).

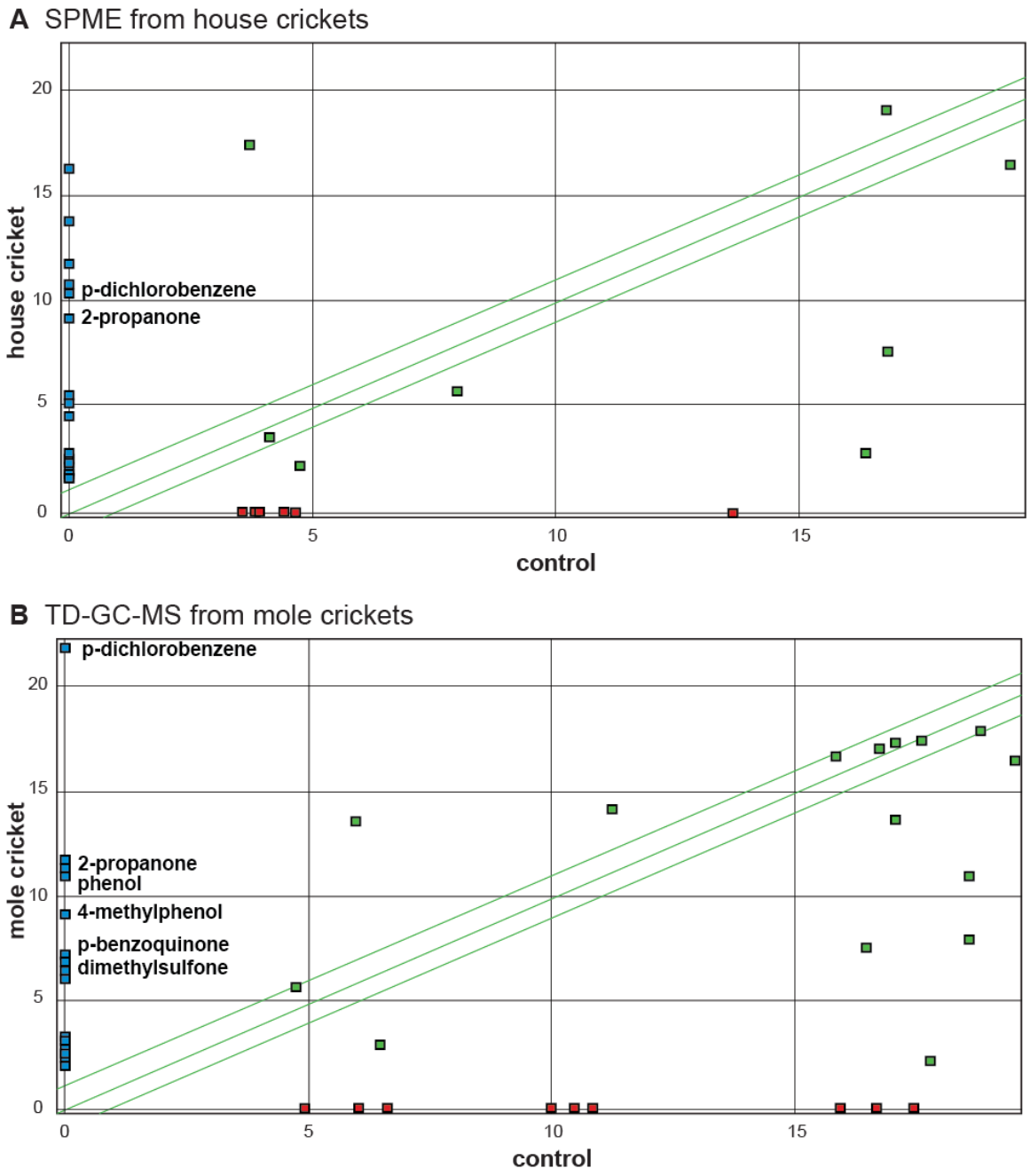


Figure 4.S4 | Identification of host-derived odorants by GC-MS. A. A representative trace showing SPME-GC-MS data obtained from live house crickets. **B.** A representative trace showing TD-GC-MS data obtained from live mole crickets. For both graphs, the x-axis indicates the relative abundance in control air, and the y-axis indicates relative abundance in host air. Odorants found

exclusively in host air are indicated in blue, odorants found exclusively in control air are indicated in red, and odorants found in both host air and control air are indicated in green. Of the odorants found exclusively in host air, only those identified in multiple experimental replicates at a relative abundance of ≥ 20,000 and with a library match of ≥ 95% confidence are labeled, with the exception of p-dichlorobenzene from house crickets, which was identified with a library match of ~ 90%.

