

# Olfaction shapes host–parasite interactions in parasitic nematodes

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Many parasitic nematodes actively seek out hosts in which to complete their lifecycles. Olfaction is thought to play an important role in the host-seeking process, with parasites following a chemical trail toward host-associated odors. 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 on 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 to individual host-derived odorants. In addition, we show that EPNs use the universal host cue CO<sub>2</sub> as well as host-specific odorants for host location, but the relative importance of CO<sub>2</sub> versus host-specific odorants varies for different parasite–host combinations and for different host-seeking behaviors. We also identified host-derived odorants by gas chromatography-mass spectrometry and found 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.

entomopathogens | chemosensation | *Heterorhabditis* | *Steinernema*

Many parasitic nematodes actively seek out hosts using sensory cues (1). Host seeking is a complex behavior that involves chemosensory, thermosensory, hygroscopic, and mechanosensory cues (1–4). Olfaction is a critical component of host-seeking behavior: Many parasitic nematodes use CO<sub>2</sub> and other host volatiles for host location (1, 2, 5–8). 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 (9)—that includes the genera *Heterorhabditis*, *Steinernema*, and *Oscheius* (10, 11). EPNs are parasites of insects that infect and kill insect larvae (10, 11). They offer a number of advantages as model systems, including small size, short generation time, and amenability to laboratory culturing and behavioral analysis (12, 13). In addition, they resemble skin-penetrating human-parasitic nematodes in that they actively seek out hosts using olfactory cues (2, 7, 13–16). EPNs also are of interest as biocontrol agents for insect pests and disease vectors and currently are 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 (17).

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 (18). After long-range host location, IJs are thought to use short-range sensory cues for

host recognition (19). IJs then infect the host either by entering through natural orifices or by penetrating through the insect cuticle (20). Following infection, IJs release a bacterial endosymbiont into the insect host and resume development (21–23). 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 (24).

EPNs use a wide range of host-seeking strategies. Some are “cruisers” that actively seek out hosts, whereas 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 using both cruising and ambushing strategies for host location (25, 26). In addition, some EPNs of the genus *Steinernema* exhibit jumping, a rare behavior among soft-bodied, limbless organisms (27, 28). Among EPNs, jumping is a highly specialized ambushing behavior in which the IJ propels itself into the air (13, 27, 29). Jumping is thought to be a short-range host-seeking strategy that facilitates attachment to the host when the host is in close proximity (27, 30, 31). In general, cruisers are most effective at infecting stationary hosts, whereas ambushers are most effective at infecting fast-moving hosts (32). Previous studies have demonstrated that EPNs are attracted to CO<sub>2</sub> as well as to a number of other odorants (13–15, 33–35). 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 on the basis of both phylogenetic and behavioral diversity (Fig. S1). These species

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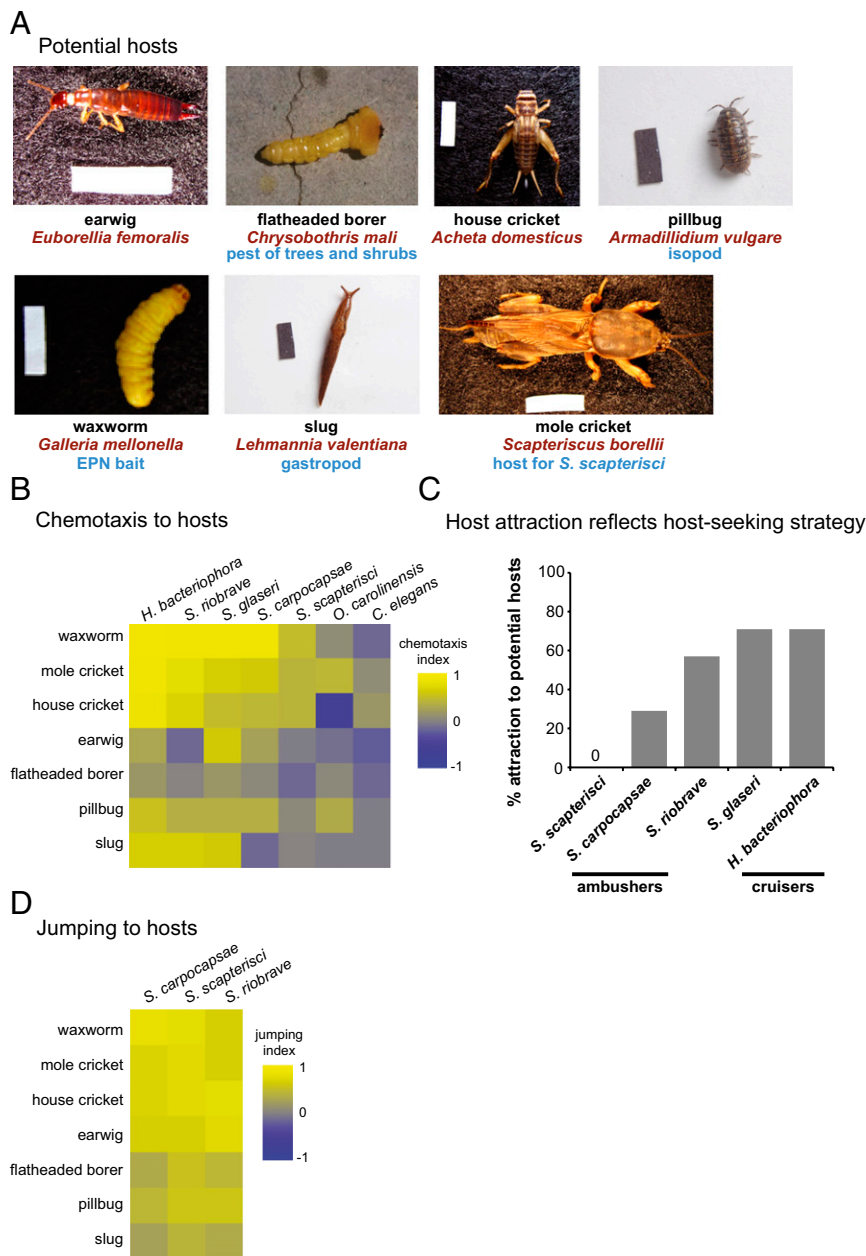
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vary greatly in their host-seeking strategies: *H. bacteriophora* and *S. glaseri* are cruisers, *S. carpocapsae* and *S. scapterisci* are ambushers, and *S. riobrave* employs an intermediate host-seeking

