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Odors and Genetic Pathways Influencing Resource-Seeking in EPNs and Free-Living Nematodes

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DEDICATION

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ABSTRACT OF THE DISSERTATION

Odors and Genetic Pathways Influencing Resource-Seeking in EPNs and Free-Living Nematodes

by

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Entomopathogenic nematodes (EPNs) are insect-killing parasitic worms that are utilized in agriculture and home-garden use against a variety of insect pests. It is the infective juvenile stage (IJ) of the EPN life cycle responsible for locating, infecting and colonizing a new insect host to continue the life cycle and produce progeny. My research revealed that EPN species within the genus *Steinernema,* responded to host volatiles, in species—specific patterns with regard to both behavioral trends to the progression of infection, as well as overall participation. Analysis of host-odor profiles- for both naive and infected hosts (including recently infected and long-term, resource depleted insect cadavers) produced a variety of odors and one of the more notable odors produced was 3-methyl-2-buten-1-ol (prenol). EPN IJs were found to be repelled by prenol at a variety of doses (ranging from 2M to 20mM) and prenol yielded age-dependent increases in dispersal, implicating prenol as a potential IJ dispersal cue. However, lack of molecular tools in EPNs prevented investigations into the molecular underpinnings of how prenol is detected and translated into the observable repulsion. To overcome this obstacle, we leveraged the model organisms: *Caenorhabditis elegans* which exhibits attraction to prenol. Through use of

natural variation, genome-wide association, and leveraging multiple genetic resources in the *C. elegans* community we identified the involvement of the AWC neuron in detection and response to prenol as well as eight genes that influence responses to prenol, including genes that have not previously been shown to affect chemotactic behaviors: *dod-17, clec-39, dcap-1* and *dcap-2*. For many of these genes, we have found EPN orthologs exist, meaning we have uncovered information that potentially could be utilized to better understand EPN behavioral ecology such that EPNs may be improved or utilized more effectively in the future.

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I. ABBREVIATIONS

(**EPN**) Entomopathogenic nematodes

(**IJ**) Infective juvenile

(**CI**) Chemotaxis index

(**dpi**) Days post infection

(**SPME**) Solid phase micro extraction

(**GCMS**) Gas chromatography and Mass spectrometry

(**VOCs**) Volatile organic compounds

(**M**, **mM or µM**) Molar, Millimolar, or micromolar respectively

(**L**, **mL or µL**) Liter, milliliter, and microliter respectively

(**AMC**) 3-Hydroxy-2-butanone- also known as Acetoin or Acetomethyl carbinol

(**C.e**) *Caenorhabditis elegans*

(**D.m.**) *Drosophila melanogaster* (fruit fly)

(**L. t.**) Levipalatum texanum-a species of free-living nematode

(**S .r.** or **Sr**) *Steinernema riobrave- and EPN*

(**S. g.** or **Sg**) *Steinernema glaseri- an EPN*

(**S. f.** or **Sf**) *Steinernema feltiae- an EPN*

(**S. c.** or **Sc**) *Steinernema carpocapsae- an EPN*

(**S. g.** or **Sg**) *Steinernema glaseri- an EPN*

(**ANOVA**) Analysis of Variance- a statistical test

(**PCR**) polymerase chain reaction

(**ns**) not significant

(**ALT**) Alternative genotype (ALT) strains of *C. elegans* wild isolates.

(**Mb**) Megabases

(**QTL**) quantitative trait loci

(***lf***) loss of function mutants

CHAPTER 1

Entomopathogenic Nematodes, Behavioral Assays and a Path to Understanding Molecular Mechanisms of Nematode Behavior

Introduction

Nematodes are microscopic round worms and are among one of the most abundant organisms that live on earth[1]. From lakes[2] and oceans[3], to soils; from Antarctic valleys[3] to deserts[4], and from free-living bacterivorous life styles to being parasites of various organisms, these worms[5] exhibit amazing diversity in the wide array of ecological habitats and niches they occupy.

Among the many nematode parasites that exist some are known as entomopathogenic nematodes (EPNs)— a special guild of insect-parasitic nematodes. Unlike many of the distantly related parasitic nematodes— which cause devastating diseases—EPNs are beneficial and are used in agriculture and home gardening as a biological control agent against a variety of insect pests[6]. Furthermore, since they are easy to culture and safe to work with, they make an excellent model for studying several aspects of host-parasite interactions.

EPN ecology is incredibly complex as there are many behavioral and environmental components that play a role in their life cycles. From dispersal — away from a depleted host, to decoding the plethora of odors and other stimuli in order to navigating the epigeal habitat; the challenge of seeking out a host to

infect is a monumental task; one that is that is only rivaled in magnitude by the complex associations, behaviors and spectrums that these worms have evolved in order to accomplish such a task.

Ecological and behavioral spectrums in EPNs

Spectrums among the behaviors can include characteristics such as host ranges; there are specialists such as *Steinernema scapterisci* and its preferred cricket hosts[7] and generalists such *Steinernema carpocapsae* which can infect a variety of insects including various fruit flies, moths and beetles[8]. Associations with bacterial symbionts can also vary among EPN species, from those that rely heavily on their symbiont and carry many colony forming units (CFUs) to EPN species which carry very few bacterial cells and do not rely heavily on the bacterial symbiont[9]. Behavioral spectrums also play a large role in EPN ecology, from ambusher and cruiser foraging styles[10,11], to the ability and proclivity to perform nictation and jumping as a way to detect the presence and adhere to a host[11,12], to sensitivity and responses to conspecific-infected hosts[13,14]; there is much variation and behavioral parameters to consider, especially if EPNs are to serve as biological control agents.

Understanding the variations and causes of behaviors in EPNs may assist future efforts to more effectively leverage these insect-killing parasites for use in agriculture and home-gardens for the purpose of pest-control. Many behaviors serve as essential factors for the survival and persistence of these worms in their

environments. Such behaviors include, dispersal away from the depleted host cadavers, host-seeking, evaluation of the host, as well as invasion and colonization of a selected hosts.

Environmental cues and nematode behavior

Nematodes— such as *C. elegans*— have been the subject of much study since the 1970s. Such studies have made great strides in elucidating many details regarding attraction, avoidance and foraging among many other behaviors. Although the body of research regarding *C. elegans* behavior may perhaps be more extensive— especially regarding mechanistic detail— behavioral research in EPNs has been ongoing for multiple decades[15]. There has been work done on many facets of EPN behavioral ecology. The topics include understanding infection strategies of EPNs, how EPNs combat the host's immune responses as well as other opportunistic competitors, and even how EPNs gain entrance to the host hemocoel (blood) in the first place[15]. However, in order for any of the above-mentioned interactions to take place, an EPN must first locate a host and evaluate if the host is worth infecting— i.e. if the host has adequate resources or has already been colonized.

