Chapter 3:

A Sensory Code For Host Seeking in Parasitic Nematodes*

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

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Abstract

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

Results and Discussion

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

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

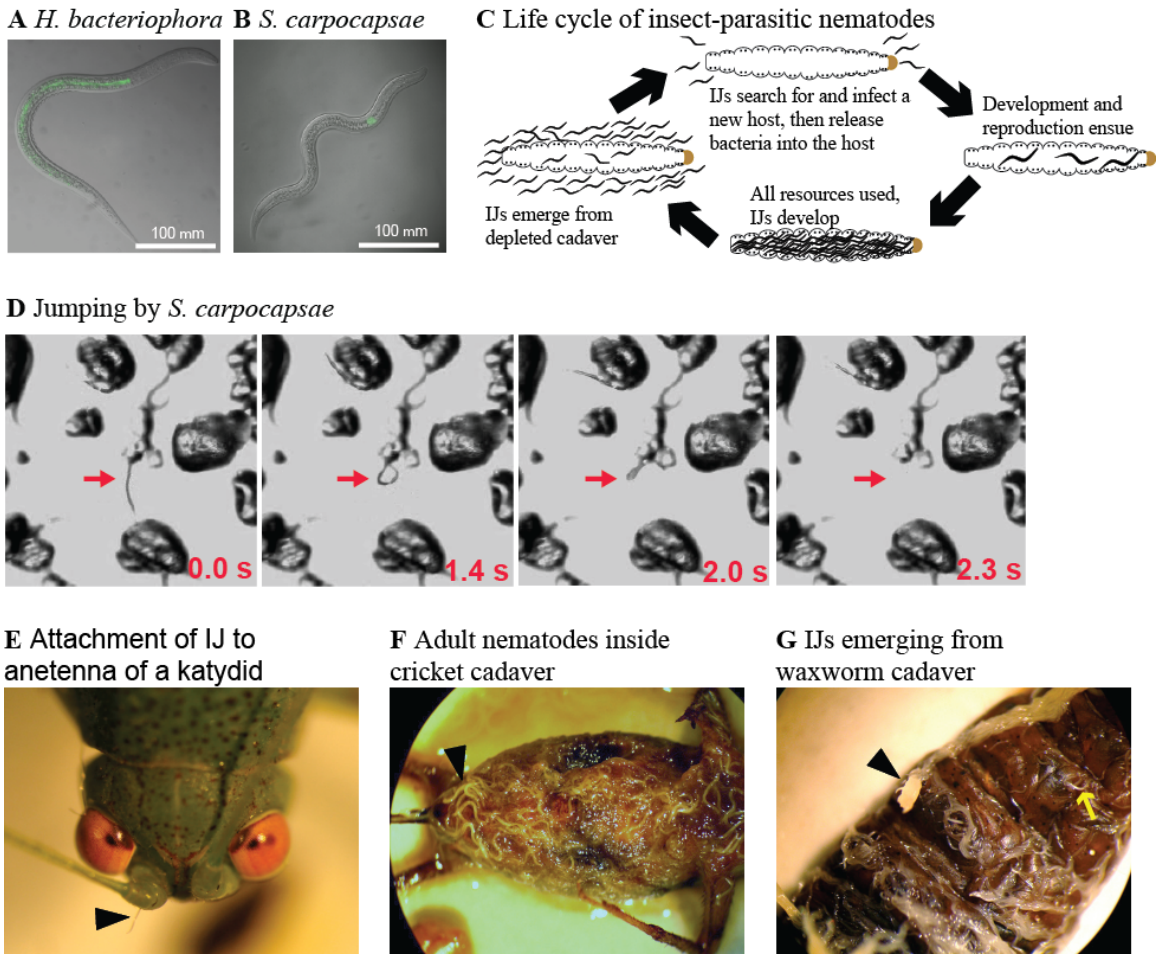

Figure 3.1 | Life cycles of insect-parasitic nematodes. A–B. Photomicrographs of an *H. bacteriophora* **(A)** and an *S. carpocapsae* (**B**) infective juvenile (IJ). Both species harbor a bacterial symbiont—*H. bacteriophora* harbors *Photorhabdus luminescens* and *S. carpocapsae* harbors *Xenorhabdus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