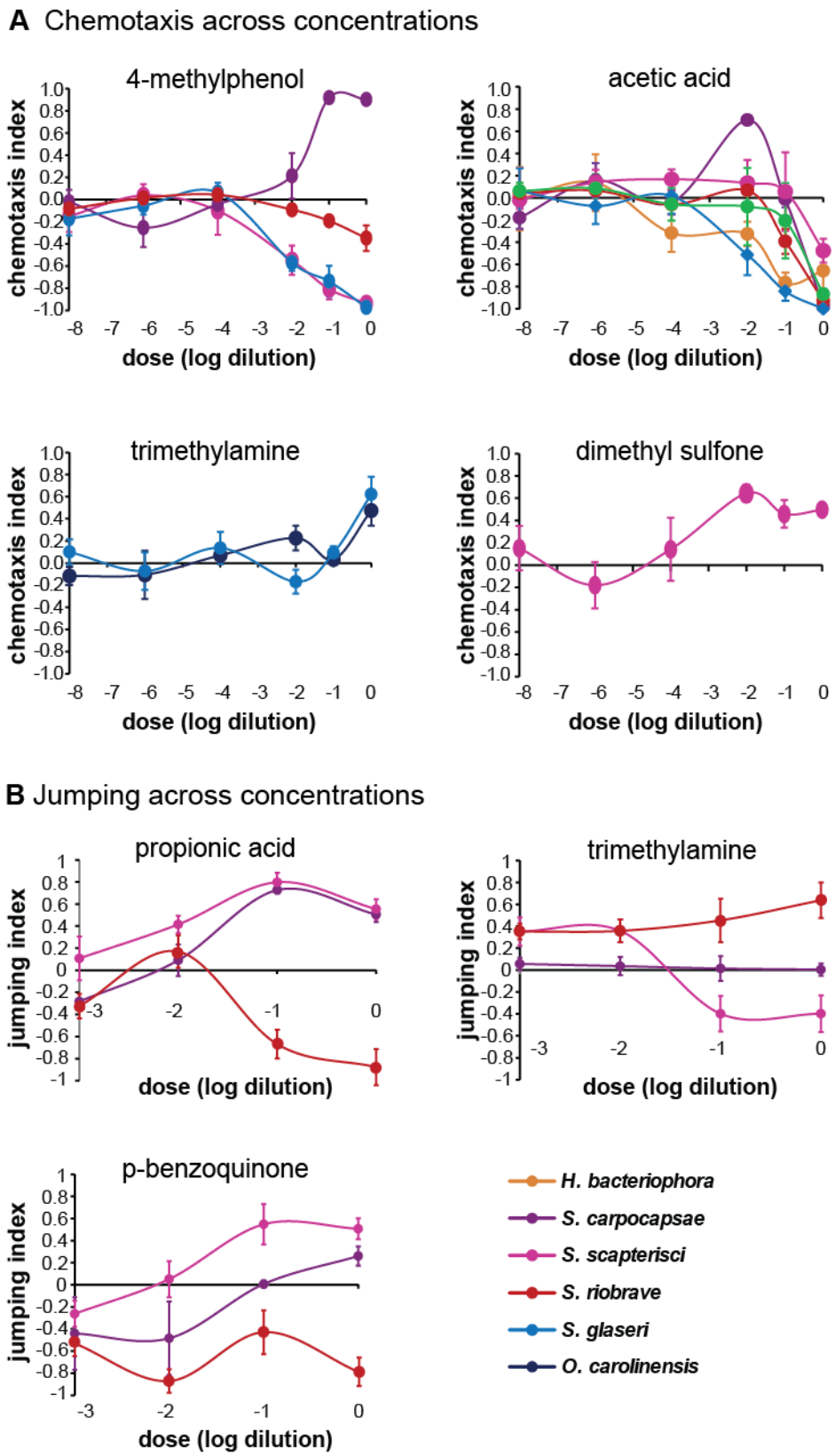


Figure 4.S5 | Dose-response analysis for selected host-derived odorants. A. Chemotaxis

behavior across concentrations. n = 4–8 trials for each EPN-odorant combination. **B.** Jumping behavior across concentrations. n = 2 trials for each EPN-odorant combination.