Studies have looked a variety of environmental cues that may play a role in EPN host-seeking and behavioral biology. Such studies have shown that a variety of chemicals, such as those from plants or damaged plant tissues[16-19], can attract or influence EPN behaviors; while ascarosides (nematode pheromones) affect both

EPN development[20] and dispersal behaviors[21]. Host-associated odors include more general cues such as CO_2 which elicits attraction from EPN species, while other more host-specific odors can elicit a range of responses between attraction to repulsion [22-24].

Methodologies of studying EPN behavior

Behavioral analysis of nematodes- and EPNs in particular- has leveraged several types of assays including jumping assays[25], dispersal assays in soil[26] or on agar[21], as well as chemotaxis assays[25]. Such assays have been routinely employed to evaluate the responses of a wide variety of nematodes to a plethora of different odors and sources of complex mixes of odors volatile compounds[13,14,22,23,27]. In my own work I have leveraged and improved upon previous techniques to explore the behavioral biology of EPNs in regards to how they respond to already-colonized hosts, and how these cues might be sensed at the neuronal and molecular level as well as how such cues play a role in the dispersal of IJs away from the depleted host cadaver.

Impacts of nematode behavioral studies

Many behavioral studies of EPNs have been done to improve our understanding of both *what* environmental cues are leveraged *how* these simple microscopic organisms are able to sense and respond to a variety of cues. In addition to being safe to work with, easy to procure, as well as inexpensive and easy to maintain; these organisms serve as an excellent model for studying several

aspects of host-parasite interactions including host-seeking. The details that are uncovered through studies on behavioral ecology could be leveraged in the future to improve field efficacy. Although there has been success in using EPNs, as in the case of controlling Diaprepes *abbreviates* (root weevil) in citrus groves, there are many cases where EPNs have not yielded effective results for reasons that are not necessarily understood[28].

Although there have been efforts to improve EPN efficacy in the past[29], progress is slow, and this is only made worse by the paucity of information regarding *how* EPNs detect environmental cues at the molecular level. Any improvement in our understanding of EPN behavioral ecology and elucidation of details as to *how* these worms might be detecting and responding to environmental cues could potentially be leveraged in the future— when more molecular tools allow for direct genetic manipulation to make improvements to EPNs to increase their usefulness as agriculture pest-control systems.

The work included in this dissertation first focuses on EPN responses to already-parasitized insect hosts and the odors that are associated with the infection. Prenol (3-methly-2-buten-1-ol) is one of the odors identified in association with EPN infected *Galleria mellonella*, much of this dissertation focuses on prenol and its behavioral effects on EPNs, as well as how prenol is detected by the model organisms *C. elegans*. Evaluations of this odor revealed it to be repulsive to EPNs, and it may even serve as a cue in the dispersal process. Since prenol is generally regarded as safe (GRAS) at low concentrations it could potentially be

leveraged as a dispersal agent to assist in EPN application. Moreover, though the goal of the studies to follow have aimed at leveraging prenol (as an EPN-infection-related odor to which EPNs respond strongly) and the model organism *C. elegans* which may provide insights into the molecular underpinnings of *how* prenol is detected and elicits responses in other nematode species. Gaining an understanding of what genes and neural circuitry underlies responses to EPN-associated odors opens up new opportunities to leverage newly-developing molecular techniques in EPNs and eventual manipulation of EPNs such that field efficacy may be improved.

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CHAPTER 2

Host Seeking Parasitic Nematodes use Specific Odors to Assess Host Resources

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Abstract

Entomopathogenic nematodes (EPNs) are insect parasites used as biological control agents. Free-living infective juveniles (IJs) of EPNs employ host-seeking behaviors to locate suitable hosts for infection. We found that EPNs can differentiate between naïve and infected hosts, and that host attractiveness changes over time in a species-specific manner. We used solid-phase microextraction and gas chromatography/ mass spectrometry to identify volatile chemical cues that may relay information about a potential host's infection status and resource availability. Among the chemicals identified from the headspace of infected hosts, 3-Methyl-2-buten-1-ol (prenol) and 3-Hydroxy-2-butanone (AMC) were selected for further behavioral assays due to their temporal correlation with the behavioral changes of IJs towards the infected hosts. Both compounds were repulsive to IJs of *Steinernema glaseri* and *S. riobrave* in a dose-dependent manner when applied on an agar substrate. Furthermore, the repulsive effects of prenol were maintained when co-presented with the uninfected host odors, overriding attraction to uninfected hosts. Prenol was attractive to dauers of some free-living nematodes and insect larvae. These data suggest that host-associated chemical cues may have several implications in EPN biology, not only as signals for avoidance and dispersal of conspecifics, but also as attractants for new potential hosts.

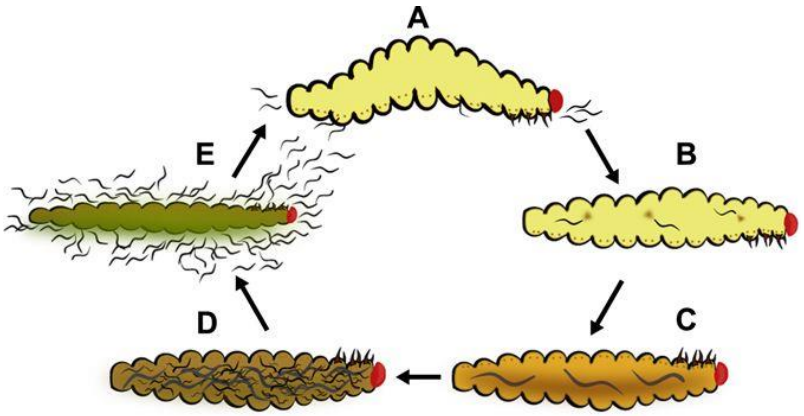


***Figure 2.1**: **The EPN life cycle**. (**A**) Uninfected host is infected with infective juveniles (IJs). (**B**) IJs invade and release symbiotic bacteria. (**C**) The bacteria proliferate and IJs mature into adulthood. (**D**) Adults produce progeny. (**E**) Eventually resources run out and newly emerging IJs disperse from depleted cadaver.*

Introduction

Entomopathogenic nematodes (EPNs) are insect-killing parasites used in biological control and are a model system for studying host-parasite interactions. EPNs can infect and kill a host within 48 hours and are commercially available for use in home gardens and industrial agriculture[1,2]. In the EPN life cycle, free-living infective-juveniles (IJs) encounter a host (either uninfected or possibly at an early stage of infection) (Fig. 2.1A), and then decide whether or not to invade the host. Once an IJ invades a host it releases symbiotic bacteria (Fig. 2.1B), which proliferate and help to both kill the host and provide a food source for the growing nematodes[3] (Fig. 2.1C).