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



**Fig. 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: 1 cm  $\times$  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 heatmap 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 posttest).  $n = 6$ –30 trials for each EPN-host combination. Mean,  $n$ , and SEM values for each assay are given in [Dataset S1](#);  $P$  values for each posttest are given in [Datasets S2 and S3](#). (C) Chemotaxis behavior reflects host-seeking strategy, so that cruisers display more overall attraction to hosts than do ambushers. The y-axis indicates the percentage of hosts that were strongly attractive (as defined by a chemotaxis index of  $\geq 0.5$ ). *S. scapterisci* and *S. carpocapsae* are ambushers, *S. glaseri* and *H. bacteriophora* are cruisers, 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, cophenetic correlation = 0.85). (D) Jumping of EPNs in response to volatiles released by live potential hosts. The order of the nematodes in the heatmap 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 posttest). 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 [Dataset S1](#);  $P$  values for each posttest are given in [Datasets S4 and S5](#). In B and D, response magnitudes are color-coded so that a chemotaxis index or jumping index of +1 is yellow, -1 is blue, and 0 is gray.

closest known EPN relative of *C. elegans* (21), has not yet been characterized.

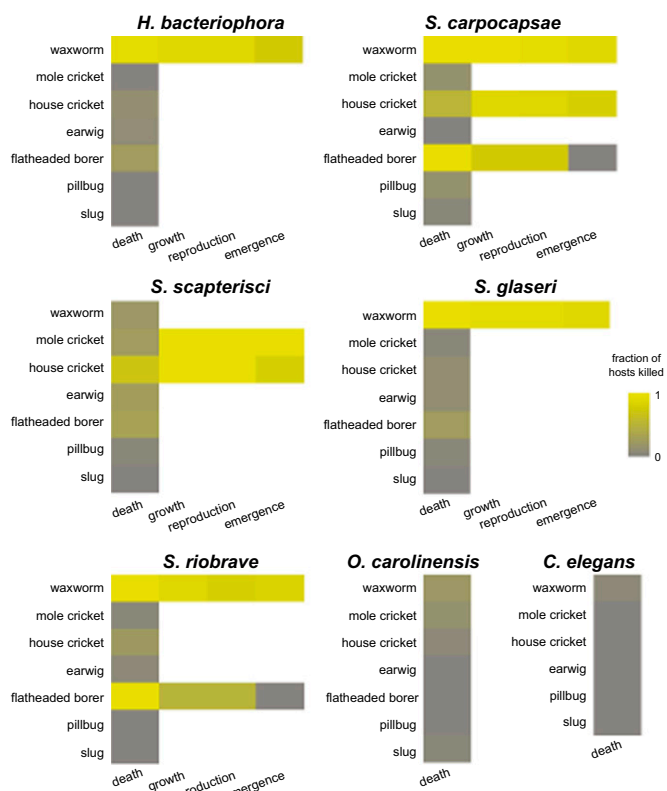
These six EPN species also were chosen because of their differing host ranges. *H. bacteriophora* and *S. carpocapsae* are thought to have very broad host ranges, with *S. carpocapsae* capable of infecting more than 250 different species of insects from 13 orders under laboratory conditions (36, 37). 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 tested as thoroughly, 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: Noctuidae) (47), *Scapteriscus vicinus* and *Scapteriscus borellii* (Orthoptera: Gryllotalpidae) (38, 48), and *Popillia japonica* (Coleoptera: Scarabaeidae) (49), respectively. Whether these species represent true natural hosts or merely opportunistic hosts remains unclear except for *S. scapterisci*, which has been used successfully for decades to control invasive species of mole crickets (40).