Materials and Methods

Nematodes. *H. bacteriophora* were from the inbred strain M31e [3–5]. *S. carpocapsae* were from the inbred strain ALL [4, 6, 7]. *C. elegans* were the wild isolate CB4856 ("Hawaii"). *O. carolinensis* were the YEW strain [8]. *S. glaseri* were from the inbred NC strain [9]. *S. scapterisci* were inbred from the FL strain [10]. *S. riobrave* were inbred from the TX strain [11].

Nematode culturing. All nematodes were cultured as previously described [4]. Briefly, 5 last instar *Galleria mellonella* larvae (American Cricket Ranch, Lakeside, CA) were placed in a 5 cm Petri dish with a 55 mm Whatman 1 filter paper acting as a pseudo-soil substrate in the bottom of the dish. ≤ 250 ml containing 500–1000 IJs suspended in water was evenly distributed on the filter paper. After 7–10 days the insect cadavers were placed on White traps [12]. *Steinernema glaseri* was placed onto a modified White trap containing plaster of Paris as previously described [13]. Emerging IJs were harvested and rinsed 3 times with water. *S. scapterisci* was also cultured by infecting house crickets and mole crickets using similar techniques. IJs were stored at either room temperature or 15°C and tested within 2 months of emergence. *C. elegans* was cultured on NGM plates seeded with *E. coli* OP50 according to standard methods [14], and dauer larvae were collected from the lids of plates from which the nematodes had exhausted their bacterial food supply (i.e., "starved plates").

Nematode phylogeny. Small subunit ribosomal DNA (SSU rDNA) sequences for the large phylogenetic analysis were obtained from GenBank for all taxa included in the present study (accession numbers: AJ920356, EU086375, AF036593, AY268117, U81584, AF083007,

AF279916, AF036604, AY284620, AY284621, AY284671, U94367, AF036588, U61761, AF036600, U60231, EU344798, X87984, AF036589, AF519234, AJ920348, FJ547240, AJ417024, U81581, and AF036639). A total of 23 nematode species and 2 outgroup taxa (a priapulid and a nematomorph) were used in the analyses for Figure 4.S1A. Sequences were aligned using ProAlign [15] with 1500 Mb of memory allotted, bandwidth set to 1500 with HMM model parameters being estimated from the data. We excluded characters aligned with posterior probability values under 60%, resulting in 1330 aligned characters for subsequent analysis. The TIM2+I+G model was selected as the best-fit model of substitution for all analyses using the AIC and BIC model selection criteria in the program jModelTest [16, 17]. Maximum likelihood and bootstrap (1000 replicates) analyses were carried out in PhyML 3.0 [18] using the parameters for base frequencies, substitution rate matrix, proportion of invariable sites, number of substitution categories, and shape distribution parameter determined as the best-fit by jModelTest (freqA = 0.2618, freqC = 0.1850, freqG = 0.2443, freqT = 0.3089, Ra(AC) = 1.4966, Rb(AG) = 2.4339, Rc(AT) = 1.4966, Rd(CG) = 1.0000, Re(CT) = 3.7721, Rf(GT) = 1.0000, p-inv = 0.1150, and gamma shape = 0.5290). Bayesian analysis was carried out using MrBayes 3.1.2 [19]. The number of substitution categories and shape was based on the parameters determined by jModelTest (as above). The parameters for base frequencies, relative rates, substitution rate matrix, and proportion of invariant sites were allowed to vary throughout the analysis. The parameters (shape, statefreq, and revmat) were unlinked to allow for more flexibility in searching tree space. Trees were sampled every 1000 generations. The burn-in value was set to 2000 trees. The total number of generations was set to 8 million. Four parallel chains (one cold and three heated) were used. A majority-rule consensus tree was reconstructed after discarding the burn-in.