Steinernema spp. are gonochoristic, requiring a male and female in order to produce offspring[4]. Within the host approximately 2-3 generations of nematodes can be produced (Fig. 2.1D), but eventually resources begin to run out inside the insect cadaver[2]. As resources are depleted, the L2 juveniles will associate once more with the bacteria and take an alternative developmental pathway, becoming

infective juveniles (IJs) rather than L3 juveniles. These IJs will then emerge and search for a new host to infect[2,5] (Fig. 2.1E). In their search for a new host there are two host-seeking strategies–employed by IJs–which represent endpoints of a continuous spectrum. The first is a cruise foraging strategy where the IJ spends the majority of its time actively moving in search of a host[6,7]. The second strategy is an ambush foraging strategy where the IJ employs a sit-and-wait approach, waiting for a potential host to pass close by, allowing the IJ to attach and invade[7,8]. Between these two endpoint foraging strategies there are species of EPNs that have been classified as intermediates[6,9]. These foraging strategies are employed by individuals within a population and it has been shown that even for a species like *S. carpocapsae,* which is described as an ambush forager, a small proportion of individuals are cruise foragers and have been referred to as "sprinters"[8]. Here, we worked with four species of *Steinernema*: *S. carpocapsae,* an ambush forager[6,8]; *S. glaseri*, a cruise forager[6,9]; and *S. riobrave* and *S. feltiae*, which are described as intermediate foragers[6,9].

EPN foraging strategies and the decision of whether or not to infect certain hosts is informed by olfactory and mechanosensory cues. Previous work has shown that volatile odorants emitted by uninfected insects elicit host-seeking behavior in EPN IJs[10-12]. IJs rely on chemical cues in the environment to locate, identify, and evaluate their hosts[7]. It has been shown that EPNs can distinguish between uninfected and infected hosts, and even between hosts with conspecific or

heterospecific infections[13]. However, the dynamics of host odorant profiles throughout the course of infection and which odorants IJs use to differentiate conspecific and heterospecific infections remains unknown. We evaluated the behavioral response of EPNs to infected hosts over time and identified odorants used by IJs to inform behavioral decisions regarding attraction to or avoidance of previously-infected hosts.

Results

Infective Juveniles can Differentiate Between Uninfected and Infected Hosts

We evaluated the responses of the IJs of four EPN species (*S. carpocapsae*, *S. feltiae, S. glaseri*, *and S. riobrave*) in response to last instar *Galleria mellonella* hosts at various stages of infection. We determined the IJ response to uninfected hosts, and hosts that had been infected for different amounts of time (1, 3, 5, 7, 9, and 16 days). To perform these evaluations, we used a 2-choice assay, designed to test the effects of volatile compounds emitted by the hosts[10,11].

Our results showed that all four species of EPNs tested were attracted to the odors of uninfected *G. mellonella* (Fig. 2.2A-D). By 9 days post-infection (dpi), attraction had been abolished, and the IJs of all four species were repelled from cadavers by 16 dpi. At late stages of infection such as 9 and 16 dpi, the

resources are often depleted, and the cadaver contains IJs ready to emerge in search of new hosts[14].

IJs Respond to Infected Cadavers in a Species-Specific Manner

IJs of *S. glaseri*, *S. carpocapsae*, *S. feltiae, and S. riobrave* can differentiate between uninfected and infected hosts, however, the trend of host-seeking behavior varied in a species-specific manner (Fig. 2.2A-D). There were two clear trends: Steady decrease in attraction, or an initial increase, followed by a

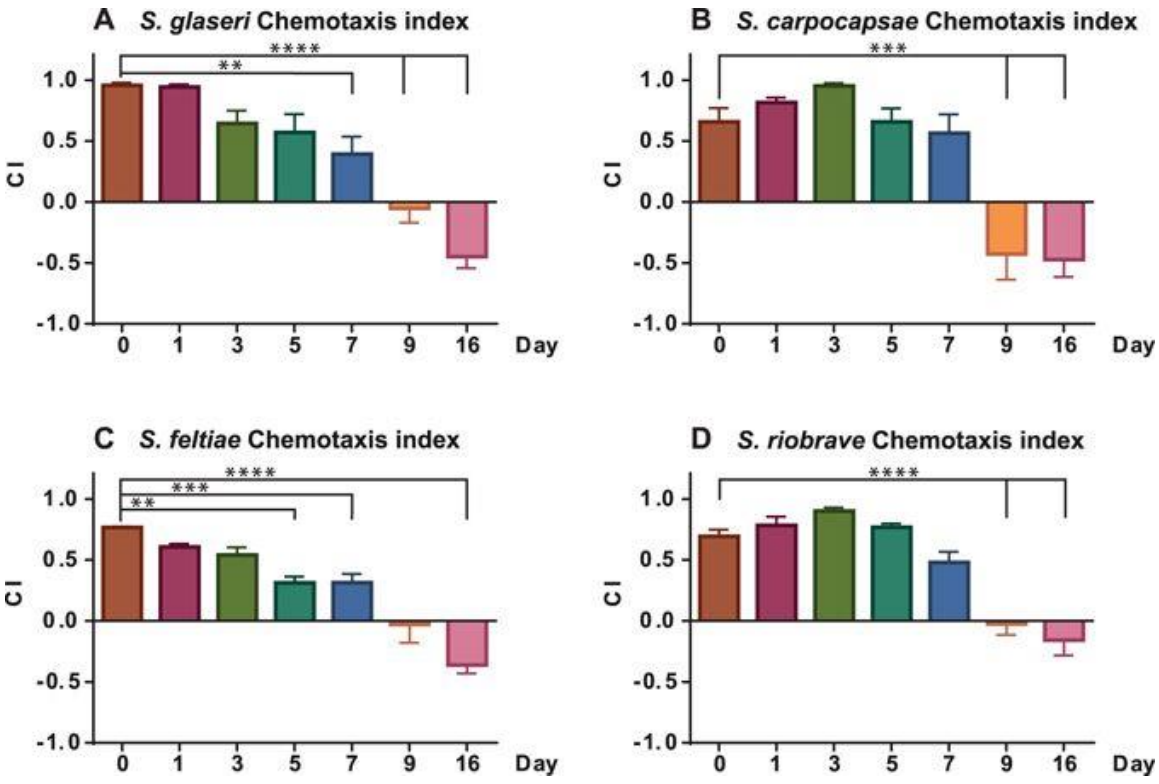


Figure 2.2: Chemotaxis results to host volatiles. Chemotaxis indices shown for the four species of Steinernema EPN IJs. CI values near: +1.0 indicate high attraction, near zero indicate indifference, and near −1.0 indicate high repulsion. Statistical significance was evaluated using an unpaired, ordinary, one-way ANOVA with Tukey's multiple comparisons post-test. Error bars represent SEM. **P < 0.01; ***P < 0.001; ****P < 0.0001.

decrease in attraction. The host-seeking behavior observed for *S. glaseri* and *S. feltiae* revealed that these species were most attracted to uninfected hosts and exhibited a general trend of reduced attraction by 3 dpi. The reduction in attraction continued over the course of the infection and by 9 dpi attraction had been fully abolished, while by 16 dpi repulsion was observed (Fig. 2.2A, C).

Steinernema carpocapsae and *S. riobrave* exhibited attraction to uninfected hosts, however the highest attraction observed was towards recently deceased cadavers at 3 dpi. The attraction then declined over the later stages of infections until repulsion was observed at 16 dpi.

