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OLFACTORY PREFERENCES OF THE PARASITIC NEMATODE *Howardula aoronymphium* AND ITS INSECT HOST *Drosophila falleni*

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Abstract

Many parasitic nematodes have an environmental infective stage that searches for hosts. Olfaction plays an important role in this process, with nematodes navigating their environment using host-emitted and environmental olfactory cues. The interactions between parasitic nematodes and their hosts are also influenced by the olfactory behaviors of the host, since host olfactory preferences drive behaviors that may facilitate or impede parasitic infection. However, how olfaction shapes parasite-host interactions is poorly understood. Here we investigated this question using the insect-parasitic nematode *Howardula aoronymphium* and its host, the mushroom fly *Drosophila falleni.* We found that both *H. aoronymphium* and *D. falleni* are attracted to mushroom odor and a subset of mushroom-derived odorants, but they have divergent olfactory preferences that are tuned to different mushroom odorants despite their shared mushroom environment. *H. aoronymphium* and *D. falleni* respond more narrowly to odorants than *Caenorhabditis elegans* and *Drosophila melanogaster,* consistent with their more specialized niches. Infection of *D. falleni* with *H. aoronymphium* alters its olfactory preferences, rendering it more narrowly tuned to mushroom odor. Our results establish *H. aoronymphium-D. falleni* as a model system for studying olfaction in the context of parasite-host interactions.

Keywords

parasitic nematodes; mushroom *Drosophila*; olfaction; *Drosophila falleni*; *Howardula aoronymphium.*

INTRODUCTION

Parasitic nematodes comprise a diverse group of roundworms that includes both harmful and beneficial species. Harmful parasitic nematodes are a major health and economic burden worldwide. Human-parasitic nematodes infect over a billion people and cause some of the most common neglected tropical diseases (Hotez et al. 2014; Pullan et al. 2014), while parasitic nematodes of crops and livestock result in billions of dollars in economic losses

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each year (Jasmer et al. 2003). Beneficial parasitic nematodes include entomopathogenic nematodes (EPNs) that infect and kill insect pests and disease vectors, and are used as biocontrol agents (Gang and Hallem 2016). The interactions between parasitic nematodes and their hosts are shaped by the olfactory responses of both the parasite and the host: many parasitic nematodes engage in odor-driven host seeking (Gang and Hallem 2016), and hosts engage in odor-driven behaviors such as foraging that expose them to parasites. However, the relationships between the olfactory responses of parasitic nematodes and their hosts are poorly understood.

The parasitic nematode *Howardula aoronymphium* Welch 1959 and its insect host *Drosophila falleni* Wheeler 1960 are a potentially powerful but unexplored model system for investigating the contribution of olfaction to parasite-host interactions. *D. falleni* is a mycophagous *Drosophila* species found in forested regions of North America that feeds on a wide variety of mushrooms (Grimaldi 1985; Markow and O'Grady 2008). *H. aoronymphium* is a parasite of multiple mycophagous *Drosophila* species (Jaenike and Perlman 2002). The infective stage of *H. aoronymphium* is the inseminated female. Inseminated females infect fly larvae by cuticular penetration, and then grow and produce progeny inside the hemocoel as the fly develops to adulthood (Figure 1A). When the infected fly contacts a mushroom, the nematode progeny exit through the ovipositor or anus onto the mushroom. The nematodes then develop and mate within the mushroom, resulting in the formation of new infective inseminated females (Figure 1A) (Jaenike and Anderson 1992; Jaenike and Perlman 2002).

The *H. aoronymphium–D. falleni* system offers a number of advantages as a model system for studying olfaction in the context of parasitism. First, *H. aoronymphium* and *D. falleni* are easy to maintain in the lab using culturing conditions similar to those used for the model fruit fly *Drosophila melanogaster* (Haselkorn et al. 2013). Second, *H. aoronymphium* and *D. falleni* larvae can be studied using similar behavioral assays (Figure 1B), allowing direct comparisons of their olfactory preferences. Third, although *H. aoronymphium* reduces *D. falleni* adult female fecundity, it does not affect egg-to-adult survival (Haselkorn et al. 2013; Jaenike and Anderson 1992), making it possible to examine the effects of chronic infection on host behavior.

Here we examined the responses of *H. aoronymphium* and *D. falleni* larvae to the natural odors of fresh or aged mushrooms and fruit, as well as a panel of mushroom-derived odorants. We found that *H. aoronymphium* and *D. falleni* are attracted to the odor of aged mushroom and some fruits, as well as a small subset of mushroom-derived odorants. Both species respond more narrowly to odors than the generalists *C. elegans* and *D. melanogaster*, consistent with their specialized mushroom niches. In addition, infected and uninfected *D. falleni* larvae show different olfactory preferences, suggesting that *H. aoronymphium* is capable of altering the olfactory behaviors of its host. Our results establish *H. aoronymphium-D. falleni* as a model system for investigating the role of olfaction in shaping parasite-host interactions and parasite manipulation of host behavior.

MATERIALS AND METHODS

Flies and Nematodes

D. falleni Wheeler 1960 and *H. aoronymphium* Welch 1959 were collected by S. Perlman from W. Hartford, CT in 2006. *C. elegans* was the wild isolate CB4856 (Hawaii). *D. melanogaster* was the wild-type strain Canton-S.

Maintenance of D. falleni and H. aoronymphium

Uninfected *D. falleni* and *D. falleni* infected with *H. aoronymphium* were maintained using a protocol modified from Haselkorn et al. (2013). *D. falleni* was maintained at room temperature in vials containing standard *D. melanogaster* media (0.012 kg agar, 0.029 kg Red Star yeast, 0.071 kb cornmeal, 0.071 kg Grandma's Unsulphured Molasses, 16 mL 10% methylparaben, 10 mL 50% propionic acid, and 1 L dH_2O). Before use, ~200 μL of dH_2O was pipetted into the fly vials and the media was scored with a wooden tongue depressor. A 4 cm × 0.5 cm slice of fresh white mushroom (*Agaricus bisporus*) and one dental cotton roll (DEFEND, product # CSX 0200) were placed separately in each fly vial, partly submerged in the food. Mushrooms were purchased commercially, stored at 15°C in a plastic box with a moist paper towel until use, and used within 3 days. Mushrooms were peeled and sliced immediately before use. Uninfected and infected stocks were maintained separately and passaged to fresh vials at least twice a week.

In the case of *D. falleni,* third-instar larvae were used for behavioral assays. In the case of *H. aoronymphium*, mixed-stage nematode larvae obtained from dissection of 1-week-old infected *D. falleni* adults were used for behavioral assays. The 1-week-old infected *D. falleni* adults were collected from a stock of infected *D. falleni* that was maintained continuously. The flies become infected with one or more inseminated female nematodes as larvae, and the infection is then maintained through the adult stage. Adult flies contain the progeny of the original inseminated female nematodes (Figure 1A). For all chemotaxis assays involving *H. aoronymphium* (see below), most of the nematodes found in the scoring regions of the chemotaxis plates (Figure 1B) were later-stage larvae, suggesting that later-stage larvae chemotax more robustly than early-stage larvae.

Stocks of infected *D. falleni* were checked every 2–4 weeks to confirm the presence of *H. aoronymphium.* To check a stock, 10 adult flies (2 weeks post-eclosion) were dissected in 1x phosphate-buffered saline (PBS) (8 g NaCl, 0.2 g KCl, 1.15 g $Na_2HPO_4{\bullet}7H_2O$, 0.2 g KH_2PO_4, and dH_2O to 1 L) under a dissecting microscope and examined for the presence of nematodes. If fewer than 5 of the dissected flies were confirmed to have nematodes, the infected stock was discarded and a new infected stock was established using a controlled infection (see below). Stocks in which at least 7 of the dissected flies were confirmed to have nematodes were used for behavioral assays involving infected *D. falleni* larvae.

