Chapter 4:

Olfaction Shapes Host-Parasite Interactions in Parasitic Nematodes*

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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

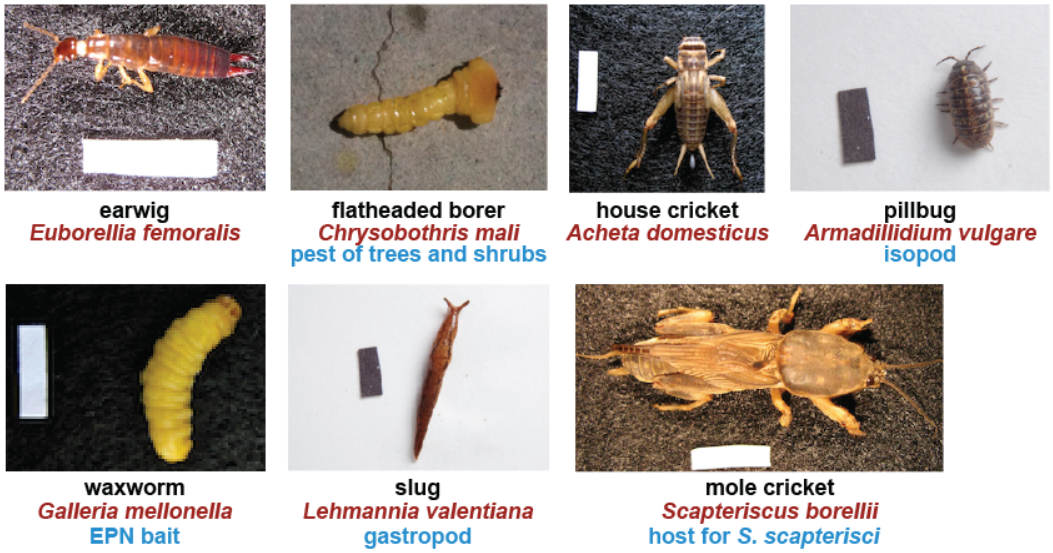
We examined the odor-evoked host-seeking behaviors of six different EPNs in response to seven potential invertebrate hosts. The EPNs—*Heterorhabditis bacteriophora, Steinernema carpocapsae, Steinernema scapterisci, Steinernema riobrave, Steinernema glaseri,* and *Oscheius* carolinensis—were chosen based 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

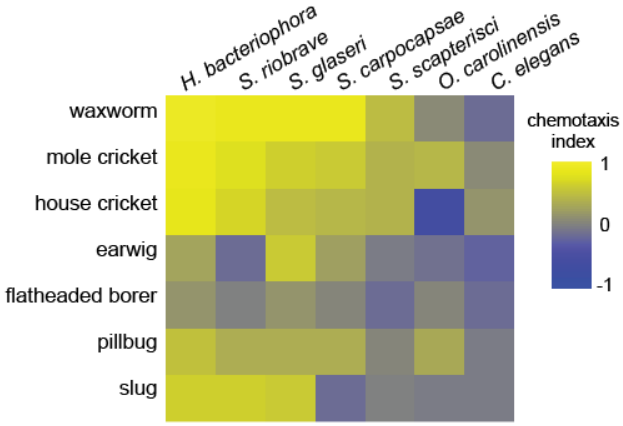
sedentary subterranean larvae, such as those of beetles [36, 41, 42]. *S. riobrave* has not been as thoroughly tested, but it is presumed to have a fairly broad host range and it has been used successfully as a biocontrol agent against both lepidopteran and coleopteran hosts [43, 44]. The host range 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].