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

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

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

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

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

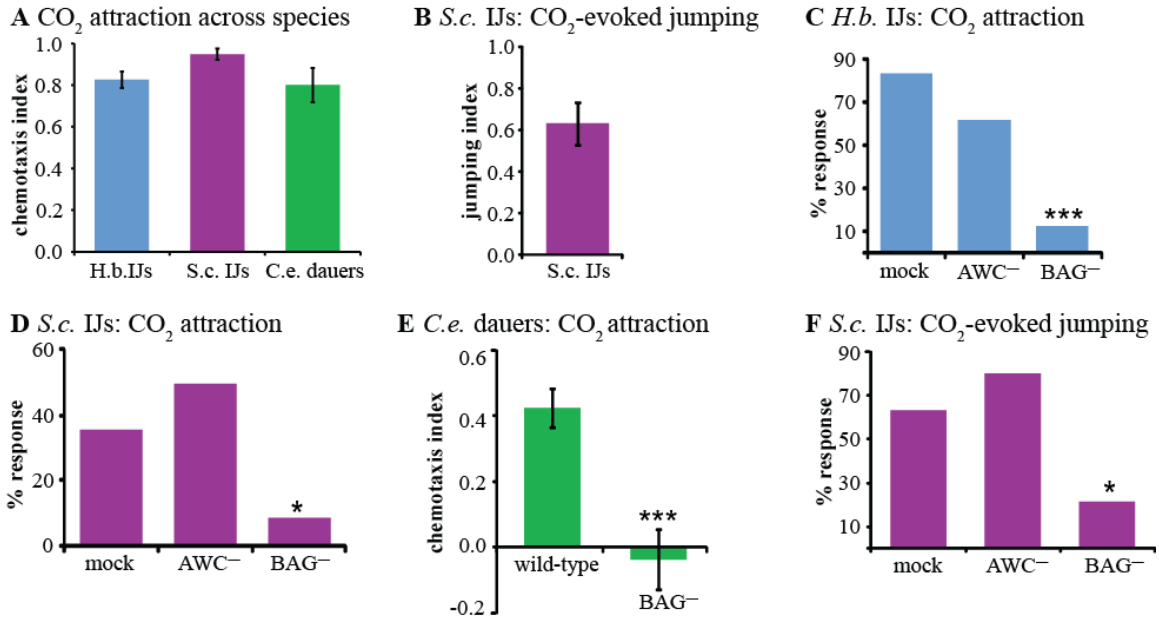

Figure 3.2 | BAG neurons are required for CO_2 response in free-living and parasitic nematodes. A. Parasitic IJs and *C. elegans* dauers are attracted to CO_2 in a chemotaxis assay (Figure S3A). n = 10–29 trials. **B.** CO_2 induces jumping by *S. carpocapsae* in a jumping assay (Figure S2B). n = 4–11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