Preparation of mushroom agar plates for controlled infection of D. falleni

Mushroom agar plates were used for *D. falleni* egg collections (see below), and were prepared as described (Hamilton et al. 2014). Mushroom agar puree contained 100 g of fresh, chopped white or cremini mushrooms; 100 mL dH_2O; 2.5 g agar; 5 g sucrose; and 0.1

g methylparaben. The mixture was blended until a slurry formed. The slurry was poured into an Erlenmeyer flask and microwaved at 1000 watts for two minutes with occasional stirring. The slurry was then poured into 6 cm Petri dishes and allowed to solidify on the bench top. After solidification, the mushroom agar plates were stored upside-down at 15°C until use.

Controlled infection of D. falleni with H. aoronymphium

In order to maintain a stock of infected *D. falleni,* controlled infections of *D. falleni* with *H. aoronymphium* were performed when the infection level of any existing stock dropped below 50%. Two controlled *D. falleni* infections were always performed in parallel to ensure a successful infection. Controlled infections were performed as described (Perlman and Jaenike 2003). To obtain fly eggs, 20–30 uninfected adult flies (2 weeks post-eclosion) were placed in a Petri dish containing mushroom agar for ~16–24 hours. To obtain nematodes, 20–30 infected adult flies (2 weeks post-eclosion) were dissected in 1x PBS. ~400 nematodes were collected from the PBS and placed onto a slice of fresh, peeled white or cremini mushroom inside a Petri dish lined with moistened Whatman paper. Nematodes were incubated in the Petri dish overnight. ~40 fly eggs from the mushroom agar were then transferred to the mushroom piece containing the nematodes using a paint brush. The mushroom was moistened with a drop of 1x PBS and then transferred to a fresh fly vial containing a mushroom slice and a dental cotton roll. As soon as adult flies emerged from the vial, 1–2 flies were dissected to confirm they were infected and the remaining adult flies were transferred to a new vial.

Maintenance of C. elegans

C. elegans was cultured on 2% or 3% Nematode Growth Medium (NGM) (3 g NaCl, 2.5 g Bacto™ Peptone, 17 g agar, and dH_2O to 1 L) plates seeded with *E. coli* OP50 using standard techniques (Brenner 1974). Young adults were used for behavioral assays.

Maintenance of D. melanogaster

D. melanogaster was maintained in fly bottles using standard techniques. Third-instar larvae were used for behavioral assays.

Natural odor chemotaxis assay for nematodes

C. elegans young adults were prepared for assays as previously described (Carrillo et al. 2013). *H. aoronymphium* larvae were obtained from 1-week-old infected *D. falleni* adults. Adult flies were dissected in droplets of 1x PBS. Nematodes were continuously pipetted into clean 1x PBS droplets and recollected until clean. To prepare fresh mushroom or fruit for chemotaxis assays, peeled fresh mushroom or fruit was mashed by hand. To prepare aged mushroom or fruit, fresh mushroom or fruit was cut into slices and placed in sealed plastic bags at room temperature for one week. Assays were performed on 10-cm chemotaxis plates (17 g agar, 1 mL 1 M $CaCl_2$, 1 mL 1 M $MgSO_4$, 5 mL 1 M KPO_4 pH 6.0, and dH_2O to 1 L) (Bargmann et al. 1993). Chemotaxis plates were prepared by affixing a 1-cm × 2-cm piece of Whatman paper to each side of the lid of the plate using double-stick tape, 1 cm from the edge of the lid. 0.25 g of fruit or mushroom was added to one piece of Whatman paper using a spatula, while 100 μL dH_2O was added to the other piece of Whatman paper. In some

cases, no dH_2O was added to the other piece of Whatman paper; no differences were observed between assays with dH_2O and assays without dH_2O. In addition, neutral responses were observed for control assays involving Whatman paper with 100 μL dH_2O on one side and dry Whatman paper on the other side (Table S2). Thus, responses to natural odors were not affected by the amount of dH_2O in the odor source.

For each assay, ~100–200 nematodes were placed in the center of the plate and plates were placed on a vibration-reducing platform for 1 h for *C. elegans* or 3 h for *H. aoronymphium* (Figure 1B). The longer assay time for *H. aoronymphium* was necessary due to their slower crawling speed. A chemotaxis index (CI) was then calculated as follows (Figure 1B):

$$CI = \frac{(\#\ worms\ at\ odor - \#\ worms\ at\ control)}{(\#\ worms\ at\ odor + control)}$$

Two or more identical assays were performed simultaneously with the odor gradient oriented in opposite directions on at least two of the plates to control for directional bias due to room vibration or other causes. The set of assays was not counted if the difference in the CIs between any two of the plates was ≥0.9. Trials in which fewer than 5 worms moved into the scoring regions were also discarded. 6–12 trials were performed for each species and condition (Table S2). For statistical analysis, the responses to odors were compared to the responses to dH_2O in the dH_2O vs. dry control assay.

Odorant chemotaxis assay for nematodes

Nematodes were prepared for assays as described above. Odorant chemotaxis assays were performed as described (Lee et al. 2016), except that sodium azide was not used as an anesthetic because sodium azide was toxic for *H. aoronymphium*. Assays were performed on 10-cm chemotaxis plates (Bargmann et al. 1993). Scoring regions consisted of 2-cm diameter circles on each side of the plate along the diameter (Figure 1B). The odorant panel consisted of odorants known to be components of mushroom odor (Table S1). Odorants (Sigma-Aldrich or Fisher) were diluted 10^{-1} in paraffin oil, with the exception of ethanol, which was diluted in dH_2O. 1 μL of the 10^{-1} dilution of odorant was placed in the center of one scoring region and 1 μL of diluent was placed in the center of the other scoring region. Assays were conducted and scored as described above for natural odor assays. 8–9 trials were performed for each species and condition (Table S2). For statistical analysis, the responses to odorants were compared to the responses in the paraffin oil vs. paraffin oil control assay.

Carbon dioxide (CO_2) chemotaxis assay for nematodes

Nematodes were prepared for assays as described above. Assays were performed as previously described for *C. elegans* (Carrillo et al. 2013) and parasitic nematodes (Castelletto et al. 2014; Dillman et al. 2012; Hallem et al. 2011a). Assays were performed on 10-cm chemotaxis plates (Bargmann et al. 1993). Gas stimuli were delivered to chemotaxis plates through holes in the plate lids. The CO_2 stimulus was a certified mix of 10% CO_2, 21% O_2, balance N_2; the air control was a certified mix of 21% O_2, balance N_2 (Airgas). *C. elegans* were tested in the standard 20 min assay previously used for *C. elegans* with a gas

flow rate of 2 mL/min (Bretscher et al. 2008; Bretscher et al. 2011; Carrillo et al. 2013); *H. aoronymphium* were tested in the standard 1 h assay previously used for parasitic nematodes with a gas flow rate of 0.5 mL/min (Castelletto et al. 2014; Dillman et al. 2012; Hallem et al. 2011a). Assays were scored as described above. 8 trials were performed for each species and condition (Table S2). CO_2 was the strongest attractive odorant we identified for *H. aoronymphium* (CI = 0.99), and the CO_2 assays had an average of 53 worms (out of ~100–200 total worms) in the scoring regions of the plates. Thus, the extent to which the *H. aoronymphium* population chemotaxed was comparable to what we previously observed with other parasitic nematode species (Castelletto et al. 2014; Dillman et al. 2012; Lee et al. 2016).