The seven potential invertebrate hosts—the mole cricket *Scapteriscus borellii*, the house cricket *Acheta domesticus*, the earwig *Euborellia femoralis*, the waxworm *Galleria mellonella*, the flat-headed borer *Chrysobothris mali*, the pillbug *Armadillidium vulgare*, and the slug *Lehmannia valentiana*—also were chosen based on their phylogenetic and ecological diversity (Fig. 1A). Mole crickets are the only known natural host for *S. scapterisci* (40), 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). Waxworms were selected because they are a common laboratory host for EPNs and typically are used as bait when collecting EPNs from soil; thus, many described EPNs are attracted to waxworms, even in complex soil environments (42, 51). 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, because they develop under the bark in the phloem of host plants (52). They represent nonnatural but potential hosts of EPNs, ones that EPNs have not evolved to find or infect. In contrast, pillbugs and slugs are noninsects that are similar in size to many potential insect hosts of EPNs and often are in the same or overlapping communities with EPNs. Pillbugs belong to the same phylum as insects (Arthropoda) but to a different order (Isopoda); 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 nonhosts or dead-end hosts for several EPNs (53–57); however, the potential for EPNs to use isopods and gastropods as alternative or reservoir hosts when insects are scarce has not been explored fully, 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 (Fig. S2).

**EPNs Respond Differently to Different Host Odors.** We examined EPN responses to odors emitted from live hosts using both chemotaxis and jumping assays (13). 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 (Fig. 1B and Datasets S1–S3). In addition, odor–response profiles differ for the different EPNs, so that some hosts are more attractive to some EPNs than to others (Fig. 1B and Datasets S1–S3). Overall, we found that host attraction reflects host-seeking strategy such that cruisers showed more robust attraction to live hosts than ambushers in our chemotaxis assay (Fig. 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 *Caenorhabditis elegans* dauers to the potential host odors; the Hawaii strain was used for this comparison because it most closely resembles wild *C. elegans* strains (58). We found that all the invertebrate odors were neutral or repulsive (chemotaxis index < 0.2) for *C. elegans* dauers (Fig. 1B and Dataset S1). 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 (Fig. 1D and Datasets S1, S4, and S5). EPNs showed significantly higher rates of jumping in response to some potential hosts than to others, and some



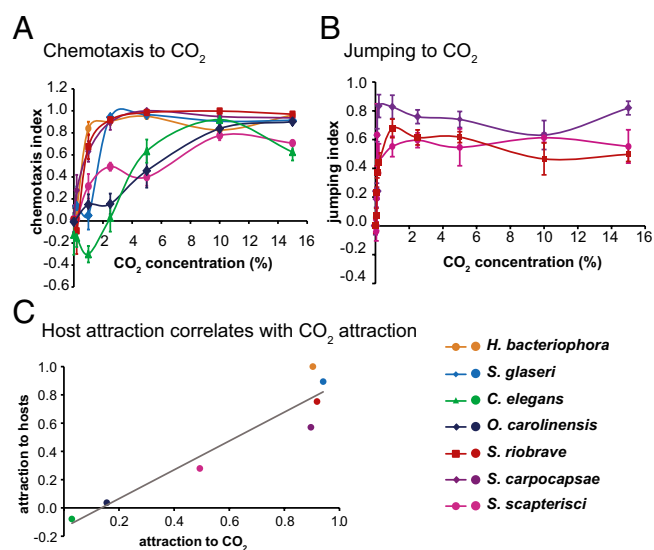
**Fig. 2.** EPNs differ in their virulence toward potential hosts. Graphs show the virulence of each nematode toward the panel of potential hosts. Values for “death” represent the fraction of hosts that died within 48 h following exposure to nematodes. Values for “growth,” “reproduction,” and “emergence” 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 100 IJs.  $n = 20$ –50 assays for all invertebrates except flatheaded borers;  $n = 8$ –12 assays for flatheaded borers because of the limited availability of these insects. For each EPN–host combination, statistical significance was determined relative to an uninfected control using a  $\chi^2$  test. Mean values for death, growth, reproduction, and emergence are given in Dataset S6.



potential hosts evoked significantly higher rates of jumping overall than others (Fig. 1D and [Datasets S1, S4, and S5](#)). 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 (Fig. 1D and [Datasets S1, S4, and S5](#)). These results suggest that chemotaxis behavior may display more species specificity than jumping behavior.

**EPNs Vary in Their Virulence Toward Potential Hosts.** We then tested the virulence, i.e., the disease-producing power (59), of the six different EPNs toward the seven potential hosts. EPN virulence usually is tested by exposing potential hosts to a defined number of IJs (typically between 1 and 1,000 per potential host) (54, 60, 61). Previous work suggests that using high doses of IJs in mortality experiments allows poor host suitability to be overcome by the 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 h. When 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 (Fig. 2 and [Dataset S6](#)). For example, *S. carpocapsae* was virulent toward three of the seven species tested, whereas *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 in parasitizing waxworms. This result 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, because most of these species have been maintained in waxworms after 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 with 71% of house crickets. However, the mole crickets that were killed successfully were the most effective hosts: 100% of the mole cricket cadavers supported *S. scapterisci* growth, reproduction, and emergence (Fig. 2 and [Dataset S6](#)). 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 (39). 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 could grow and reproduce inside borer cadavers, emergence of IJs from borer cadavers was never observed (Fig. 2 and [Dataset S6](#)). None of the EPNs was able to kill earwigs, pillbugs, or slugs successfully in our assay (Fig. 2 and [Dataset S6](#)). Thus, at this inoculum (100 IJs per host), EPNs differ in their range of hosts.