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

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

BAG sensory neurons are required for CO_2 attraction

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

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

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

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

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

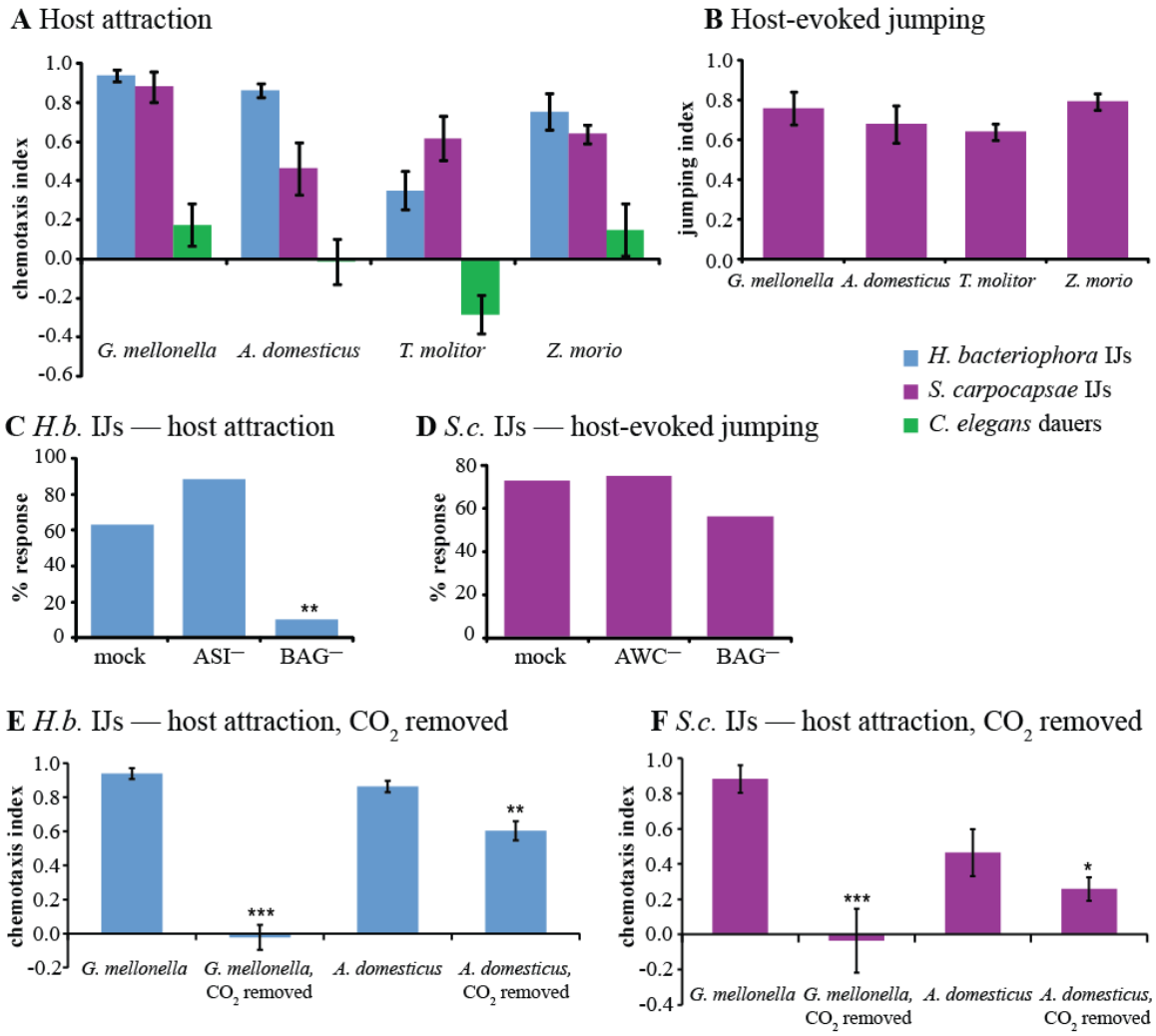

Figure 3.3 | BAG neurons are required for some but not all host-seeking behaviors.