Natural odor chemotaxis assay for fly larvae

Third-instar fly larvae were collected using a 15% sucrose solution (Nichols et al. 2012). The sucrose solution was poured into the fly bottle or vial, and a spatula was used to gently disrupt the surface of the media to release any larvae embedded in the media. The larvae were allowed to float up to the surface of the bottle/vial and were then collected using a 25-ml pipet with a truncated tip. The larvae were allowed to float up to the top of the pipet; the liquid under the larvae was discarded (Nichols et al. 2012). The larvae were then transferred to a watch glass. Some vials with infected *D. falleni* had fewer than 15 third-instar larvae; for these vials, larvae were transferred individually to a watch glass using a paint brush. Larvae were washed 2–3x in 15% sucrose, and then starved for 30 min in a watch glass on top of the 15% sucrose solution. Note that the fly larvae do not submerge in the sucrose solution but rather float on top of it. Larvae were starved by flotation on sucrose to prevent them from escaping during the starvation period. Larvae were starved prior to assays because *D. falleni* larvae showed more consistent and robust chemotaxis behavior following starvation. Prior to testing, the larvae were washed with 1x PBS. The larvae were then transferred to chemotaxis plates using a spatula. For assays with uninfected *D. falleni* larvae, ~40–80 fly larvae were used per assay. For assays involving infected *D. falleni* larvae, ~20–30 fly larvae were used per assay. We did not observe any differences in olfactory preferences based on the number of fly larvae used per assay.

Assays were performed on 10-cm chemotaxis plates (Bargmann et al. 1993). Assay plates were prepared as described above for natural odor chemotaxis assays for nematodes. Fly larvae were placed in the center of the plate, and plates were placed on a vibration-reducing platform for 5 min (Kreher et al. 2008). Scoring regions consisted of each half of the plate, with the exception of a 2-cm wide region in the center of the plate (Figure 1B). A CI was calculated according to the formula described above. At least two identical assays were performed simultaneously with the odor gradient oriented in opposite directions on at least two of the plates to control for directional bias due to room vibration or other causes. The set of assays was not counted if the difference in the CIs between any two of the plates was ≥0.9. When testing infected *D. falleni,* fly larvae were removed from the chemotaxis plate after the assay and dissected individually in 1x PBS droplets to confirm the presence of nematodes. Only fly larvae that were confirmed to be infected were included in the chemotaxis index calculation; assays were discarded if fewer than 5 larvae were confirmed to be infected. 6–8 trials were performed for each species and condition (Tables S3 and S4).

For statistical analysis, the responses to natural odors were compared to the responses to dH_2O in the dH_2O vs. dry control assay.

Odorant chemotaxis assay for fly larvae

Fly larvae were prepared for odorant chemotaxis assays as described above for natural odor chemotaxis assays. Assays were performed on 10-cm chemotaxis plates (Bargmann et al. 1993). A 2.5 mm circle of Whatman paper was placed on each side of the chemotaxis plate along the diameter, with the edge of the circle 1.5 cm from the edge of the plate (Figure 1B). 2 μL of undiluted odorant was placed onto one Whatman paper circle, and 2 μL of paraffin oil (or dH_2O when testing ethanol) control was placed onto the other circle. Assays were then conducted and scored as described above for natural odor chemotaxis assays for fly larvae. 6–8 trials were performed for each species and condition (Tables S3 and S4). For statistical analysis, the responses to odorants were compared to the responses in a paraffin oil vs. paraffin oil control assay.

Motility assay for uninfected and infected D. falleni larvae

D. falleni larvae were collected from vials using forceps or a paint brush, washed 2–3x in 15% sucrose in a watch glass, and starved for 30 min in a watch glass on top of the 15% sucrose solution. Prior to testing, the larvae were washed with 1x PBS. For each motility assay, 10 fly larvae were placed in the center of a 12-cm × 12-cm square chemotaxis plate on a vibration-reducing platform. The larvae were allowed to distribute on the plate for 10 minutes. The percentage of fly larvae in each of four concentric zones (Figure 5D) was then calculated. When testing infected *D. falleni,* fly larvae were removed from the chemotaxis plate after the assay and dissected to confirm the presence of nematodes. Only fly larvae confirmed to be infected were included in the analysis.

Data Analysis

Standard statistical tests were performed using GraphPad Prism. Cluster analysis was performed using PAST (Hammer et al. 2001). Heatmaps were generated using Heatmap Builder (King et al. 2005). The chemotaxis experiments that are described in Figures 2–3 were analyzed using a Kruskal-Wallis test with Dunn's post-test. The comparisons of olfactory preferences across species that are described in Figure 4 were analyzed using a two-way ANOVA with Tukey's post-test. The comparisons of olfactory preferences of uninfected vs. infected *D. falleni* larvae that are described in Figure 5 were analyzed using a two-way ANOVA with Sidak's post-test. The motility experiment described in Figure 5D was analyzed using a chi-square test.

RESULTS

H. aoronymphium is attracted to mushroom odor

We examined the olfactory behavior of *H. aoronymphium* in response to natural odors and mushroom-derived odorants using a chemotaxis assay (Figure 1B). The natural odor panel included fresh and aged white mushroom -- a food source for *H. aoronymphium* and *D. falleni* -- as well as a variety of fresh and aged fruits. The mushroom-derived odorant panel consisted of odorants emitted from a wide variety of mushrooms (Table S1). We found that

H. aoronymphium was significantly attracted to aged mushroom odor as well as the mushroom-derived ketones 3-octanone and 1-octen-3-one (Figure 2A–B). *H. aoronymphium* was also significantly attracted to the odors of aged banana, fresh strawberry, and aged strawberry, indicating that its attraction to mushroom odor is not entirely specific (Figure 2A).

A comparison of the olfactory responses of *H. aoronymphium* and *C. elegans* revealed that *H. aoronymphium* is more narrowly tuned to odorants than *C. elegans* (Figure 2). *H. aoronymphium* was significantly attracted to four of the eight natural odors tested (aged mushroom, aged banana, fresh strawberry, and aged strawberry), while *C. elegans* was significantly attracted to six of the eight natural odors tested (aged mushroom, fresh banana, aged banana, fresh orange, fresh strawberry, and aged strawberry). In addition, *H. aoronymphium* was significantly attracted to two of the twelve odorants tested (3-octanone and 1-octen-3-one), while *C. elegans* was significantly attracted to seven of the odorants (ethyl acetate, 1-pentanol, 3-penten-2-ol, 3-octanone, 1-octen-3-one, 3-methyl-1-butanol, and benzaldehyde) (Figure 2B, D). These results are consistent with the more specialized ecological niche of *H. aoronymphium* as compared to *C. elegans*: the free-living stages of *H. aoronymphium* are specifically associated with mushrooms (Jaenike and Perlman 2002), whereas *C. elegans* is associated with a wide variety of rotting plant material (Felix and Duveau 2012).

H. aoronymphium is attracted to CO_2

H. aoronymphium actively invades *D. falleni* larvae, suggesting that like other actively invading parasites, it is likely to use host-emitted sensory cues to locate hosts (Gang and Hallem 2016). One possible cue is CO_2. CO_2 is emitted by all aerobic organisms as a byproduct of respiration and is a critical host cue for many parasites, including entomopathogenic nematodes (Dillman et al. 2012; Gaugler et al. 1991; Hallem et al. 2011a; Koppenhofer and Fuzy 2008; Robinson 1995). We found that *H. aoronymphium* was strongly attracted to CO_2 (Figure 2B), raising the possibility that it uses CO_2 to identify fly larvae to infect. By contrast, the *C. elegans* Hawaii strain, a wild isolate, was neutral to CO_2 under the conditions tested (Figure 2D) (Carrillo et al. 2013; Kodama-Namba et al. 2013). However, we note that the *H. aoronymphium* larvae we tested are pre-infective, and we cannot exclude the possibility that infective *H. aoronymphium* inseminated females respond differently to CO_2.

D. falleni and H. aoronymphium are attracted to distinct mushroom odorants

We then examined the olfactory preferences of *D. falleni* larvae. Like *H. aoronymphium, D. falleni* was significantly attracted to aged mushroom odor (Figure 3A). *D. falleni* was also significantly attracted to aged banana odor and aged orange odor (Figure 3A). Of the mushroom-derived odorants, *D. falleni* was attracted to 1-pentanol, 1-octen-3-ol, and 3-methyl-1-butanol (Figure 3B). These mushroom odorants are distinct from those that attract *H. aoronymphium*, suggesting that *H. aoronymphium* and *D. falleni* utilize different mushroom-derived olfactory cues. In contrast to *D. falleni* larvae, *D. melanogaster* larvae showed significant attraction to the majority of natural odors and mushroom-derived odorants tested (Figure 3C–D).