**CO<sub>2</sub> 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<sub>2</sub>, 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 (2, 8, 62). To examine the chemotactic response to CO<sub>2</sub>, we used a CO<sub>2</sub> chemotaxis assay in which worms were allowed to distribute on a plate in a CO<sub>2</sub> concentration gradient (13). We found that all the tested EPNs are attracted to CO<sub>2</sub> (Fig. 3A and [Dataset S7](#)), and all three of the jumping species jumped in response to CO<sub>2</sub> (Fig. 3B and [Dataset S7](#)). However, the attractiveness of CO<sub>2</sub> varied among EPNs, with *S. scapterisci* and *O. carolinensis* showing less attraction to low concentrations of CO<sub>2</sub> than the other species (Fig. 3A and [Dataset S7](#)). Re-

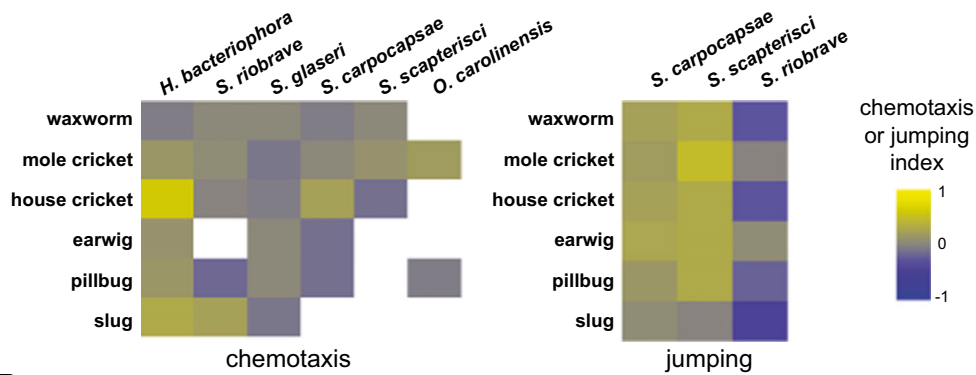


**Fig. 3.** CO<sub>2</sub> stimulates host-seeking behavior of EPNs. (A) Chemotaxis of EPN IJs and *C. elegans* dauers to CO<sub>2</sub>.  $n = 5\text{--}23$  trials. Data for *H. bacteriophora* and *S. carpocapsae* are from Hallem et al. (13). (B) Jumping of EPNs to CO<sub>2</sub>.  $n = 2\text{--}7$  trials. (C) Host attraction correlates with CO<sub>2</sub> attraction. The x-axis indicates the chemotaxis index in response to 2.5% CO<sub>2</sub>; 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 [Dataset S7](#).

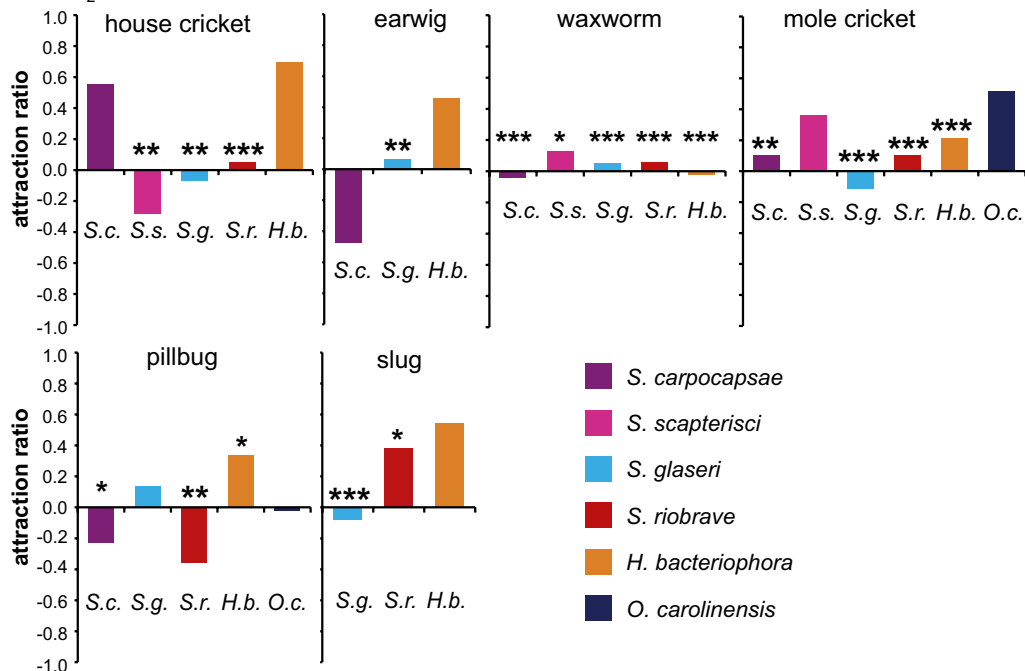
sponses to low CO<sub>2</sub> concentrations were highly correlated with overall host attraction, suggesting that differences in overall host attraction may be attributable to differences in CO<sub>2</sub> sensitivity among EPNs (Fig. 3C). Thus, CO<sub>2</sub> is an important host-seeking cue for both specialist and generalist EPNs.