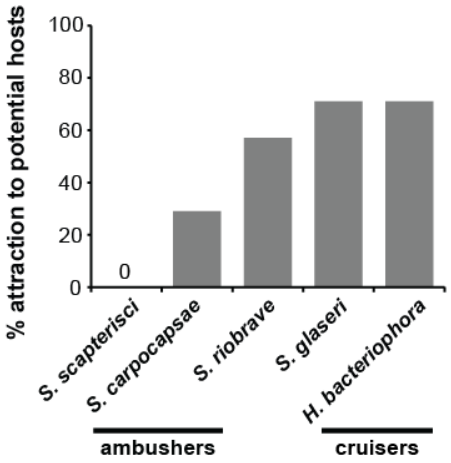
A Potential hosts



B Chemotaxis to hosts



C Host attraction reflects host-seeking strategy



D Jumping to hosts

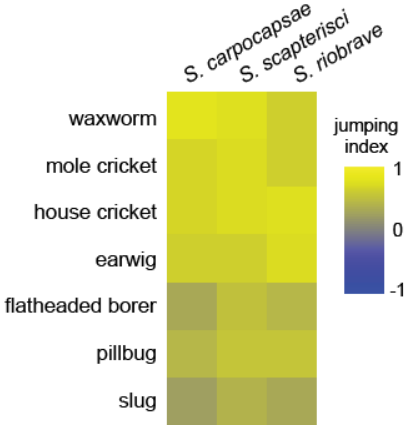


Figure 4.1 | EPNs respond differently to different potential hosts. A. Potential invertebrate hosts tested. Mole crickets, earwigs, flatheaded borers, pillbugs, and slugs were collected from the greater Los Angeles area. Waxworms and house crickets were purchased commercially. Scale bars are 1 cm x 2.5 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.

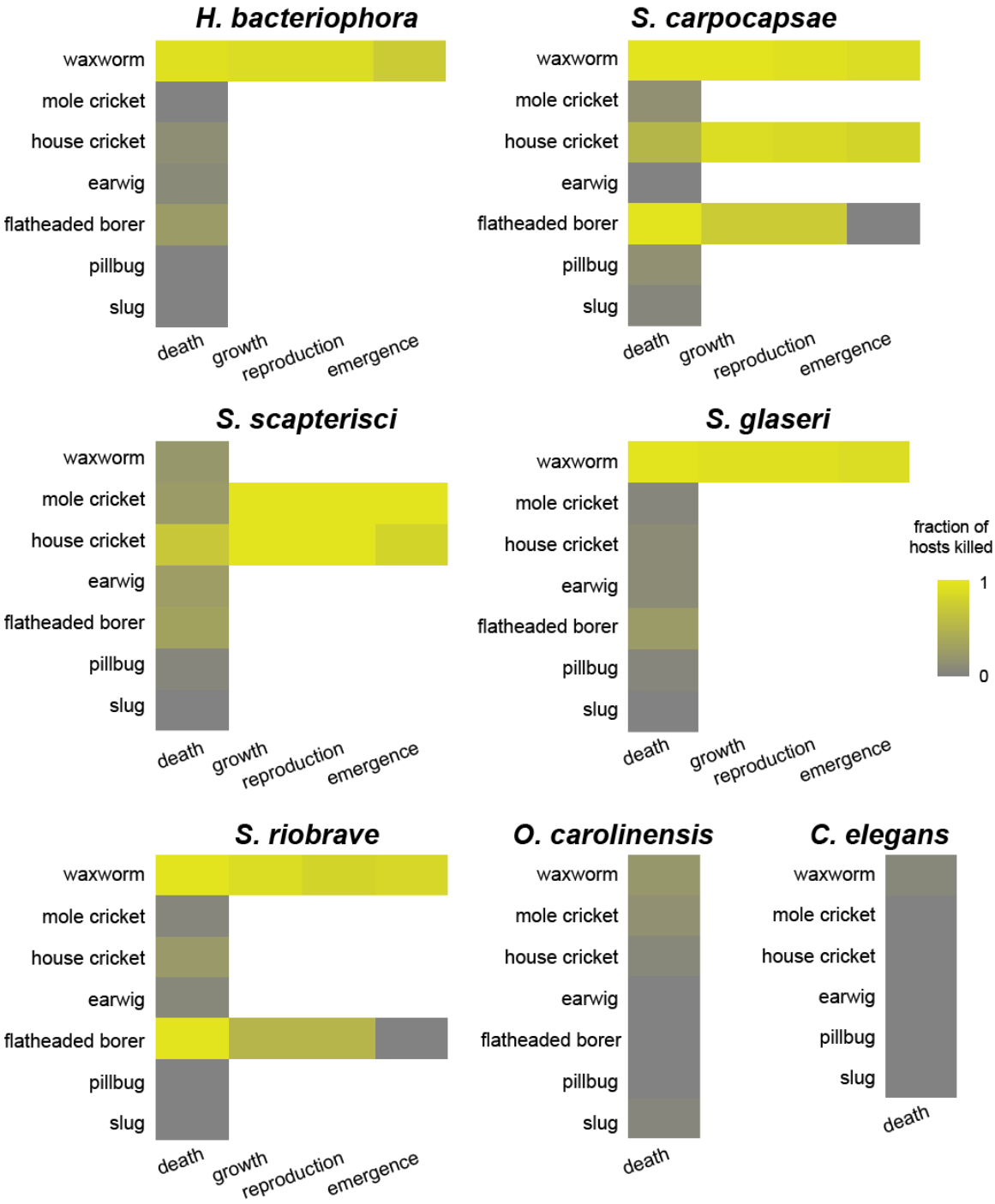


Figure 4.2 | EPNs differ in their virulence toward potential hosts. Graphs show the virulence of each nematode toward the panel of potential hosts. Values for "death" represent the

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.