Some EPNs Chemotax More Readily than Others

Although chemotaxis indices are robust and informative[10,11,15], we observed interesting patterns of IJ participation that were not captured using a chemotaxis index (CI) (Fig 2.3A-D). To better characterize the movements of the population of IJs in the testing arena, we divided our scoring templates into three sections (Fig. 2.4). Nematodes that moved out of the initial placement (center region) were scored as moving either toward the host or toward the control. The nematodes that remained close to the center of the arena were counted as remaining in the middle section (i.e. had not moved directionally).

For *S. glaseri* we observed the highest participation of IJs in chemotactic behavior in response to uninfected hosts, where 82.1% of the IJs were moving

away from their initial placement (Fig. 2.3A). This is calculated by combining the proportion of the population moving towards the host side (67.3%) and the proportion moving towards the control side of the plate (14.8%). The lowest participation of *S. glaseri* IJs was in response to 7 dpi cadavers, where we observed 69.5% of the population moving directionally. On average we observed 73% of *S. glaseri* IJs moving directionally across all time points. This indicates that the chemotaxis data is representative of the majority of the population and that most *S. glaseri* IJs participate in chemotaxis behavior.

In contrast to *S. glaseri*, *S. carpocapsae* IJs demonstrated poor participation in chemotaxis assays (Fig. 2.3B). Participation was highest in response to 9 dpi cadavers, yielding a participation of 37.4%. The lowest participation value exhibited by *S. carpocapsae* IJs was in response to uninfected hosts, where only 15.6% participated in chemotaxis. On average we observed an overall participation of only 24.8% across all time points tested. These data support the description *of S. carpocapsae* as an ambush forager and supports previous claims that *S. carpocapsae* does not respond well to uninfected hosts, as measured by chemotaxis[13,16]. Although we did record robust CI data for *S. carpocapsae*, the participation data reveal that the CI measures only a small proportion of the population, likely the "sprinters" previously described[8].

We found that *S. feltiae,* despite being classified as an intermediate forager, behaves more like an ambush forager (Fig. 2.3C). Although the general trends in attraction differ between *S. feltiae* and *S. carpocapsae*, we found that on average the participation of *S. feltiae* is lower than that of either *S. riobrave* or *S. glaseri*. We found that *S. feltiae* exhibited highest attraction to hosts at 1dpi, with 44.6% of the population moving directionally. The lowest participation we observed for *S. feltiae* was in response to 5 dpi cadavers, where only 26.5% of the population moved directionally. The average participation for *S. feltiae* was only 38.7% across all time points. This indicates that while more *S. feltiae* IJs participate in chemotaxis than *S. carpocapsae* IJs, the CIs represent slightly more than one third of the population. These data suggest that within the spectrum of cruise and ambush foraging strategies, *S. feltiae* behaves more like an ambusher.

We found that *S. riobrave* IJs had high participation in chemotaxis assays, only slightly lower than *S. glaseri* (Fig. 2.3D). For *S. riobrave* maximum participation was 80.5% of the population in response to cadavers at 3 dpi. The lowest observed participation was 55.4% in response to cadavers at 7 dpi, while the average participation across all time points was 66.4%. These data demonstrate that *S. riobrave* had participation resembling that of *S. glaseri.* Thus despite being classified as an intermediate forager, it could be argued that *S. riobrave* displays cruiser-type foraging in response to volatile host cues.

Because previous research has demonstrated that the response of nematode dauers and infective juveniles is age dependent[17,18], we evaluated the effects of IJ age on the responses of *S. glaseri* and *S. riobrave* to uninfected hosts and 16 dpi cadavers (supplementary Figs. S1 and S2). *Steinernema glaseri* and S. riobrave were selected for evaluation due to their high level of participation. We found that the response of *S. glaseri* IJs to uninfected hosts was not age dependent (Fig. S2.1). In response to 16 dpi cadavers we observed that older IJs

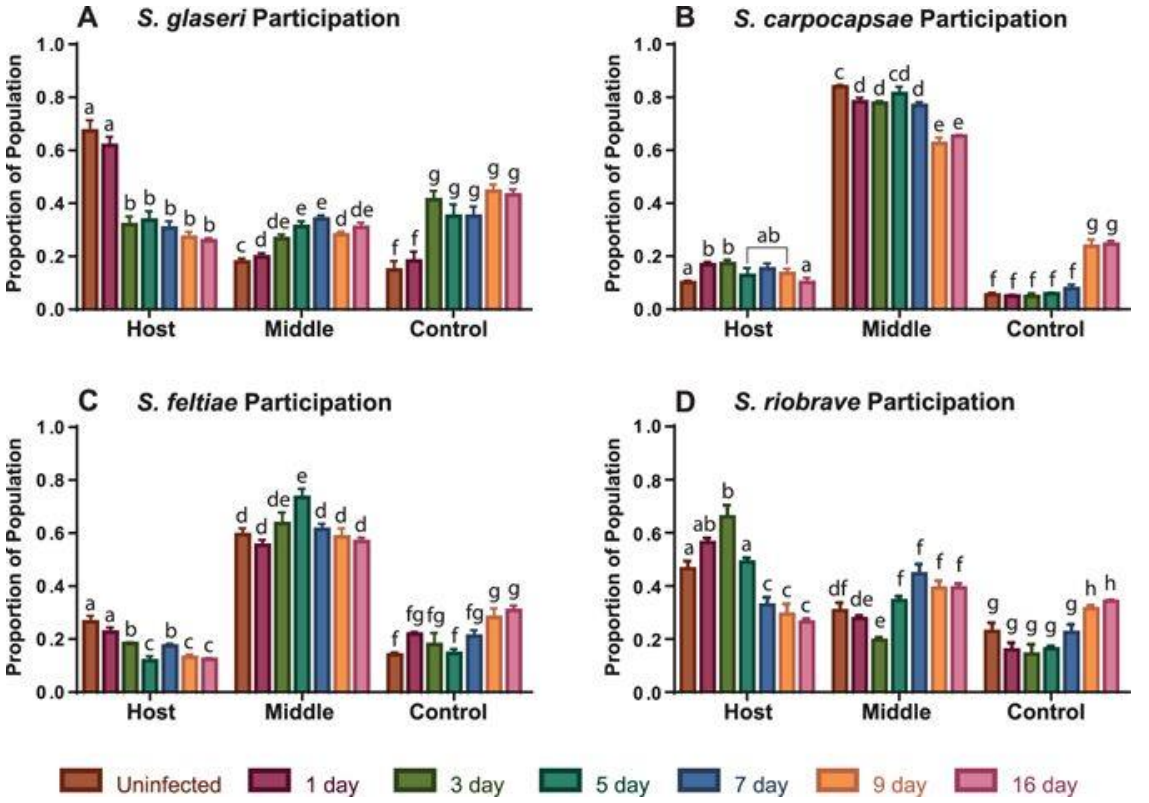


***Figure 2.3:* Participation results to host volatiles.** *Participation values for four species of Steinernema EPN IJs. Participation values were derived from separating the plate into three sections and scoring nematodes that had moved directionally 1 cm either towards the side where host volatiles or control air was being delivered. Those that did not move directionally out of the center (by at least 1 cm) were scored as remaining in the middle. Statistical analysis was done using an unpaired, ordinary one-way ANOVA evaluation for data points within–but not between–each group of "Host", "Middle", and "Control". Bars with the same letter values are not significantly different. For breakdown of the scoring template please see Fig. 4. Error bars represent SEM*

were not as repulsed by the odors, though this observation was not statistically significant (Fig. S2.2). The participation of *S. glaseri* IJs in chemotactic behaviors in response to 16 dpi cadavers did not differ significantly between age groups. However, general trends revealed slight increases in the number of IJs heading towards 16dpi cadaver odors and those IJs remaining in the middle, along with a slight decrease in the proportion of IJs traveling towards the control side of plate (Fig. S2.1).