For the four *Steinernema* species phylogeny (Figure 4.S1B), large subunit ribosomal DNA (LSU rDNA) sequences were obtained from GenBank (AF331908, AF331898, AF331893, AF331900,

and DQ145647). Sequences were aligned using ProAlign [15] with 1050 Mb of memory allotted, bandwidth set to 1000 with HMM model parameters being estimated from the data. We excluded characters aligned with posterior probability values under 60%, resulting in 883 aligned characters for subsequent analysis. The TIM3+G model was selected as the best-fit model of substitution for all analyses using both the AIC and BIC model selection criteria in the program jModelTest [16, 17]. Maximum likelihood and bootstrap (1000 replicates) analyses were carried out in PhyML 3.0 [18] using the parameters for substitution rate matrix, proportion of invariable sites, number of substitution categories, and shape distribution parameter determined as the best-fit by jModelTest (Ra(AC) = 0.3610, Rb(AG) = 1.1251, Rc(AT) = 1.0, Rd(CG) = 0.3610, Re(CT) = 3.9194, Rf(GT) = 1.0000, gamma shape = 0.5.650). Base frequencies were estimated empirically and the p-invar parameter was optimized from the data. Bayesian analysis was carried out using MrBayes 3.1.2 [19]. The number of substitution categories was based on the parameters determined by jModelTest (as above). Other parameters, such as base frequency, relative rates, substitution rate matrix, and proportion of invariant sites were allowed to vary throughout the analysis. The parameters (shape, statefreq, and revmat) were unlinked to allow for more flexibility in searching tree space. Trees were sampled every 1000 generations. The burn-in value was set to 2000 trees. The total number of generations was set to 8 million. Four parallel chains (one cold and three heated) were used. A majority-rule consensus tree was reconstructed after discarding the burn-in.

Collection of potential hosts. Mole crickets, earwigs, flatheaded borers, pillbugs, and slugs were collected from their natural habitats in the greater Los Angeles area and tested within a few weeks of collection. The majority of the earwigs, flatheaded borers, pillbugs, and slugs were collected from the campus of the California Institute of Technology (Figure 4.S2). Mole crickets were collected from the Rio Hondo golf course in Downey, California. Waxworms and house crickets were purchased commercially from either American Cricket Ranch or Petco®. For

potential hosts collected from natural habitats, species identities were confirmed by analysis of 18S ribosomal DNA sequence, knowledge of habitat distributions in Southern California, and analysis of diagnostic external morphological features.

Chemotaxis assays. Host, CO_2, and odorant chemotaxis assays were performed as previously described [4]. Briefly, assays were performed on standard chemotaxis assay plates [20]. Scoring regions consisted of 2 cm diameter circles on each side of the plate along the diameter, with the center of the circle 1 cm from the edge of the plate. For host chemotaxis assays, live hosts (1 animal in the case of mole crickets, and 4–6 animals for all other hosts) were placed into a 50 ml gastight syringe, and a control syringe was filled with room air. Syringes were depressed at a rate of 0.5 ml/min using a syringe pump. Host air was delivered to one side of the assay plate and room air was delivered to the other side of the assay plate through holes drilled into the plate lids directly above the center of the scoring regions. For CO_2 chemotaxis assays, gastight syringes were instead filled with either a certified CO_2 mixture containing the test concentration of CO_2, 10% O_2, and the balance N_2, or a control air mixture containing 10% O_2 and 90% N_2. For odorant chemotaxis assays, 1 ml of 1 M sodium azide was placed in the center of each scoring region as an anesthetic. 5 ml of odorant was then placed in the center of one scoring region, while 5 ml of a control (either paraffin oil, dH_2O, or ethanol) was placed in the center of the other scoring region. For all assays, ~ 2 ml of worm pellet containing ~ 50–150 nematodes was then placed in the center of the assay plate. Assay plates were left undisturbed on a vibration-reducing platform and scored after either 1 hour (for host and CO_2 chemotaxis assays) or 3 hours (for odorant assays). If at least 3 worms moved into the scoring regions, a chemotaxis index was then calculated as C.I. = (# worms at CO_2 - # worms at air)/(# worms at CO_2 + # worms at air). For the soda lime host chemotaxis assay, gas mixtures were passed through a 6 inch column

containing 2–5 mm soda lime pellets (Sigma-Aldrich 72073) before entering the assay plate, as previously described [4]. Solid odorants were dissolved as follows: 3-hydroxy-2-butanone and dimethyl sulfone, 1 g in 4 ml dH_2O; 4-methylphenol and p-dichlorobenzene, 0.1 g in 5 ml paraffin oil; and p-benzoquinone, 0.1 g in 5 ml ethanol.