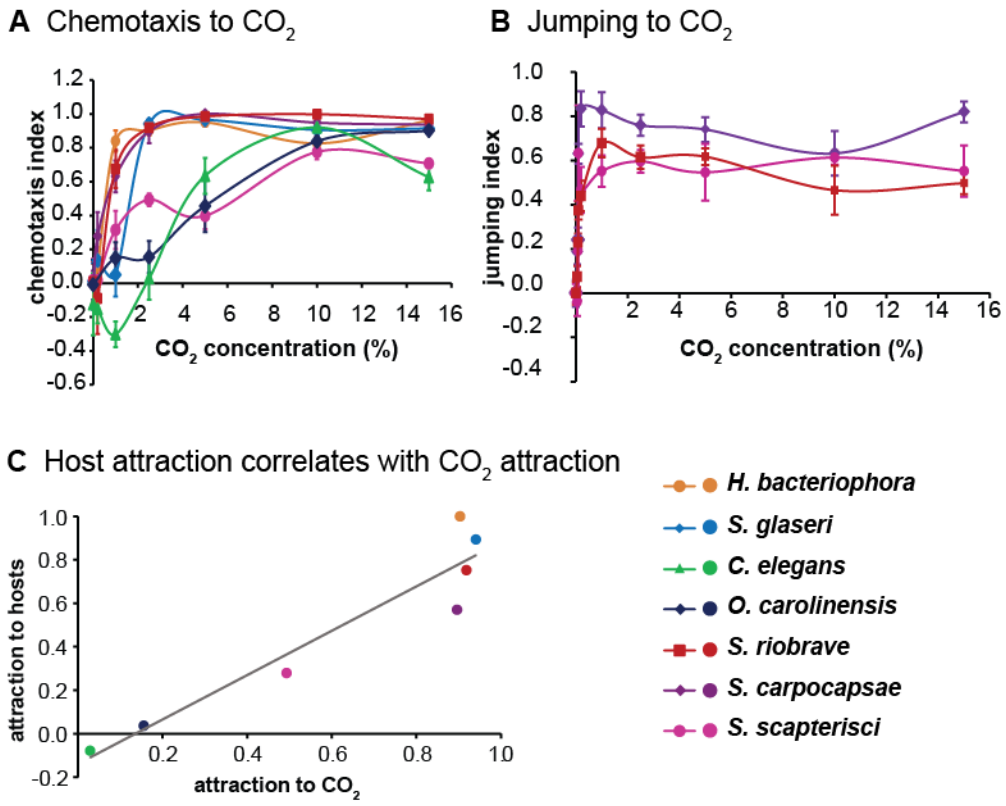


Figure 4.3 | CO_2 stimulates host-seeking behavior of EPNs. A. Chemotaxis of EPN IJs and *C. elegans* dauers to CO_2. n = 5–23 trials. Data for *H. bacteriophora* and *S. carpocapsae* are from Hallem *et al.,* 2011 [3]. **B.** Jumping of EPNs to CO_2. n = 43–192 animals. **C.** Host attraction correlates 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.