**Requirement for CO<sub>2</sub> Varies for Different EPN–Host Combinations.** To test whether CO<sub>2</sub> is required for host attraction, we assayed the response to live hosts in the presence of soda lime, which removes CO<sub>2</sub> (13). We found that for all EPN–host combinations, chemotaxis was reduced in the absence of CO<sub>2</sub> (Fig. 4A and [Datasets S1 and S8](#)). 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<sub>2</sub>, whereas mole crickets, house crickets, and earwigs were still attractive to some EPNs but not to others (Fig. 4B and [Dataset S8](#)). Removal of CO<sub>2</sub> did not render any hosts significantly repulsive (chemotaxis index  $\leq -0.2$ ) (Fig. 4A). Host-evoked jumping also was reduced in the absence of CO<sub>2</sub>, and, as with chemotaxis, the requirement for CO<sub>2</sub> differed for different EPN–host combinations (Fig. 4A and C and [Datasets S1 and S9](#)). Thus, although CO<sub>2</sub> is sufficient for eliciting host-seeking behavior from all EPNs, it is both necessary and sufficient for some EPN–host combinations but not for others. To test further the role of CO<sub>2</sub> versus host-specific odors in host seeking, we performed a chemotaxis competition experiment with *S. carpocapsae* in which CO<sub>2</sub> was introduced into one side of the chemotaxis plate and odor from a single mole cricket was introduced into the other side (Fig. S3). We found that *S. carpocapsae* prefers live mole crickets to 1% CO<sub>2</sub> (Fig. S3), even though 1% CO<sub>2</sub> is highly attractive to *S. carpocapsae* and attraction of *S. carpocapsae* to mole crickets is reduced greatly in the absence of CO<sub>2</sub> (Fig. 4A). However, higher concentrations of CO<sub>2</sub> are more attractive than mole crickets (Fig. S3). These results demonstrate that EPNs use both CO<sub>2</sub> and host-specific odorants for host location.

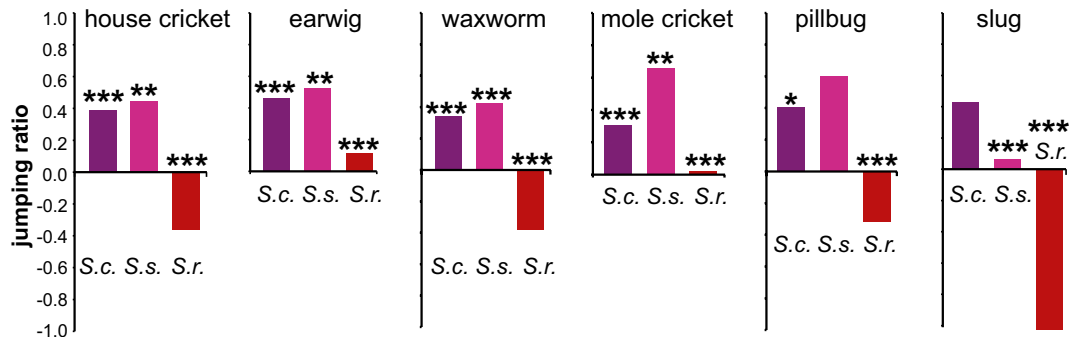
**A** Host-seeking behaviors in the absence of CO<sub>2</sub>



**B** CO<sub>2</sub>-independent chemotaxis



**C** CO<sub>2</sub>-independent jumping



**Fig. 4.** Host-seeking behavior is reduced in the absence of CO<sub>2</sub>. (A) Chemotaxis to live hosts is reduced significantly when CO<sub>2</sub> is removed from the host airstream using soda lime ( $P < 0.0001$  for all species except *O. carolinensis*;  $P < 0.05$  for *O. carolinensis*; two-factor ANOVA with replication). Chemotaxis with CO<sub>2</sub> removed was tested only for EPN–host combinations in which host attraction was observed initially. Jumping to live hosts also is reduced when CO<sub>2</sub> is removed from the host airstream using soda lime ( $P < 0.001$ , two-factor ANOVA with replication).  $n = 6$ –22 trials for chemotaxis and two to seven trials for jumping for each EPN–host combination. (B) Levels of CO<sub>2</sub>-independent attraction to potential hosts. Attraction ratios indicate the chemotaxis index for host attraction with CO<sub>2</sub> removed divided by the chemotaxis index for host attraction with CO<sub>2</sub>. (C) Levels of CO<sub>2</sub>-independent jumping to potential hosts. Jumping ratios indicate the jumping index for host-evoked jumping with CO<sub>2</sub> removed divided by the jumping index for host-evoked jumping with CO<sub>2</sub>. In B and C, asterisks indicate cases where the response to host with CO<sub>2</sub> removed was significantly different from the response to host with CO<sub>2</sub> present. \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; \* $P < 0.05$ , two-factor ANOVA with replication with a Bonferroni posttest. Mean,  $n$ , and SEM values for each assay in A are given in [Dataset S1](#);  $P$  values for each posttest are given in [Datasets S8 and S9](#).