A comparison of the olfactory preferences of *H. aoronymphium, C. elegans, D. falleni,* and *D. melanogaster* demonstrated that each species has a unique odor response profile (Figure 4A–B). Clustering the species based on their odor responses revealed that the olfactory preferences of *C. elegans* and *D. melanogaster* are most similar, while the olfactory preferences of *H. aoronymphium* and *D. falleni* are highly dissimilar (Figure 4C). Thus, parasitic nematodes and their hosts can show very different olfactory preferences despite their shared environmental niche. A comparison of tuning across species revealed that *H. aoronymphium* and *D. falleni* were both attracted to 30% of tested odors, whereas *C. elegans* and *D. melanogaster* were both attracted to 65% of tested odors (Figure 4D). Thus, the specialists responded to approximately half as many tested odors as the generalists. None of the species showed significant repulsion from any of the tested odors (Figure 2–4).

Nematode infection alters the olfactory preferences of D. falleni

Many parasites, from intracellular parasites to parasitic worms to parasitoid wasps, appear to manipulate the behaviors of their hosts in ways that promote parasite survival and transmission (Adamo 2013; Thomas et al. 2011). We compared the olfactory behaviors of uninfected and infected *D. falleni,* and found that nematode infection results in specific changes in the olfactory preferences of *D. falleni* larvae in response to both natural odors and mushroom-derived odorants (Figure 5A–B). Aged banana and aged orange odor were attractive to uninfected fly larvae but neutral to infected fly larvae (Figure 5A). In addition, ethyl acetate and 1-pentanol were attractive to uninfected fly larvae but repulsive or neutral to infected fly larvae, while 1-nonanal showed the opposite change (Figure 5B). To determine whether the observed changes in olfactory preferences generalized to structurally similar odorants, we tested an additional panel consisting of two odorants that are structurally similar to ethyl acetate (methyl acetate and propyl acetate) and two odorants that are structurally similar to 1-nonanal (1-octanal and 1-decanal). We found that three of the four additional odorants – methyl acetate, propyl acetate, and 1-octanal – elicited different responses from uninfected versus infected fly larvae (Figure 5C). Thus, the behavioral responses of *D. falleni* larvae to certain groups of odorants, such as short-chain acetates and long-chain aldehydes, are altered as a result of nematode infection. By contrast, nematode infection did not impair larval motility (Figure 5D), suggesting that the behavioral changes induced by nematode infection are not a result of general morbidity but rather are a result of specific changes in olfactory preferences.

DISCUSSION

We have found that both *H. aoronymphium* and *D. falleni* larvae are attracted to aged mushroom odor and a small subset of mushroom-derived odorants (Figure 2–3). Fresh mushroom was not attractive to either species, indicating a strong preference for rotting mushroom. These results are consistent with the fact that *H. aoronymphium* and *D. falleni* larvae inhabit a shared mushroom environment (Markow and O'Grady 2008). Despite their shared attraction to aged mushrooms, *H. aoronymphium* and *D. falleni* larvae showed very different overall odor response profiles (Figure 4A–C), suggesting they have evolved different strategies for responding to mushroom odor.

We have shown that *D. falleni* larvae respond behaviorally to a narrower range of odorants than *D. melanogaster* larvae (Figure 4), consistent with the fact that *D. melanogaster* is a generalist while *D. falleni* is a mushroom specialist, although it does not specialize on particular mushroom species (Jaenike and Perlman 2002; Markow 2015; Sokolowski 1985). Previous studies of insect generalists and specialists have revealed a number of mechanisms that enable specialists to engage in specific interactions with their food sources or oviposition substrates. For example, a comparison of *D. melanogaster* and *Drosophila sechellia*, a specialist that oviposits only on morinda fruit, revealed that *D. sechellia* shows enhanced attraction to a subset of morinda-derived odorants and increased abundance of the antennal neurons that respond to these odorants (Dekker et al. 2006). Similar changes have been observed for other *Drosophila* species, mosquitos, and bees (Burger et al. 2013; Crowley-Gall et al. 2016; Linz et al. 2013; Syed and Leal 2009). In addition to showing enhanced responses to odorants emitted by their food sources or oviposition substrates, some specialists also show reduced responses to odorants emitted by other sources. For example, specialist bees respond to fewer odorants than generalist bees, a difference that is accompanied by a reduction in the number of olfactory glomeruli, or functional processing units, in the antennal lobes of the specialists (Burger et al. 2013). Similarly, the herbivorous specialist Drosophilid *Scaptomyza flava* responds to fewer yeast odorants than *D. melanogaster*, a difference that is accompanied by a loss of odorant receptor genes (Goldman-Huertas et al. 2015). The evolutionary changes in olfactory system function and organization that underlie the behavioral differences between *D. melanogaster* and *D. falleni* remain to be determined.

While we have identified substantial differences in olfactory preferences between *D. melanogaster* and *D. falleni* larvae, these differences may be even more pronounced at the adult stage since adult flies engage in long-range environmental navigation. Whereas *D. falleni* larvae navigate primarily within mushrooms, *D. falleni* adults navigate between mushrooms (Jaenike and Perlman 2002). In future studies, it will be interesting to examine the olfactory responses of *D. falleni* adults to mushroom versus non-mushroom odors to determine how *D. falleni* adults select mushrooms over other potential breeding sites. Interestingly, mushroom-feeding *Drosophila* (Jaenike et al. 1983) but not *Howardula* nematodes (Jaenike 1985) are resistant to α-amanitin, the deadly toxin in *Amanita* mushrooms; yet neither uninfected nor worm-infected flies appear to prefer amanitin-containing mushrooms (Debban and Dyer 2013). In addition, while *D. melanogaster* larvae exposed to parasitoid wasps self-medicate against wasp infection by increasing their preference for ethanol-containing food (Milan et al. 2012), *D. falleni* larvae infected with *H. aoronymphium* do not show increased attraction to ethanol (Figure 5B), suggesting they do not employ a similar strategy to combat *H. aoronymphium* infection.

In the case of parasitic nematodes, their olfactory behaviors have been shown to reflect their host ranges and infection modes, suggesting that olfaction plays an important role in host seeking (Castelletto et al. 2014; Dillman et al. 2012; Hallem et al. 2011a). We have found that *H. aoronymphium* is strongly attracted to CO_2 (Figure 2B), suggesting that like EPNs (Gang and Hallem 2016), *H. aoronymphium* uses CO_2 in combination with host-specific odorants to locate hosts. However, we note that the *H. aoronymphium* larvae we tested were pre-infective, and at this developmental stage the nematodes may respond primarily to

mushroom odors rather than host odors. Future studies of infective inseminated females will provide insight into how they use CO_2 and host-specific odorants to locate fly larvae, whether they use olfaction to distinguish among the many species of *Drosophila* larvae that are often present in the same mushroom (Jaenike and Perlman 2002), and whether their olfactory preferences differ from those of pre-infective stage nematodes to support the processes of host finding and host selection.

At the neuronal level, the only olfactory neurons that have so far been identified in parasitic nematodes are the CO_2-sensing BAG neurons, which play a conserved role in mediating CO_2 response in the free-living nematode *C. elegans*, the necromenic nematode *Pristionchus pacificus*, and the entomopathogenic nematodes *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* (Bretscher et al. 2008; Bretscher et al. 2011; Carrillo et al. 2013; Hallem et al. 2011a; Hallem et al. 2011b; Hallem and Sternberg 2008). However, because sensory neuroanatomy is generally conserved across nematode species, the positional analogs of the *C. elegans* AWA, AWB, and AWC olfactory neurons are likely to mediate olfactory behaviors in parasitic nematodes (Gang and Hallem 2016; Rengarajan and Hallem 2016). Differences in olfactory behavior across species may arise from species-specific differences in the functional properties of olfactory microcircuit neurons and/or the wiring of these neurons. At the molecular level, functional differences could arise from species-specific differences in the expression patterns, ligand specificities, or identities of odorant receptors or other olfactory signaling components.