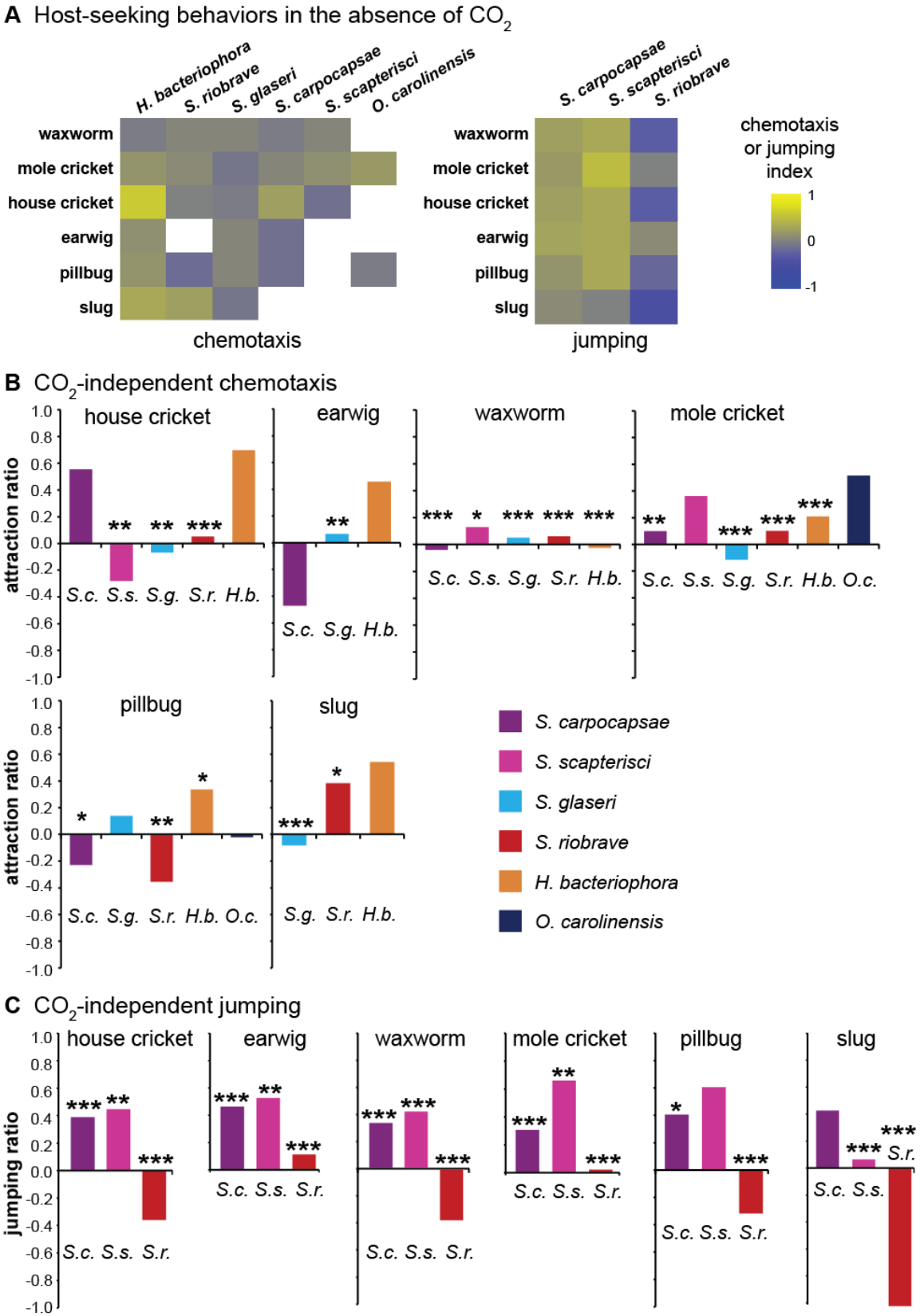

A Host-seeking behaviors in the absence of CO_2
H. bacteriophora
S. riobrave
S. glaseri
S. carpocapsae
S. scapterisci
O. carolinensis
waxworm
mole cricket
house cricket
earwig
pillbug
slug
chemotaxis
S. carpocapsae
S. scapterisci
S. riobrave
jumping
chemotaxis or jumping index
1
0
-1
B CO_2-independent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