**Diverse Array of Host-Derived Odorants Stimulates 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 (13). We now have extended this analysis to all seven potential invertebrate hosts using TD-GC-MS and solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS) (63). Overall, we identified 21 odorants emitted consistently and at relatively high abundance by the potential hosts (Fig. 5 and Fig. 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 (Fig. 5). The fact that we identified more odorants from crickets than from waxworms is consistent with our finding that crickets evoke higher levels of CO<sub>2</sub>-independent attraction than waxworms (Fig. 4B) and suggests that the relative contributions to host seeking by CO<sub>2</sub> versus host-specific odorants may depend, in part, on the number of odorants the host emits.

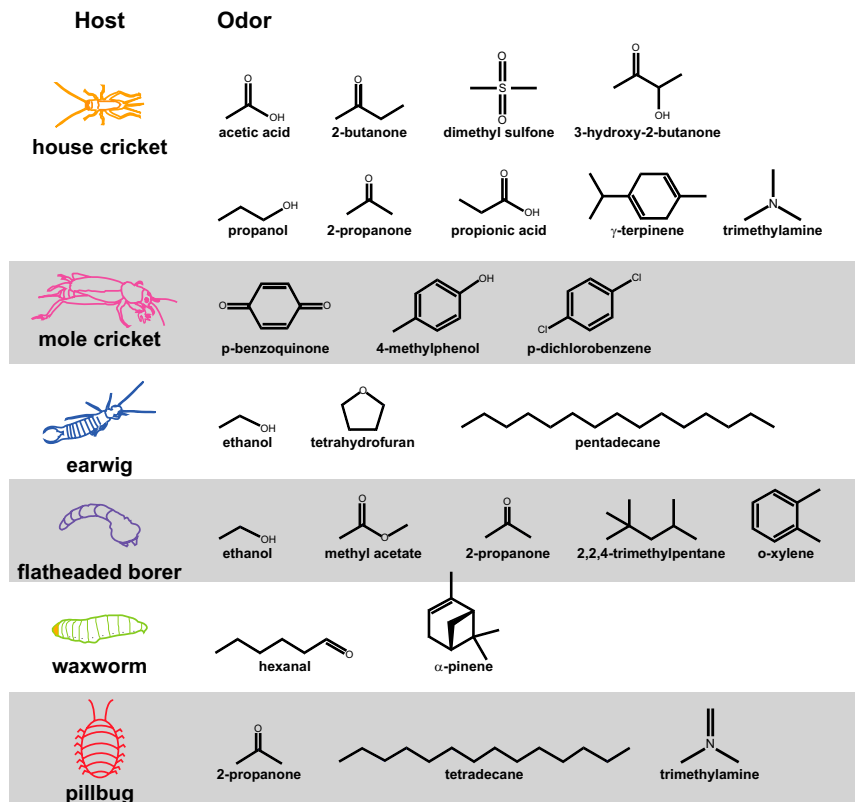
We then examined the behavioral responses to these odorants and found that many odorants strongly stimulated host-seeking behaviors (Fig. 6 and Dataset S10). Overall, we observed strong responses to at least one odorant identified from each of the tested invertebrates (with the exception of slugs, for 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 were repulsive or neutral for the

other species (Fig. 6 and Dataset S10). 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 (Fig. 6 and Datasets S11 and S12). Thus, most EPNs display species-specific responses to host-derived odorants.