A comparison of the olfactory behavior of uninfected versus infected *D. falleni* revealed that infection with parasitic nematodes causes specific changes in the olfactory preferences of *D. falleni* larvae for both natural odors and mushroom odorants (Figure 5). Infection of other insects with parasitic nematodes has also been shown to alter insect behavior. For example, the human-parasitic filarial nematode *Brugia malayi* alters the behavior of the female mosquito, its intermediate host (Gleave et al. 2016). Mosquitoes harboring *B. malayi* are less likely to blood feed while the nematodes are developing but are more likely to blood feed once the nematodes are infective. This behavioral change may promote parasite transmission by limiting risky behaviors such as host seeking and blood feeding before the parasite can be transmitted and enhancing them when the parasite can be transmitted (Gleave et al. 2016). Similar behavioral changes occur when mosquitoes are infected with *Plasmodium* and correspond to changes in the sensitivity of olfactory neurons on the maxillary palp (Cator et al. 2013). However, similar behavioral changes also occur when mosquitoes are exposed to heat-killed *Escherichia coli,* suggesting that these changes are a generalized response to immune challenge (Cator et al. 2013). Whether the changes we observe with *D. falleni* enhance *H. aoronymphium* survival, and whether they are a specific response to *H. aoronymphium*, a generalized response to immune challenge, or a consequence of some other aspect of nematode exposure remains to be determined. In addition, co-infection of mushroom *Drosophila* with *Spiroplasma* bacteria and *H. aoronymphium* reduces nematode fitness (Haselkorn et al. 2013; Jaenike et al. 2010), and in future studies it will be interesting to determine whether bacterial infection restores wild-type olfactory preferences to nematode-infected fly larvae.

In summary, we have described a new model system for investigating the olfactory behaviors of parasitic nematodes and their hosts, and the poorly understood effects of parasitic nematode infection on host sensory behaviors. The model system consists of the parasitic nematode *H. aoronymphium* and its insect host *D. falleni,* both of which are easy to maintain and manipulate in the lab and are amenable to quantitative behavioral analysis. The olfactory system of *D. falleni* is also likely amenable to the same type of electrophysiological characterization as other *Drosophila* species and other insects (Pellegrino et al. 2010). Thus, future studies of the *H. aoronymphium–D. falleni* system should greatly enhance our understanding of the mechanisms underlying parasite-induced changes in host olfactory function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

Adamo SA. Parasites: evolution’s neurobiologists. J Exp Biol. 2013; 216:3–10. [PubMed: 23225861]

Bargmann CI, Hartwieg E, Horvitz HR. Odorant-selective genes and neurons mediate olfaction in *C. elegans*. Cell. 1993; 74:515–527. [PubMed: 8348618]

Brenner S. The genetics of *Caenorhabditis elegans*. Genetics. 1974; 77:71–94. [PubMed: 4366476]

Bretscher AJ, Busch KE, de Bono M. A carbon dioxide avoidance behavior is integrated with responses to ambient oxygen and food in *Caenorhabditis elegans*. Proc Natl Acad Sci USA. 2008; 105:8044–8049. [PubMed: 18524954]

Bretscher AJ, Kodama-Namba E, Busch KE, Murphy RJ, Soltesz Z, Laurent P, et al. Temperature, oxygen, and salt-sensing neurons in *C. elegans* are carbon dioxide sensors that control avoidance behavior. Neuron. 2011; 69:1099–1113. [PubMed: 21435556]

Burger H, Ayasse M, Dotterl S, Kreissl S, Galizia CG. Perception of floral volatiles involved in host-plant finding behaviour: comparison of a bee specialist and generalist. J Comp Physiol A Neuroethol Sens Neural Behav Physiol. 2013; 199:751–761. [PubMed: 23793798]

Carrillo MA, Guillermin ML, Rengarajan S, Okubo R, Hallem EA. O_2-sensing neurons control CO_2 response in *C. elegans*. J Neurosci. 2013; 33:9675–9683. [PubMed: 23739964]

Castelletto ML, Gang SS, Okubo RP, Tselikova AA, Nolan TJ, Platzer EG, et al. Diverse host-seeking behaviors of skin-penetrating nematodes. PLoS Pathog. 2014; 10:e1004305. [PubMed: 25121736]

Cator LJ, George J, Blanford S, Murdock CC, Baker TC, Read AF, et al. ‘Manipulation’ without the parasite: altered feeding behaviour of mosquitoes is not dependent on infection with malaria parasites. Proc R Soc B. 2013; 280:20130711.

Crowley-Gall A, Date P, Han C, Rhodes N, Andolfatto P, Layne JE, et al. Population differences in olfaction accompany host shift in *Drosophila mojavensis*. Proc Biol Sci. 2016; 283:20161562. [PubMed: 27581882]

Debban CL, Dyer KA. No evidence for behavioural adaptations to nematode parasitism by the fly *Drosophila putrida*. J Evol Biol. 2013; 26:1646–1654. [PubMed: 23663194]

Dekker T, Ibba I, Siju KP, Stensmyr MC, Hansson BS. Olfactory shifts parallel superspecialism for toxic fruit in *Drosophila melanogaster* sibling, *D. sechellia*. Curr Biol. 2006; 16:101–109. [PubMed: 16401429]

Dillman AR, Guillermin ML, Lee JH, Kim B, Sternberg PW, Hallem EA. Olfaction shapes host-parasite interactions in parasitic nematodes. Proc Natl Acad Sci USA. 2012; 109:E2324–2333. [PubMed: 22851767]

Felix MA, Duveau F. Population dynamics and habitat sharing of natural populations of *Caenorhabditis elegans* and *C. briggsae*. BMC Biol. 2012; 10:59. [PubMed: 22731941]

Gang SS, Hallem EA. Mechanisms of host seeking by parasitic nematodes. Mol Biochem Parasitol. 2016; 208:23–32. [PubMed: 27211240]

Gaugler R, Campbell JF, Gupta P. Characterization and basis of enhanced host-finding in a genetically improved strain of *Steinernema carpocapsae*. J Invert Pathol. 1991; 57:234–241.

Gleave K, Cook D, Taylor MJ, Reimer LJ. Filarial infection influences mosquito behaviour and fecundity. Sci Rep. 2016; 6:36319. [PubMed: 27796352]

Goldman-Huertas B, Mitchell RF, Lapoint RT, Faucher CP, Hildebrand JG, Whiteman NK. Evolution of herbivory in Drosophilidae linked to loss of behaviors, antennal responses, odorant receptors, and ancestral diet. Proc Natl Acad Sci USA. 2015; 112:3026–3031. [PubMed: 25624509]

Grimaldi D. Niche separation and competitive coexistance in mycophagous *Drosophila* (Diptera: Drosophilidae). Proc Entomol Soc Wash. 1985; 87:498–511.

Hallem EA, Dillman AR, Hong AV, Zhang Y, Yano JM, DeMarco SF, et al. A sensory code for host seeking in parasitic nematodes. Curr Biol. 2011a; 21:377–383. [PubMed: 21353558]

Hallem EA, Spencer WC, McWhirter RD, Zeller G, Henz SR, Ratsch G, et al. Receptor-type guanylate cyclase is required for carbon dioxide sensation by *Caenorhabditis elegans*. Proc Natl Acad Sci USA. 2011b; 108:254–259. [PubMed: 21173231]

Hallem EA, Sternberg PW. Acute carbon dioxide avoidance in *Caenorhabditis elegans*. Proc Natl Acad Sci USA. 2008; 105:8038–8043. [PubMed: 18524955]

Hamilton PT, Leong JS, Koop BF, Perlman SJ. Transcriptional responses in a *Drosophila* defensive symbiosis. Mol Ecol. 2014; 23:1558–1570. [PubMed: 24274471]

Hammer Ø, Harper DAT, Ryan PD. PAST: Paleontological statistics software package for education and data analysis. Palaeontol Electronica. 2001; 4:9.