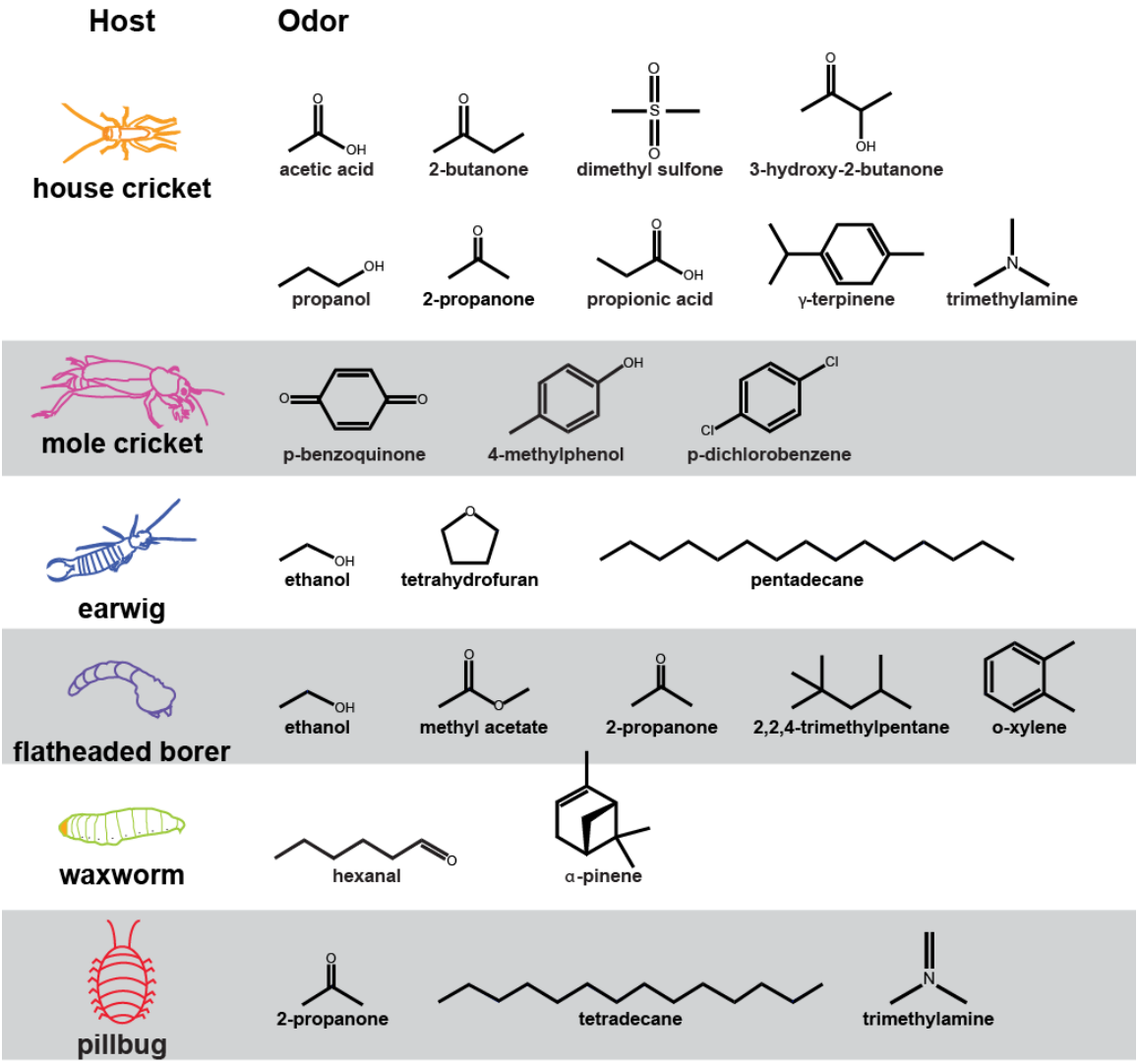


Figure 5. Host-derived odorants identified by TD-GC-MS and SPME-GC-MS. Each listed odorant was identified in at least two different experimental replicates at a relative abundance of ≥ 20,000 and with library matches of at least 95% confidence. Odorants identified from earwigs, flatheaded borers, and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