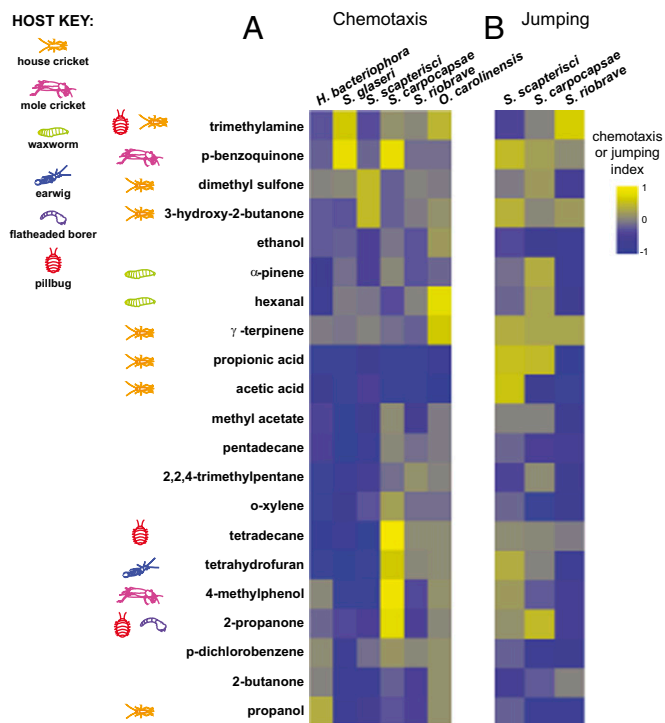
In the case of the cricket specialist *S. scapterisci*, we found that all the odorants that elicited a strong response (as defined by a chemotaxis or jumping index of  $\geq 0.5$ ) were cricket derived, and 7 of the 11 cricket-derived odorants elicited a positive chemotactic or jumping response (as defined by a chemotaxis or jumping index of  $\geq 0.2$ ). Thus, the odor-response profile of *S. scapterisci* appears to reflect its specialized host range.

Dose-response analysis indicated that, for chemotaxis behavior, most odorants were consistent attractants or repellants across concentrations (Fig. S5A and Dataset S13). The one exception was acetic acid, which was repulsive to *S. carpocapsae* at high concentrations but attractive at lower concentrations (Fig. S5A and Dataset S13). 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 (Fig. S5B and Dataset S14). These results suggest that EPNs may use olfactory cues to encode information about host proximity as well as host identity.

To explore further 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 that we identified from



**Fig. 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  $\geq 20,000$  and with library matches of at least 95% confidence. Odorants identified from earwigs, flatheaded borers, and pillbugs and 2-propanone identified from house crickets were identified by SPME-GC-MS; all other odorants were identified by TD-GC-MS.



**Fig. 6.** EPN host-seeking behavior is stimulated by a wide variety of host-derived odorants. (A) Chemotaxis of EPNs to host-derived odorants. The order of both the nematodes and odorants in the heatmap 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 posttest), with the exception of *S. riobrave* and *O. carolinensis*, which did not differ from each other significantly.  $n = 4$ –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,  $\alpha$ -pinene, propanol, propionic acid,  $\gamma$ -terpinene, and trimethylamine are from Hallem et al. (13). Mean,  $n$ , and SEM values for each assay are given in [Dataset S10](#);  $P$  values for each posttest are given in [Dataset S11](#). (B) Jumping of EPNs to host-derived odorants. The order of nematodes in the heatmap 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 the three species display unique jumping odor-response profiles ( $P < 0.001$ ).  $n = 2$ –11 trials for each EPN-odorant combination. Mean,  $n$ , and SEM values for each assay are given in [Dataset S10](#);  $P$  values for each posttest are listed in [Dataset S12](#).

hosts but that did not elicit a response when tested individually) (Fig. 6) or soil odor. We found that host-derived odorants that attracted EPNs when tested individually still were attractive in the presence of both the neutral odorant mixture and the soil odor (Fig. 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.

## Discussion

*Heterorhabditis*, *Steinernema*, and *Oscheius* are phylogenetically distant genera of EPNs that convergently have 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 (11). The convergence of three separate genera in the EPN guild therefore is 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.

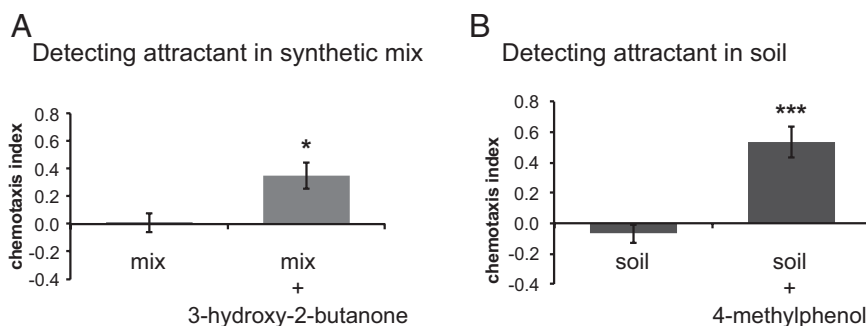
Overall, we found that chemotaxis behaviors exhibit more species specificity than jumping behaviors. For example, the relative attractiveness of different potential hosts in a chemotaxis assay varied for different EPN species (Fig. 1B). By contrast, all the jumping species tested displayed the same relative 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 the reverse was also true (Fig. 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 therefore may serve different functions in the host-seeking process. The evolution of jumping behavior likely played a major role in niche partitioning among EPNs, because jumping ambushers are found primarily in epigeal (soil-air interface) habitats, whereas cruisers often are found deeper in the soil column (64). However, our results suggest that odor-driven chemotaxis behavior may have played a more important role than odor-driven jumping behavior in further partitioning of the epigeal niche among jumping species. This suggestion 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, whereas chemotaxis before jumping and tactile or other cues subsequent to jumping are used for host discrimination. However, it is possible that jumping also can 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 appeared to respond primarily to crickets and cricket-derived odorants (Figs. 1, 2, and 6). In addition, we found that *S. scapterisci* showed a reduced response to low concentrations ( $\leq 1\%$ ) of  $\text{CO}_2$  compared with most EPNs in a chemotaxis assay but not in a jumping assay (Fig. 3), and the response of *S. scapterisci* to mole crickets in a chemotaxis assay was not significantly different when  $\text{CO}_2$  was removed from the host airstream (Fig. 4A and [Dataset S8](#)). Thus, *S. scapterisci* may rely more than generalist EPN species on host-specific cues and less on  $\text{CO}_2$  for long-range host seeking. 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 (Fig. 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 (Fig. 5) suggests either that *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, also was identified from mole crickets but did not meet our stringent criteria for inclusion in our analysis (Fig. S4). Dimethyl sulfone elicited behavioral responses from *S. scapterisci* even at low concentrations (Fig. 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*, the attraction of *O. carolinensis* to  $\text{CO}_2$  declined at concentrations around 1% (Figs. 1B and 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 (11, 21, 65). Thus, the olfactory system of