Haselkorn TS, Cockburn SN, Hamilton PT, Perlman SJ, Jaenike J. Infectious adaptation: potential host range of a defensive endosymbiont in *Drosophila*. Evolution. 2013; 67:934–945. [PubMed: 23550746]

Hotez PJ, Alvarado M, Basanez MG, Bolliger I, Bourne R, Boussinesq M, et al. The global burden of disease study 2010: interpretation and implications for the neglected tropical diseases. PLoS Negl Trop Dis. 2014; 8:e2865. [PubMed: 25058013]

Jaenike J. Parasite pressure and the evolution of amanitin tolerance in *Drosophila*. Evolution. 1985; 39:1295–1301. [PubMed: 28564265]

Jaenike J, Anderson TJC. Dynamics of host-parasite interactions: the *Drosophila-Howardula* system. Oikos. 1992; 64:533–540.

Jaenike J, Grimaldi DA, Sluder AE, Greenleaf AL. α-Amanitin tolerance in mycophagous *Drosophila*. Science. 1983; 221:165–167. [PubMed: 17769215]

Jaenike J, Perlman SJ. Ecology and evolution of host-parasite associations: mycophagous *Drosophila* and their parasitic nematodes. Am Nat. 2002; 160(Suppl 4):S23–39. [PubMed: 18707451]

Jaenike J, Unckless R, Cockburn SN, Boelio LM, Perlman SJ. Adaptation via symbiosis: recent spread of a *Drosophila* defensive symbiont. Science. 2010; 329:212–215. [PubMed: 20616278]

Jasmer DP, Goverse A, Smant G. Parasitic nematode interactions with mammals and plants. Annu Rev Phytopathol. 2003; 41:245–270. [PubMed: 14527330]

King JY, Ferrara R, Tabibiazar R, Spin JM, Chen MM, Kuchinsky A, et al. Pathway analysis of coronary atherosclerosis. Physiol Genomics. 2005; 23:103–118. [PubMed: 15942018]

Kodama-Namba E, Fenk LA, Bretscher AJ, Gross E, Busch KE, de Bono M. Cross-modulation of homeostatic responses to temperature, oxygen and carbon dioxide in *C. elegans*. PLoS Genet. 2013; 9:e1004011. [PubMed: 24385919]

Koppenhofer AM, Fuzy EM. Attraction of four entomopathogenic nematodes to four white grub species. J Invert Path. 2008; 99:227–234.

Kreher SA, Mathew D, Kim J, Carlson JR. Translation of sensory input into behavioral output via an olfactory system. Neuron. 2008; 59:110–124. [PubMed: 18614033]

Lee J, Dillman AR, Hallem EA. Temperature-dependent changes in the host-seeking behaviors of parasitic nematodes. BMC Biol. 2016; 14:36. [PubMed: 27154502]

Linz J, Baschwitz A, Strutz A, Dweck HK, Sachse S, Hansson BS, et al. Host plant-driven sensory specialization in *Drosophila erecta*. Proc Biol Sci. 2013; 280:20130626. [PubMed: 23595274]

Markow TA. The secret lives of *Drosophila* flies. Elife. 2015:4.

Markow TA, O'Grady P. Reproductive ecology of *Drosophila*. Funct Ecol. 2008; 22:747–759.

Milan NF, Kacsoh BZ, Schlenke TA. Alcohol consumption as self-medication against blood-borne parasites in the fruit fly. Curr Biol. 2012; 22:488–493. [PubMed: 22342747]

Nichols CD, Becnel J, Pandey UB. Methods to assay *Drosophila* behavior. J Vis Exp. 2012; 61:3795.

Pellegrino M, Nakagawa T, Vosshall LB. Single sensillum recordings in the insects *Drosophila melanogaster* and *Anopheles gambiae*. J Vis Exp. 2010:1–5. [PubMed: 20164822]

Perlman SJ, Jaenike J. Infection success in novel hosts: an experimental and phylogenetic study of *Drosophila*-parasitic nematodes. Evolution. 2003; 57:544–557. [PubMed: 12703944]

Pullan RL, Smith JL, Jasrasaria R, Brooker SJ. Global numbers of infection and disease burden of soil transmitted helminth infections in 2010. Parasit Vectors. 2014; 7:37. [PubMed: 24447578]

Rengarajan S, Hallem EA. Olfactory circuits and behaviors of nematodes. Curr Opin Neurobiol. 2016; 41:136–148. [PubMed: 27668755]

Robinson AF. Optimal release rates for attracting *Meloidogyne incognita, Rotylenchulus reniformis*, and other nematodes to carbon dioxide in sand. J Nematol. 1995; 27:42–50. [PubMed: 19277260]

Sokolowski MB. Genetics and ecology of *Drosophila melanogaster* larval foraging and pupation behavior. J Insect Physiol. 1985; 31:857–864.

Syed Z, Leal WS. Acute olfactory response of *Culex* mosquitoes to a human- and bird-derived attractant. Proc Natl Acad Sci USA. 2009; 106:18803–18808. [PubMed: 19858490]

Thomas F, Brodeur J, Maure F, Franceschi N, Blanchet S, Rigaud T. Intraspecific variability in host manipulation by parasites. Infect Genet Evol. 2011; 11:262–269. [PubMed: 21211573]

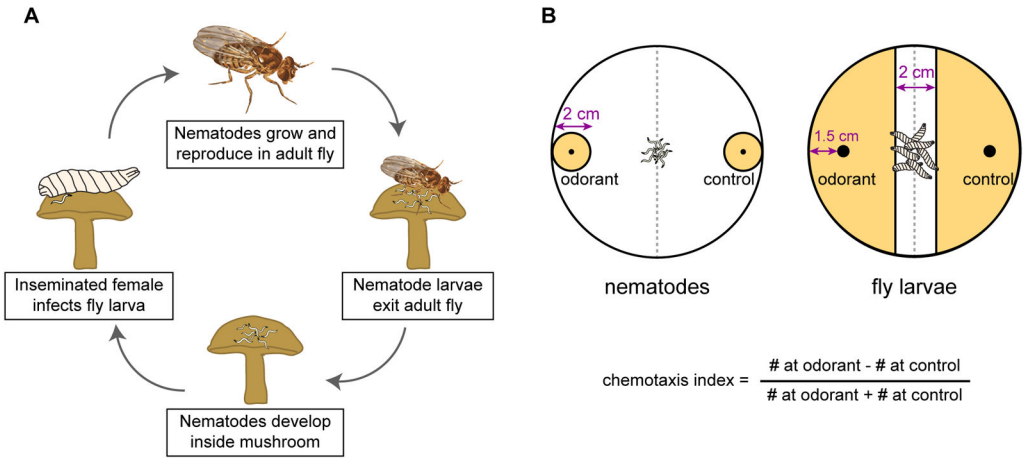

Fig. 1.

The *H. aoronymphium–D. falleni* model system. **A.** The life cycle of *H. aoronymphium.* The infective stage of *H. aoronymphium* is the inseminated female, which infects fly larvae. The nematodes remain in the host through metamorphosis, and grow and reproduce in the fly hemocoel. Juvenile progeny exit the adult fly through the ovipositor or anus when the fly contacts a mushroom. The nematodes develop and mate inside the mushroom, resulting in new infective inseminated females. Adapted from Jaenike and Perlman (2002). **B.** Chemotaxis assays for nematodes (left) and fly larvae (right). For both assays, an odorant was placed on one side of the plate and a control on the other side. Nematodes or fly larvae were placed in the center of a plate and allowed to distribute in the odor gradient. The number of animals in each scoring region (beige) was then counted, and a chemotaxis index (CI) was calculated as indicated. The CI ranges from +1 (maximal attraction) to −1 (maximal repulsion).