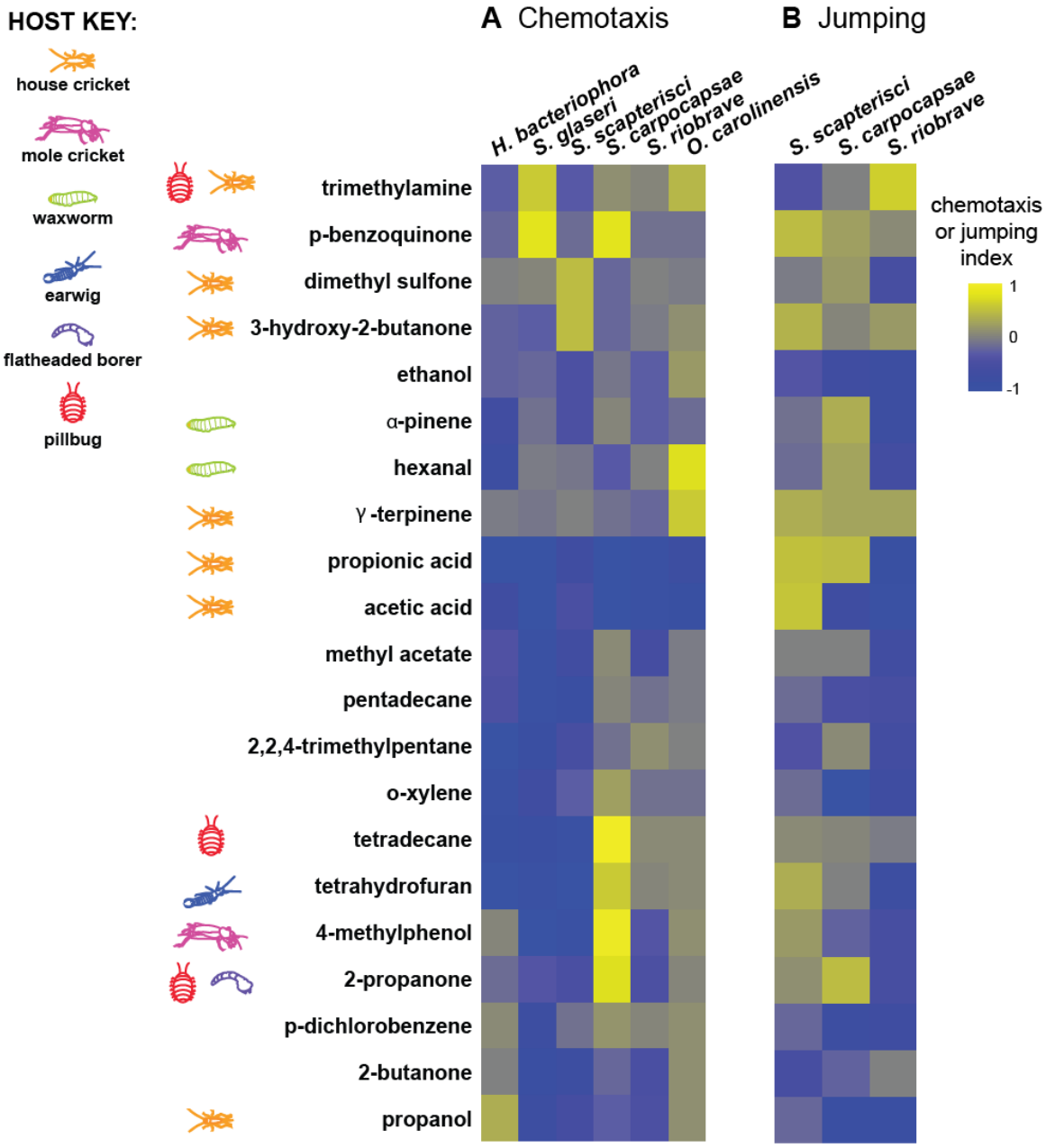


Figure 4.6 | A wide variety of host-derived odorants stimulate host-seeking behavior by EPNs. A. Chemotaxis of EPNs to host-derived odorants. The order of both the nematodes and odorants in the heat map was determined by hierarchical cluster analysis

(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.

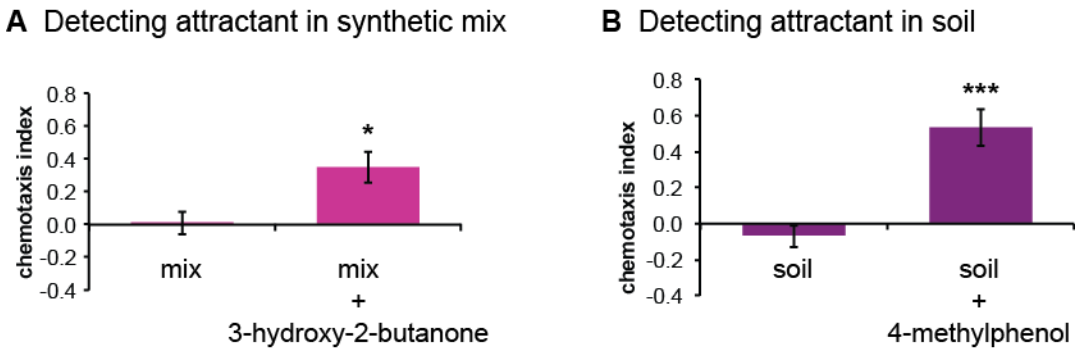


Figure 4.7 | EPNs detect and respond to host-derived odorants in the presence of complex odor mixtures. A. Response of *S. scapterisci* IJs to a 10^{-1} dilution of the cricket-derived odorant 3-hydroxy-2-butanone in the presence of a synthetic mix containing 10^{-1} dilutions of hexanal, γ-terpinene, 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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