Steinernema riobrave IJs did not exhibit any significant age-dependent shifts in chemotaxis index values in response to either uninfected or 16 dpi cadavers (supplementary Fig. S2.3). We did observe an increase in older IJs traveling towards the control side of the plate in response to uninfected hosts, but there were no other significant shifts in proportion of IJs heading towards the host odors or remaining in the middle in response to uninfected host odors (Fig. S2.3).

A Diverse Array of Odorants Influence Host-Seeking Behavior.

Having identified discrete changes in host-seeking behavior throughout the course of infection, we next worked to identify odorants that might mediate these changes in behavior. To address this, we evaluated the headspace of *G. mellonella* hosts, both uninfected and infected with either *S. glaseri* or *S. riobrave*, using solid phase microextraction (SPME) followed by gas chromatography and mass spectrometry (GCMS).

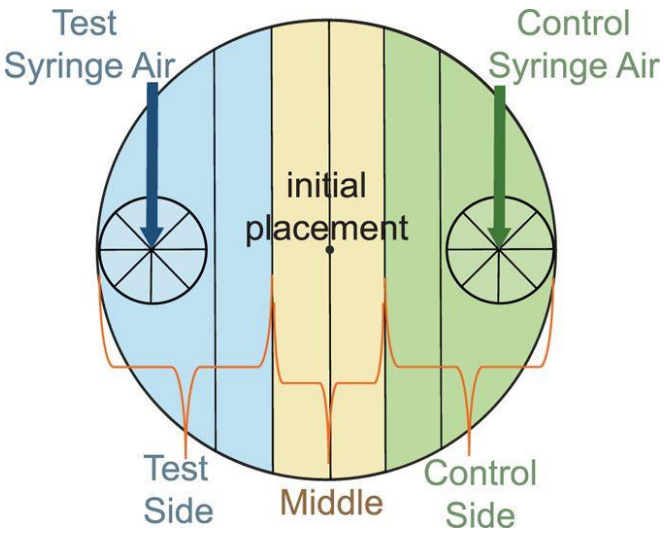


Figure 2.4: ***Chemotaxis assay scoring template***. *The scoring circles indicate where volatiles were delivered (for insect-odor response assays) or where odorants were applied (for chemical response assays). Scoring circles were used to count nematodes and calculate chemotaxis indices (CI values), while the 3 designated sides (test, middle and control) were used to determine participation.*

Our aim was to identify odorants that might be responsible for the behavioral shifts we observed in EPN IJs. We sampled the headspace of uninfected and infected insects at 1, 3, and 16 dpi. The uninfected and 1 dpi time points were associated with attraction for both *S. glaseri and S. riobrave* (Fig. 2.2A, D). Whereas recently deceased hosts, at 3 dpi, were associated with a general increase in attraction for *S. riobrave* and a general decrease in attraction for *S. glaseri* (Fig. 2.2A, D). Lastly, 16 dpi was associated with IJ repulsion. We expected that odorants present in the headspace of uninfected hosts might decrease in abundance or disappear, while other chemicals appeared and increased in abundance during the progression of the infection.