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

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

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

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

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

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

Identification of volatiles emitted by insect larval hosts

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

Olfactory behavior in free-living versus parasitic nematodes

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

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

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

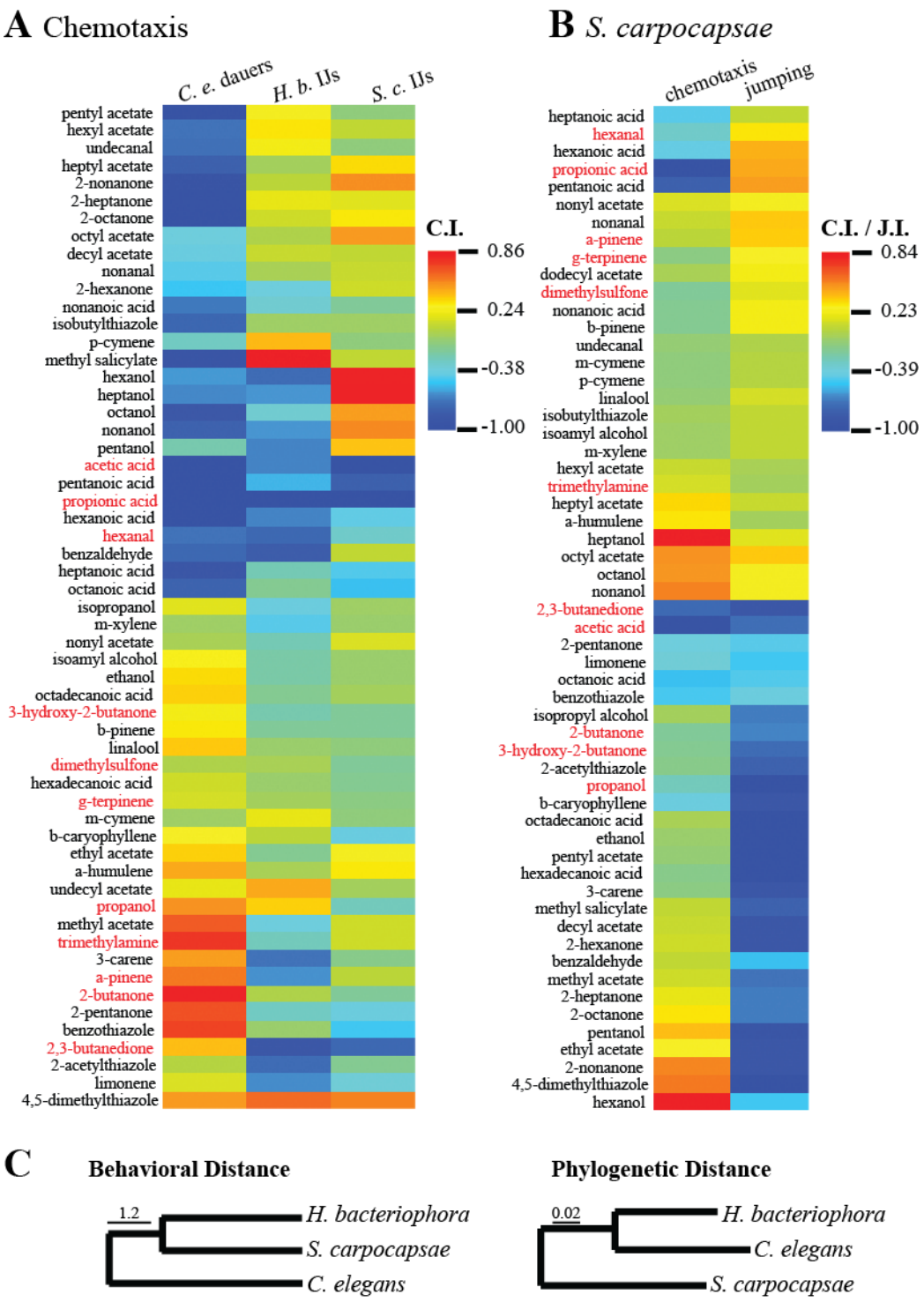

Figure 3.4 | Odor response profiles of free-living and parasitic nematodes. A. Odor response profiles of *C. elegans* dauers, *H. bacteriophora* IJs, and *S. carpocapsae* IJs. n = 5–33 trials for each odorant. **B.** A comparison of odorant-evoked chemotaxis and jumping by

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

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

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