For the mixture assay shown in Figure 4.7A, the control assay (left bar) had 5 ml of an odorant mix containing 10^{-1} dilutions of p-dichlorobenzene, hexanal, and γ-terpinene on one side of the chemotaxis plate and 5 ml of paraffin oil control on the other side. The experimental assay (right bar) had 5 ml of odorant mix containing 10^{-1} dilutions of p-dichlorobenzene, hexanal, and γ-terpinene on one side of the chemotaxis plate and 5 ml of odorant mix containing 10^{-1} dilutions of p-dichlorobenzene, hexanal, γ-terpinene, and 3-hydroxy-2-butanone on the other side. The soil assay shown in Figure 4.7B used a modified version of the CO_2 and host chemotaxis assays. For the control assay (left bar), one syringe contained 3 g of soil (collected from the sampling site shown in Figure 4.S2) and the other syringe contained air. For the experimental assay (right bar), one syringe contained 3 g of soil + 5 ml paraffin oil on a small piece of filter paper and the other syringe contained 3 g of soil + 5 ml of 4-methylphenol (dissolved as described above) on a small piece of filter paper.

Jumping assay. Jumping assays were performed as previously described [4]. Briefly, 100 IJs suspended in 200 ml water were evenly distributed onto a 55 mm Whatman 1 filter paper on the bottom of a 5 cm Petri dish. For host jumping assays, a single live host was placed into a 10 ml gastight syringe and a control syringe was filled with room air. For CO_2 jumping assays, syringes were filled with either a certified CO_2 mixture or air control as described above. For odorant jumping assays, a small piece of filter paper containing 5 ml of undiluted odorant was placed inside the syringe. The needle from the syringe was inserted through a 1.25 mm hole in the

side of the dish such that the tip of the needle was within ~ 2 mm of a standing IJ. A small puff of air from the syringe (~ 0.5 ml volume) was then administered directly at the IJ, and a jumping response was scored if the IJ jumped within 8 s. ~ 20 IJs were tested from the same arena. A normalized jumping index (J.I.) that ranged from -1 to +1 was then calculated. For stimuli that evoked higher levels of jumping than the control, the J.I. and SEM were calculated as J.I. = (fraction jumped to stimulus – fraction jumped to control)/(1 – fraction jumped to control) and SEM = $\sqrt{[(\text{SEM for stimulus})^2 - (\text{SEM for control})^2]}$/(1 – fraction jumped to control). For stimuli that evoked lower levels of jumping than the control, the J.I. and SEM were calculated as J.I. = (fraction jumped to stimulus – fraction jumped to control)/(fraction jumped to control) and SEM = $\sqrt{[(\text{SEM for stimulus})^2 - (\text{SEM for control})^2]}$/(fraction jumped to control). For soda lime host jumping assays, the assay setup is as described above, but gas mixtures were passed through a 2 inch column of Nalgene (8050–0250) FTP 3/16" OD tubing containing 2–5 mm soda lime pellets (Sigma-Aldrich 72073) before entering the assay arena. The column was held between 2 female-ended Swagelok compression fittings. To securely attach the column to the syringe and needle, the Swagelok fittings were filled with a male (on the needle end) and female (on the syringe end) biomedical luer fitting.