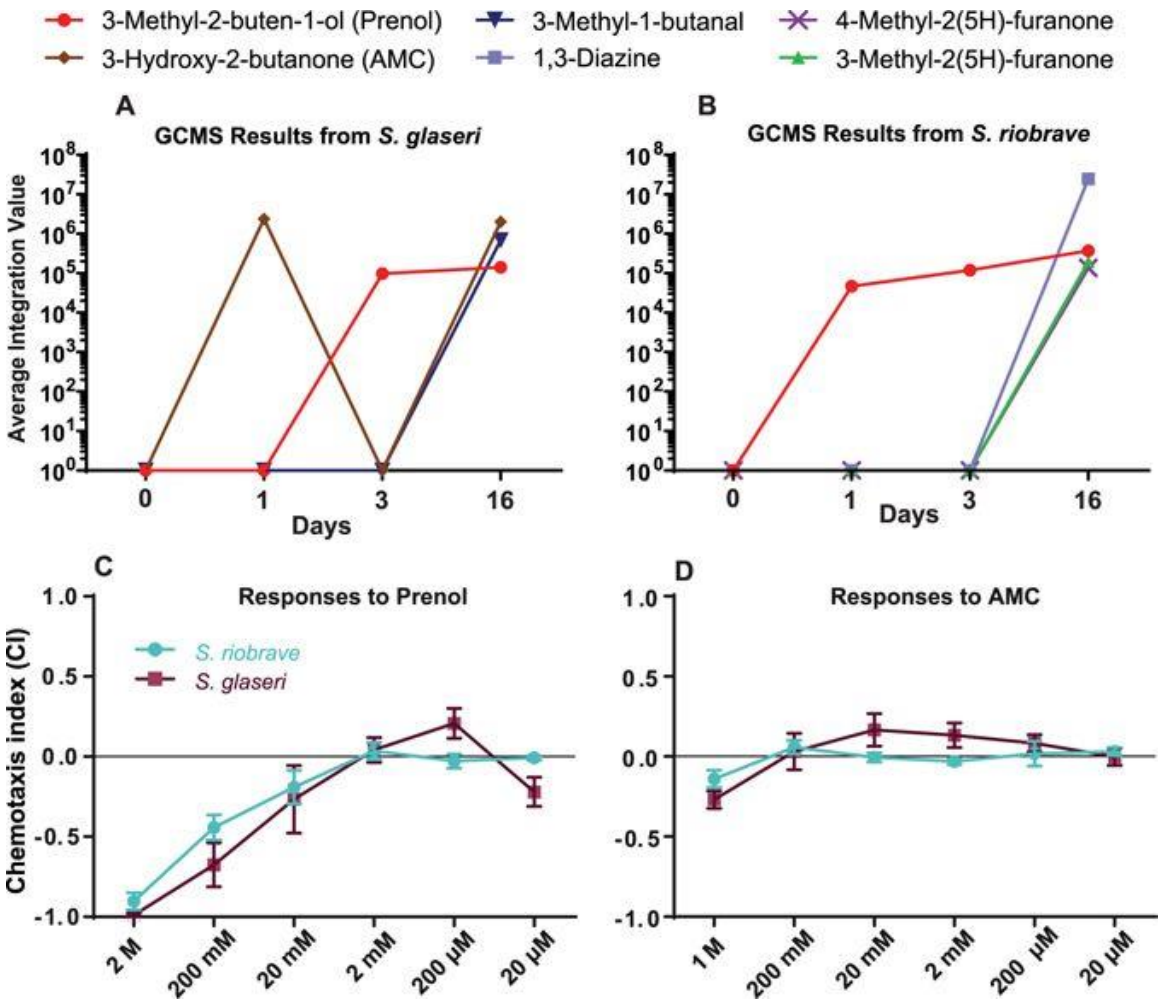


Figure 2.5: **GCMS results and responses to two odors.** Panels A, B: Results from GC-MS of (**A**) *S. glaseri* and (**B**) *S. riobrave* infected *G. mellonella* hosts. Numbers represent integration values of peaks. This reflects the overall relative abundance of individual odors. Here we found that most odors are appeared in highest abundance at 16 days post infection (dpi), however two chemicals–AMC and prenol–stood out by appearing at earlier time points. 3-Hydroxy-2-butanone (AMC) is found in association with *S. glaseri*-infected hosts at 1 dpi and 16 dpi. 3-Methyl-2-buten-1-ol (prenol) is found in both *S. riobrave* and *S. glaseri* infected hosts and appears at 1dpi (for S. riobrave-infected hosts only) as well as at 3 dpi, and 16 dpi in both S. riobrave and S. glaseri-infected hosts. Panels C, D: Results of dose response curves for (**C**) 3-Methyl-2-buten-1-ol (prenol) and (**D**) 3-Hydroxy-2-butanone (AMC). These show the responses of both S. glaseri and S. riobrave IJs to various concentrations of these chemicals. It is worth noting that the concentrations listed are what was applied to the experimental arena but are certainly higher than the concentration experienced by the nematodes. Error Bars represent SEM.

From our headspace analysis we found several chemicals: α-pinene, β-pinene, nonanoic acid, butanoic acid, and longifolene, which had been previously associated with uninfected *G. mellonella* hosts[10,11,19]. However, one of our

controls–SPME-GCMS experiments examining the odors emitted by the pine chips used as packing material for *G. mellonella*–showed that the likely source of these odorants was the wood chips rather than the larvae.

Using GCMS we identified 3 consistent odorants from *S. glaseri*-infected hosts, and 4 consistent odorants from *S. riobrave*-infected hosts. From *S. glaseri*-infected larvae we found 3-Methyl-2-buten-1-ol, Butanal-3-methyl, and 3-Hydroxy-2-butanone (Fig. 2.5A). From *S. riobrave*-infected larvae we found 3-Methyl-2-buten-1-ol, 4-Methyl-2(5H)-Furanone, 3-Methyl-2(5H)-Furanone, 3-methyl, and 1,3-Diazine (Fig. 2.5B). Odors were identified by comparing spectral analysis of the sampled headspace against chemical profiles in a database. The majority of the odorants we found came from 16 dpi cadavers, and all of the odorants identified were found at highest relative abundance at this time point (Fig. 2.5A-B).

Two odorants, 3-Methyl-2-buten-1-ol (prenol), and 3-Hydroxy-2-butanone (acetyl methyl carbinol or AMC), were chosen for behavioral evaluation due to their appearances at time points associated with IJ attraction (i.e. 1dpi -3 dpi cadavers) as well as time points associated with repulsion (i.e. 16 dpi cadavers). All other odorants appeared only on 16 dpi. We reasoned that AMC and prenol were the odorants most likely to elicit dose-dependent behaviors from IJs due to their appearance at the time points associated with both attraction (1-3 dpi) and repulsion (16 dpi).

Both AMC and prenol retention times were compared to synthetic standards to confirm retention time matches (see methods information for standards used). AMC (3-Hydroxy-2-butanone) was chosen for appearance at both 3 dpi–a time point associated with IJ attraction and 16 dpi–a time point associated with IJ repulsion/ reduced attraction (Fig. 2.5A). However, it should be noted that AMC was only present in *S. glaseri*-infected cadavers. Similarly, prenol (3-Methyl-2-buten-1-ol) was chosen for its appearance in both *S. glaseri* and *S. riobrave* infected cadavers. Additionally, prenol was present at time points associated with IJ attraction (1 dpi for *S. riobrave* and 3 dpi–for both *S. riobrave* and *S. glaseri*) as well as time points associated with IJ repulsion (16 dpi for both *S. riobrave* and *S. glaseri*.) (Fig. 2.5A-B).

S. glaseri and _S. riobrave_ IJs are Repelled by Prenol and AMC

Having identified prenol and AMC as potentially important cues for IJs, we measured the response of *S. glaseri* and *S. riobrave* IJs to these odorants. Since these chemicals were identified at time points associated with both attraction (at 1dpi and 3 dpi) and repulsion (at 16 dpi), we hypothesized that low amounts of these odorants would elicit attraction responses from EPNs, while higher amounts would elicit repulsion. Prenol elicited strong repulsion responses for

both *S. glaseri* and *S. riobrave* in a dose-dependent manner (Fig. 2.5C), while AMC elicited mild repulsion behavior from both *S. glaseri* and *S. riobrave* in high abundance (Fig. 2.5D). However, we found no statistically significant difference between the responses to varying doses of AMC.

We note that although in these assays we applied doses of 2M, 200mM, 20mM etc., the actual concentrations experienced by the nematodes were probably much lower than these initial concentrations, as the chemicals diffused into the surrounding air and agar. The results overall indicate that both *S. glaseri* and *S. riobrave* IJs are sensitive to prenol. It is important to note that the exact concentrations of these odors that are emitted by the hosts are not known, and can vary from host to host, as well as over time. Methods for determining ecologically-relevant concentrations have yet to be adequately established in this field, and as such we tested a range of concentrations that we thought were appropriate.

Additionally, we tested whether or not IJ age would have an effect on the responses of *S. glaseri* and S. riobrave IJs to prenol (supplementary Figs. S1 and S3). We found that the responses by *S. riobrave* did not differ between younger and older IJs either as measured by chemotaxis index or participation. *S. glaseri* IJs did not show a significant change in chemotaxis index but did show significant shifts in participation. We observed a significant increase in the proportion of older *S. glaseri* IJs that remained in the middle, and a significant

decrease in the proportion of older IJs chemotaxing towards the control side of the plate (supplementary Fig. S2.1). This indicates that although the overall response to prenol did not change, the sensitivity of prenol may be age dependent.