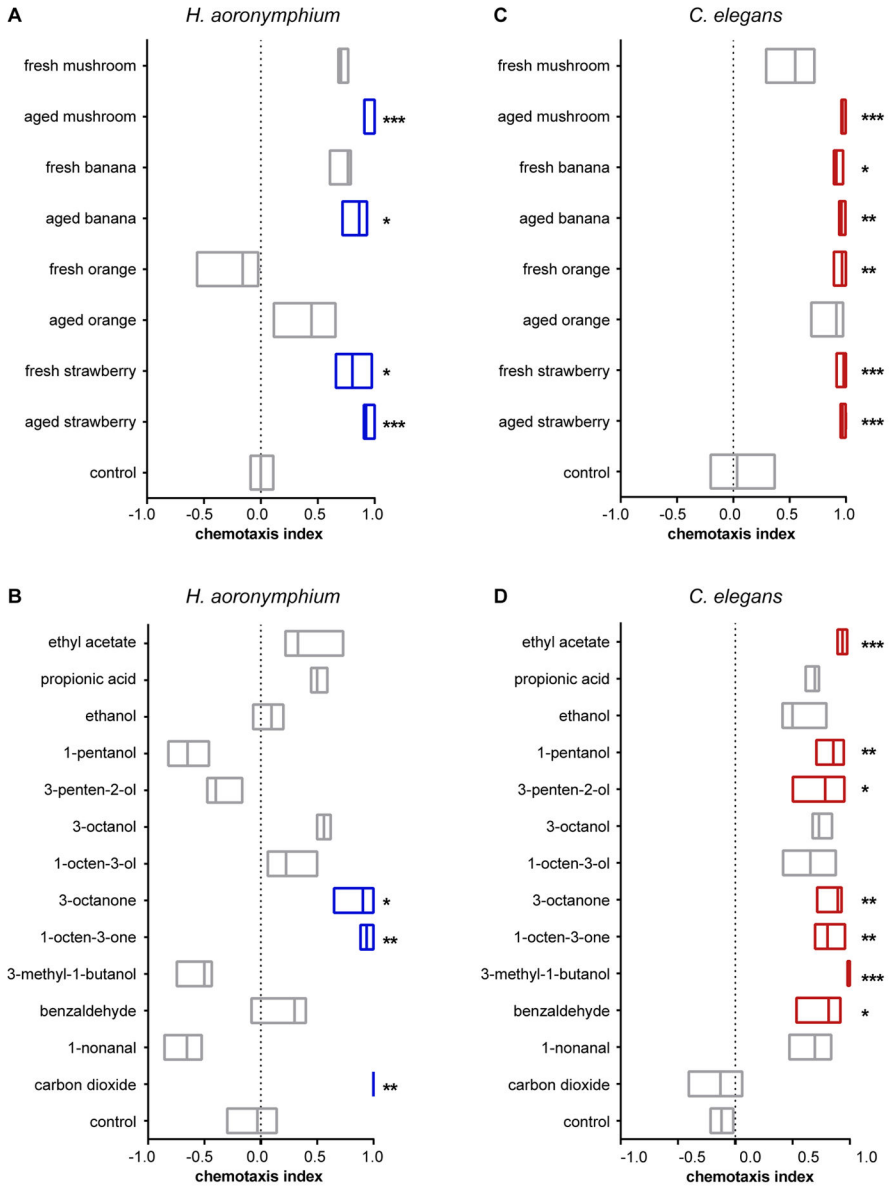

Fig. 2.

Olfactory behaviors of *H. aoronymphium* and *C. elegans*. **A–B.** Responses of *H. aoronymphium* to natural odors (A) and mushroom-derived odorants (B) in a chemotaxis assay. **C–D.** Responses of *C. elegans* to natural odors (C) and mushroom-derived odorants (D) in a chemotaxis assay. Attractive responses are colored. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Kruskal-Wallis test with Dunn's post-test. n = 6–9 trials for each condition. Graphs show medians and interquartile ranges. Medians, quartiles, and n values for each condition are listed in Table S2.

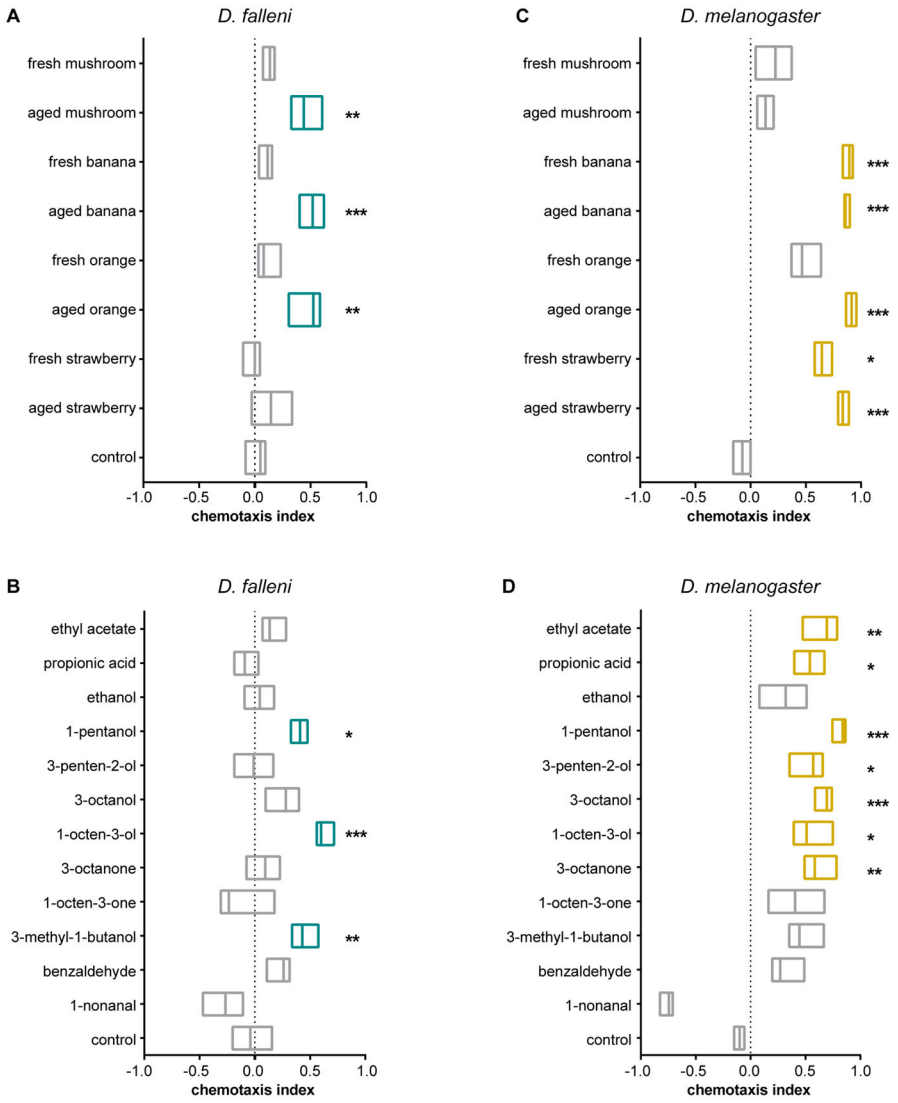

Fig. 3.