Virulence assay. Individual hosts were placed into either 5 cm Petri dishes (all hosts except mole crickets) or small glass baby food jars with an air hole drilled into the lid (mole crickets) containing a 55 mm Whatman 1 filter paper at the bottom. 100 IJs suspended in 200 ml water were then evenly distributed onto the filter paper. Hosts were exposed to IJs for 48 hours at room temperature, and host survival was then scored by response to gentle prodding. To assay EPN growth and reproduction in host cadavers, the cadavers were dissected at 5 days post-exposure and scored for the presence of either adult EPNs only (growth but not reproduction) or adults and

young larvae (growth and reproduction). To assay emergence from host cadavers, cadavers were placed onto standard White traps [12] at either 10 days post-exposure (all hosts except house crickets) or 5 days post-exposure (house crickets) and scored for the presence of IJs in the trap at 20 days post-exposure. For potential hosts that desiccate easily (mole crickets, house crickets, pillbugs, and slugs), 200 ml water was added to the filter paper each day to prevent desiccation.

Identification of host-derived odorants by TD-GC-MS and SPME-GC-MS. TD-GC-MS was performed as previously described [4]. TD-GC-MS data for waxworms and house crickets was from Hallem et al., 2011 [4]. Both the collection of volatile organic compounds (VOCs) and subsequent solid phase microextraction (SPME) analysis were modified from Villaverde et al., 2007 [21]. Briefly, VOCs were collected for SPME analysis by placing insects into 10 ml glass vials, sealed with a Teflon septum (SUPLECO 27529). The larger and potentially cannibalistic insects (mole crickets and house crickets) were placed individually into sampling vials whereas all other, smaller species (waxworms, flat-headed borers, pillbugs, and earwigs) were sampled with four individuals per sampling vial. Experiments were done in pairs and replicated 3 times, with an empty control sampling vial being run each time. Clean, sterile vials were used each time. After 12 hrs, volatiles secreted were sampled from the head space, corresponding to the gaseous phase in contact with the insect sample. VOCs were sampled for 15 minutes using carboxen/polydimethylsiloxane (CAR/PDMS) fiber (75 mm film thickness) (SUPELCO 504831). Selection of fibers was based on manufacturer's recommendations for sampling volatiles of low to intermediate polarity and from data reported by Villaverde et al., 2007 [21]. Fibers were preconditioned in accordance with the manufacturer's instructions. Quantitative analysis was performed using a Hewlett Packard 6890 GC–5973 MS gas chromatograph–mass spectrometer (Agilent Technologies, US) employing a non-polar DB-5 capillary column (30m x 0.25mm, 0.25 micron film thickness) (Agilent). The injector was operated in the splitless mode at 250°C and

the oven temperature was programmed (40°C for 3 min, 5°C/min to 80°C, 20°C/min to 150°C, and 30°C/min to 250°C, with a holding time of 10 min at the final temperature). The transfer line temperature was set at 280°C and the ion source was held at 250°C. VOC identification was performed by CGC–MS analysis with an Eclipse 4660 purge and trap sampler with chromatographic conditions similar to the CGC; the ion source was set at 200°C and the transfer line at 275°C. VOC were tentatively identified by interpretation of their mass spectral fragmentation. Data was analyzed with both Chemstation and Masshunter software. Mass spectra were also compared to data from the Wiley library (275,000 spectra) of electron impact mass spectra. Only compounds that were found in multiple traces (≥ 2) with a relative abundance ≥ 20,000, were not present in the control traces, and had library matches of ≥ 95% were considered in this study.

Data analysis. Statistical analysis was performed using either GraphPad InStat, GraphPad Prism, or PAST [22]. Two-factor ANOVAs with Bonferroni post-tests were used to compare the responses of the different EPNs to the different hosts or host-derived odorants. *P* values from the ANOVAs (factor 1, factor 2, and the interaction between the factors) are given in the figure legends; *P* values from the post-tests are given in the supplemental tables. For example, when examining the responses of the different EPNs to the different hosts, we show that EPNs respond differently to different hosts ($P < 0.0001$ for factor one), different hosts evoke different overall responses from EPNs ($P < 0.0001$ for factor two), and different EPNs show different odor response profiles ($P < 0.0001$ for the interaction). Heatmaps were generated using Heatmap Builder [23].

Supplemental Data. The original published version of this work, Dillman et al. [24], has 14 supplemental data sets associated with it. Those data sets are available online (http://www.pnas.org/content/109/35/E2324/suppl/DCSupplemental).

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