**Fig. 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,  $\gamma$ -terpinene, and p-dichlorobenzene. The left bar represents the response to the synthetic mix vs. a paraffin oil control. The right bar represents 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. The left bar represents the response to soil odor vs. an air control. The right bar represents response to 4-methylphenol plus soil odor vs. soil odor alone.  $n = 6$  trials for each condition. The response to 4-methylphenol plus 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 [Dataset S10](#).

*O. carolinensis* may be less highly specialized for insect parasitism than those of the more anciently evolved EPNs. It also is 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 *Oscheius necromenus* is associated with millipedes, which are noninsect 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 able to infect some insects better than others (Fig. 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, at high doses, of infecting a wide variety of insect larvae and even some noninsect invertebrates (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 were 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 (Figs. 1 and 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 nonhost insects and noninsect invertebrates such as isopods and earthworms (72–74). Attraction to nonhosts in the absence of hosts may offer a survival advantage to EPNs by facilitating dispersal to more favorable environmental niches. It also is possible that in some cases olfactory preferences can lead EPNs to pursue nonhosts 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 (19, 57). 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 examined responses to  $\text{CO}_2$  and other host-derived odorants. We found

that all EPNs tested are attracted to  $\text{CO}_2$  and that  $\text{CO}_2$  sensitivity is positively correlated with overall host attraction (Fig. 3). Thus,  $\text{CO}_2$  is a critical host-seeking cue for EPNs regardless of host-seeking strategy or host range. However, the importance of  $\text{CO}_2$  as a host-seeking cue varies for different hosts. For example,  $\text{CO}_2$  appears to be more important for attraction to waxworms than to crickets: Waxworms were no longer attractive to any of the EPNs in the absence of  $\text{CO}_2$ , but crickets were still attractive to some but not all EPNs (Fig. 4). In addition, *S. carpocapsae* preferred mole cricket odor to 1%  $\text{CO}_2$  in a competition chemotaxis assay, demonstrating that at least some live hosts are more attractive than low concentrations of  $\text{CO}_2$  alone (Fig. S3). The importance of  $\text{CO}_2$  also varies for different EPNs. For example, in the absence of  $\text{CO}_2$ , *S. riobrave* responded to slugs only, and, in fact, host-evoked chemotaxis and jumping were suppressed in many cases in the absence of  $\text{CO}_2$  (Fig. 4). Consistent with the reliance of *S. riobrave* on  $\text{CO}_2$ , 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 (Fig. 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  $\text{CO}_2$  for host location, but others use  $\text{CO}_2$  in combination with host-specific odorants. We also found that at least some EPNs are attracted to host-specific odorants even in the presence of complex mixtures (Fig. 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). Because of their strikingly diverse biogeography, EPNs are promising biocontrol agents for nearly all climates and locales and have been used successfully throughout the world to control a wide variety of insect pests (81). However, the commercial success of EPNs as biocontrol agents often is unpredictable. For example, *S. scapterisci* has proven to be as effective as chemical pesticides for the control of mole crickets and now is used widely on golf courses, pastures, and other grassy terrains subject to mole cricket infestation (40, 81). In 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 for 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.

## Materials and Methods

All nematode strains were cultured as previously described (13). Mole crickets, earwigs, flatheaded borers, pillbugs, and slugs were collected from their natural habitats in the greater Los Angeles area (Fig. S2); waxworms and house crickets were purchased commercially from American Cricket Ranch or Petco. Chemotaxis and jumping assays were performed as previously described (13). For virulence assays, individual hosts were placed in Petri dishes lined with filter paper containing 100 lJs. Survival was scored after 48 h, growth and reproduction in host cadavers was scored after 5 d, and emergence from host cadavers was scored after 10 d for all hosts except house crickets, for which survival was scored after 5 d. TD-GC-MS was performed as previously described (13), and the procedure for SPME-GC-MS was

modified from Villaverde et al. (63). A detailed description of all materials and methods used in this study is provided in *SI Materials and Methods*.

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