Prenol Elicits Different Behaviors from Organisms of Different Ecological Niches

Of the two odorants tested, prenol elicited the strongest response (Fig. 2.5C). A paucity of information regarding prenol and its overall ecological relevance raises questions about how other organisms might respond to it. To gain a better understanding of the possible ecological relevance of prenol to other organisms, we evaluated the responses of dauer-stage nematodes for two free-living nematode species: *C. elegans* dauers and *Levipalatum texanum* dauers[20]. Additionally, we evaluated *Drosophila melanogaster* larvae to prenol. *C. elegans* dauers were highly attracted to prenol and also exhibited very high participation (Fig. 2.6A, B). We observed that approximately 61.8% of the population of *C. elegans* chemotaxed towards prenol, with approximately 34.7% remaining in the middle and a mere 3.4% moving towards the control side of the plate. The attractive response of *C. elegans* and the association of prenol with EPN-infected cadavers merits further study. *Levipalatum* texanum dauers–another free-living nematode–was evaluated and exhibited fairly high attraction to prenol (Fig 6A).

However, participation of *L. texanum* was much lower than that of *C. elegans.* Only 28.7% of the population chemotaxed towards prenol, 54.6% remained in the middle, and 16.7% moved towards the control side of the plate (Fig. 2.6C). *D. melanogaster* larvae also exhibited high attraction to prenol. However, participation data revealed that the movement of larvae overall was primarily stochastic. The pattern of attraction revealed that there was random movement by the larvae until they came within a certain range of this odorant (Fig. 2.6C).

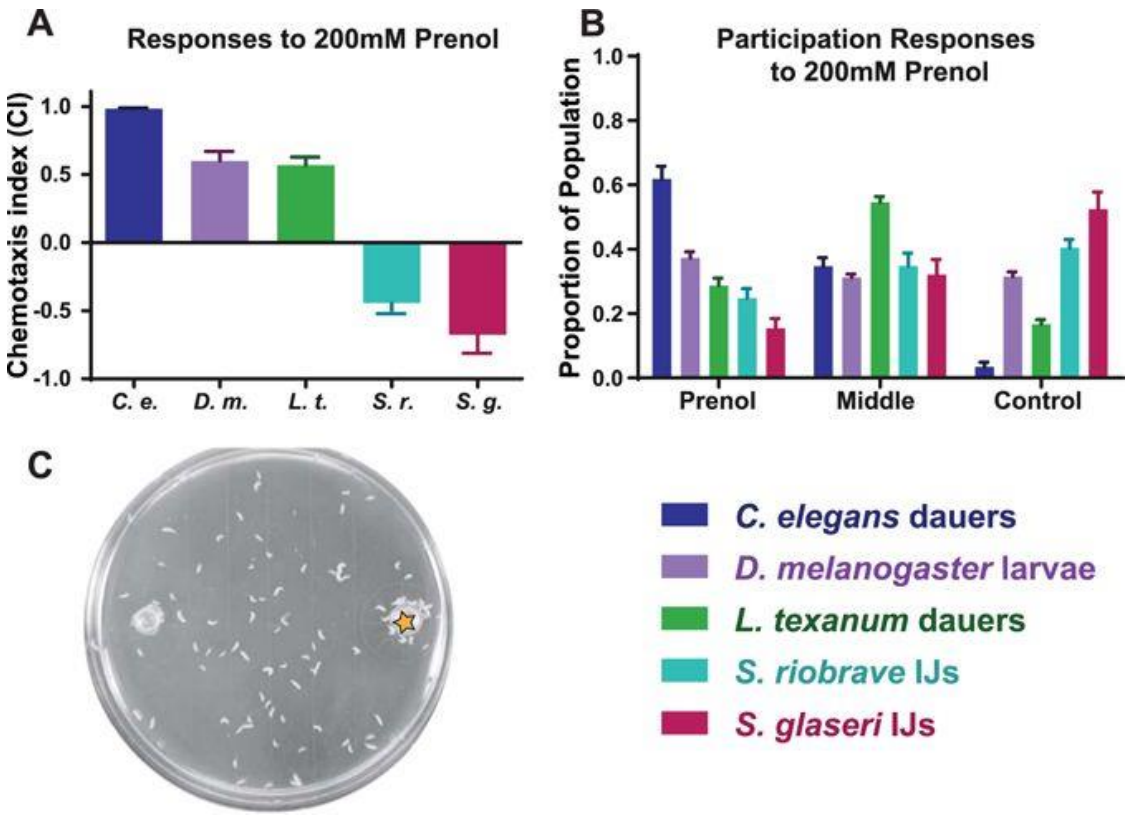


***Figure 2.6:* Multi-Species response to prenol**. (**A**) Chemotaxis indices for Caenorhabditis elegans (dauers) (C. e.) Drosophila melanogaster(larvae) (D. m.), Levipalatum texanum (dauers) (L.t.), S. riobrave IJs (S. r.), S. glaseri IJs (S. g.). (**B**) The participation values of each of the species tested in (**A**). (**C**) A representative photo of D. melanogaster larvae in response to 200 mM prenol. Star indicates location of prenol (diluted to 200 mM in paraffin oil).

Prenol Drives EPN Repulsion from Uninfected Cadavers

To evaluate the potential ecological relevance of the repulsive behavior caused by prenol, we evaluated the response of *S. glaseri* to uninfected host volatiles in combination with prenol. As noted above, *S. glaseri* is highly attracted to uninfected host volatiles (Fig. 2.2A). In our initial assessments we found that behavioral experiments with *S. glaseri* and uninfected hosts yielded a chemotaxis index of 0.955 (extremely high attraction). In contrast, when exposed to 2M prenol, we observed near-perfect

repulsion, yielding a chemotaxis index value of -0.989 (Fig. 2.5C). The hybrid assay set-up–using both uninfected host volatiles and prenol–revealed that prenol completely abolished *S. glaseri* attraction to uninfected waxworm hosts and instead caused very strong repulsion (Fig. 2.7A-C).