Olfactory behaviors of *D. falleni* and *D. melanogaster.* **A–B.** Responses of *D. falleni* larvae to natural odors (A) and mushroom-derived odorants (B) in a chemotaxis assay. **C–D.** Responses of *D. melanogaster* larvae to natural odors (C) and mushroom-derived odorants (D) in a chemotaxis assay. Attractive responses are colored. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, Kruskal-Wallis test with Dunn's post-test. n = 6–8 trials for each condition. Graphs show medians and interquartile ranges. Medians, quartiles, and n values for each condition are listed in Table S3.

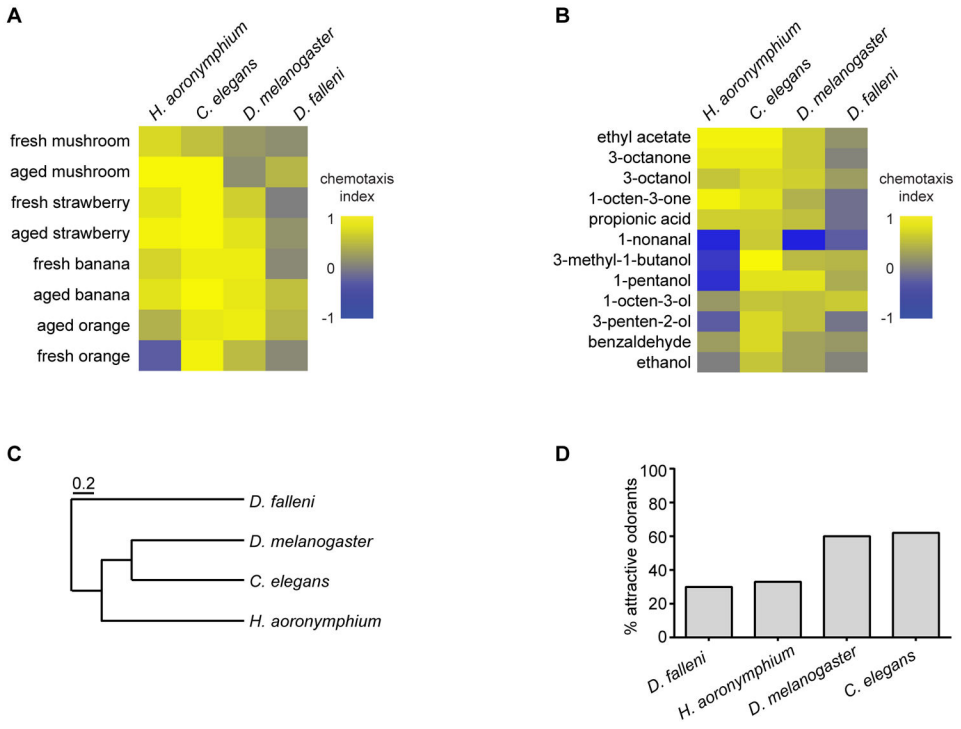

Fig. 4.

A comparison of olfactory behaviors across species. **A–B.** A comparison of responses to natural odors (A) and mushroom-derived odorants (B) across species. Responses are represented as heatmaps according to the scale shown; odors and species are ordered based on hierarchical cluster analysis. Each species responds differently to the odor panels (two-way ANOVA with Tukey's post-test, $P < 0.001$). **C.** A behavioral dendrogram in which species are clustered based on their responses to the natural odor panel. Hierarchical cluster analysis was performed using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) with Euclidean distance as a similarity index. Coph. corr. = 0.82. **D.** A comparison of the percentage of olfactory stimuli that elicited significant responses for each species. *D. falleni* and *H. aoronymphium* responded to fewer odorants than *D. melanogaster* and *C. elegans* ($P < 0.001$, chi-square test). Data are from Figures 2–3.

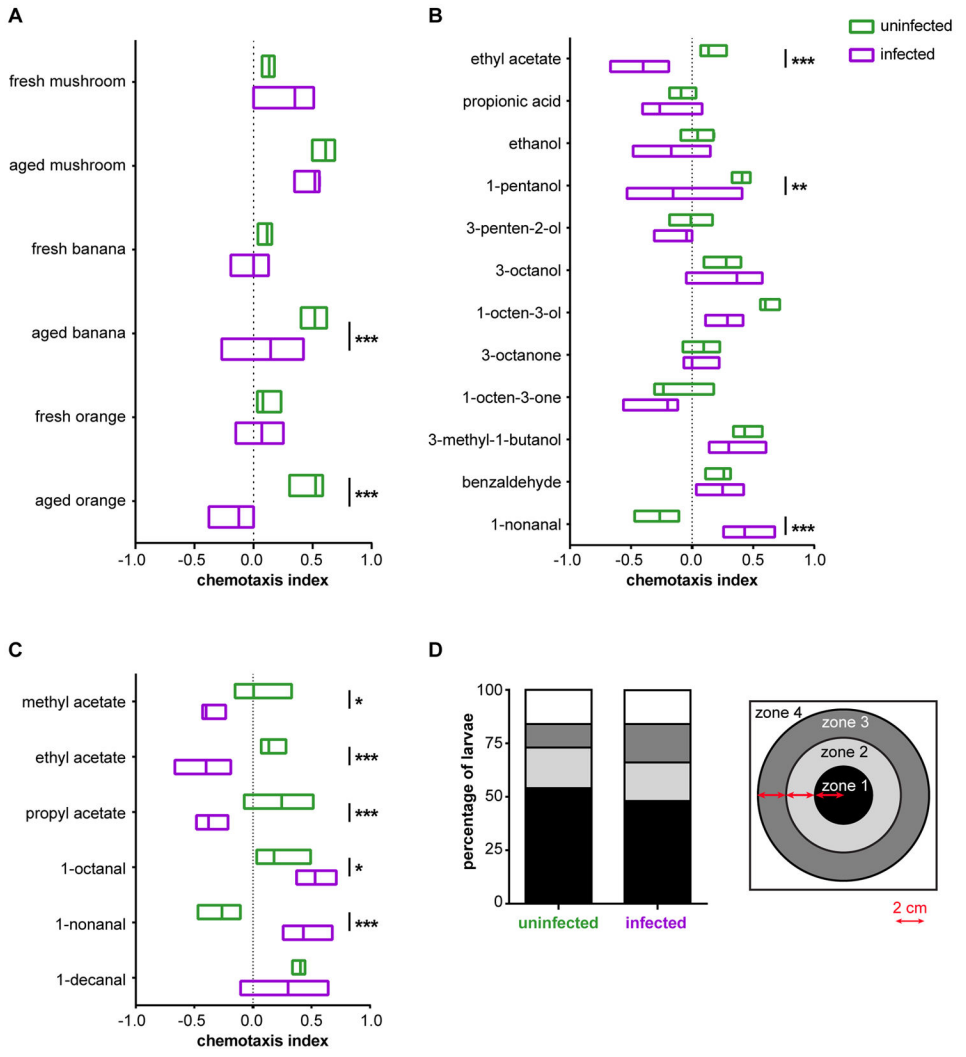

Fig. 5.

Uninfected and infected *D. falleni* larvae have different olfactory preferences. **A–B.** Responses of uninfected (green) vs. infected (purple) *D. falleni* larvae to natural odors (A) and mushroom-derived odorants (B). **C.** Responses of uninfected vs. infected larvae to an additional odorant panel chosen to include odorants that are structurally similar to ethyl acetate or 1-nonanal, two of the odorants that elicited different responses from uninfected vs. infected larvae. For A–C, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; two-way ANOVA with Sidak's post-test. n = 6–8 trials for each condition. Graphs show medians and interquartile ranges. Medians, quartiles, and n values for each condition are listed in Table S4. **D.** Motility of uninfected vs. infected larvae in the absence of olfactory stimuli. Larvae were allowed to distribute on a plate for 10 min, and the number of larvae in each of the four zones indicated was counted. No significant differences were observed between the motility of uninfected vs. infected larvae ($P = 0.5383$, chi-square test). n = 7–8 trials for each condition, with up to 10 larvae per trial. Data were analyzed in a contingency table as the total percentage of larvae in each zone from all trials combined.