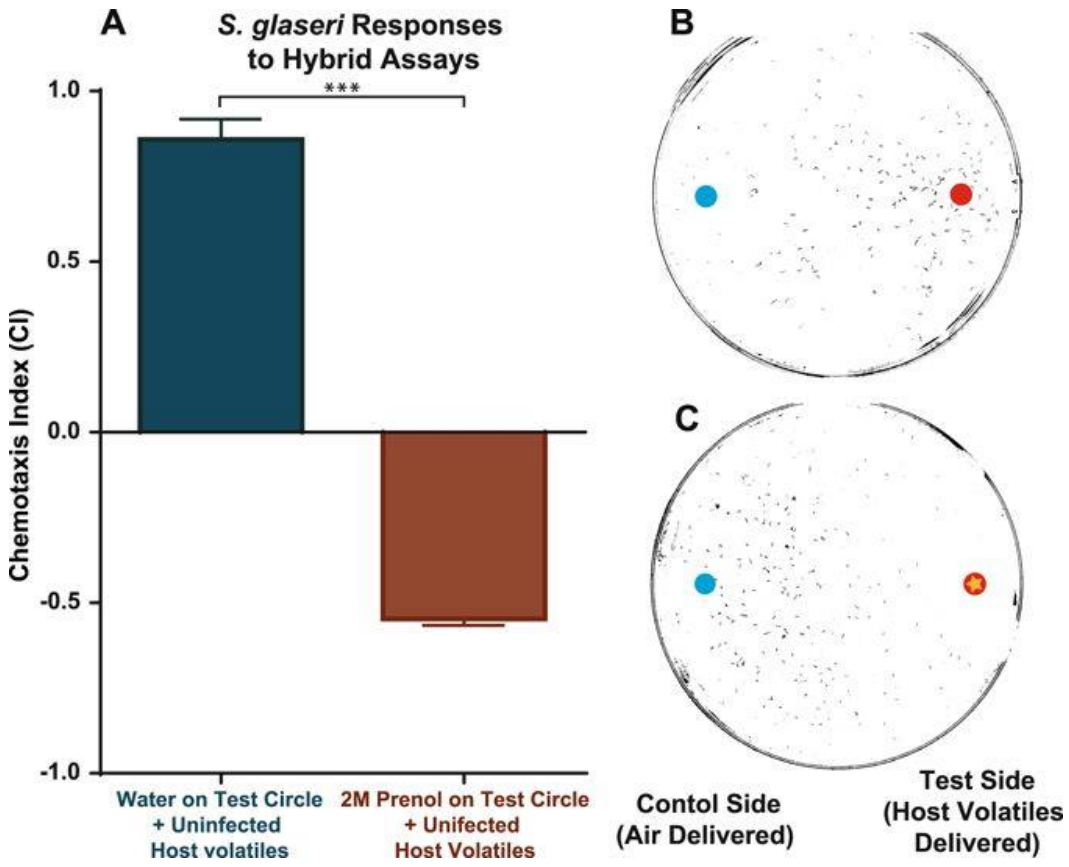


*Figure 2.7: Hybrid Assay results. (**A**) Chemotaxis index for hybrid assays done with* S. glaseri *IJs. Statistical analysis done using a two-tailed, paired, parametric t-test. Error bars represent SEM, ***P < 0.001 (**B**) A representative photo of a control hybrid assay using volatile and soluble cues, in this experiment 5 µl of ultrapure water was placed on the agar plate underneath where uninfected host volatiles were being delivered. The blue dot (on left side of the plate) represents the location of where air from control syringe was delivered, and the red dot (on right side of the plate) represents where volatiles from uninfected hosts was delivered. (**C**) A representative photo of a hybrid assay where 5 µl 2 M prenol–diluted in ultra-pure water–was added to the test circle–where uninfected host volatiles were being delivered. The combination of prenol and uninfected host volatiles is indicated by red dot with gold star. The blue dot (on left side of plate) indicates where air from control syringe was being delivered. Error bars represent SEM. ***P < 0.001.*

Discussion

Our initial assessments of the data revealed that the IJs of all four EPN species were repelled by late-stage infected hosts (9 and 16 days post infection). The repulsion observed indicates that using olfaction alone, IJs can detect odors associated with late-stage-infected host cadavers–which are no longer suitable for infection. Our results suggest that the infection process leads to changes in host health such as a reduction in available resources within the host. These changes affect the suitability of a host, which can greatly influence the behavior of free-living IJs[13,14]. Furthermore, our results support previous reports that the IJs of some EPN species are more attracted to hosts that have been recently parasitized by conspecifics, than to uninfected hosts[21]. It should be noted that previous work has shown that hosts can be infected even if the host has already been parasitized or is deceased[22]. Our results also indicate that volatile cues (including, but not limited to CO_2) can elicit behavioral responses that change dynamically as the infection progresses[7,10,11] Our participation results supported the previous characterization that *S. glaseri* is a cruiser-type nematode and participates well in chemotaxis assays[14,16,23]. Similarly our results reinforce previous reports that *S. carpocapsae* IJs behave more like ambush foragers, where most of the IJs did not participate in chemotaxis behavior, at least not in the time frame of the assays we used[23]. Furthermore, we observed that while we could calculate repeatable CIs for all four species of EPNs, a large proportion of *S. carpocapsae* and *S. feltiae* IJs did not move from their starting position in our

assays. This meant that the CI was based on only a small subset of the population. For *S. carpocapsae* in particular, the population contains “sprinters” which have been previously observed and characterized as utilizing a cruiser-type foraging strategy rather than the ambusher type strategy most *S. carpocapsae* IJs use[8,24]. We believe that our CI values reflect the behaviors of these sprinters, rather than the general behaviors elicited by *S. carpocapsae.* Additionally, the participation measure indicated that nematodes classified as intermediates can, as a population, lean towards a particular end of the foraging spectrum, between cruise and ambush foraging. For example, *S. riobrave*, which is classified as an intermediate[11,23], exhibited behavioral responses that mirrored those of *S. glaseri* IJs. *S. feltiae* has been described as a cruise forager[25,26], and an intermediate forager when compared to EPNs from various genera[27], however our results indicated that *S. feltiae* participation was similar to that of an ambusher type foragers such as *S. carpocapsae*. By evaluating participation data, we could more accurately establish where a particular species of host-seeking nematode may fall within the spectrum of cruiser to ambusher. Although the CI provides some information regarding host-seeking behavior, the resolution is limited. Adding participation data remedies this issue and relays a clearer picture of the behavioral responses of IJs to the progression of the infection.

It is known that some behavioral responses of nematode dauers and IJs are age dependent[17,18]. Our experiments revealed some age-dependent differences in *S.*

glaseri IJs responding to late-stage, resource depleted cadavers, where younger IJs displayed stronger repulsion to these cadavers than older IJs. The participation data revealed a similar trend in the response of *S. glaseri* IJs to prenol, where a higher proportion of young IJs were repelled by prenol than older IJs. These findings lead us to speculate that younger IJs may be more sensitive to dispersal cues, which would be in high abundance in the late-stage infected cadavers, and the age-dependent participation of IJs responding to prenol support our hypothesis that prenol may be a dispersal cue.

It is worth noting that previous work has shown some behavioral trends that differ from what we have reported here. For example, Grewal et al. 1997[13] indicated that *S. riobrave* is more attracted to uninfected hosts than infected ones, that *S. glaseri* is more attracted to infected hosts than naïve hosts, and that the responses of *S. feltiae* towards uninfected and infected hosts did not differ. Our results suggest the contrary; we observed that *S. riobrave* appeared to have slightly increased attraction to hosts at 3 days post infection compared to uninfected hosts (Fig.2B). *S. glaseri* was most attracted to uninfected hosts, with attraction decreasing over time (Fig. 2.2A). Additionally, we found that *S. feltiae* does exhibit altered behavior towards infected hosts, showing highest attraction to uninfected hosts, with attraction decreasing over time. These differences between our results and previously published work may be due to differences in the methods employed. Grewal et al. employed assays that used a static

environment, they infected hosts with a higher density of nematodes, and they focused mainly on hosts infected for 4 and 24 hours[13].

The results of our SPME-GCMS work revealed a set of six odors, two of which we explored: Prenol (also known as 3-Methyl-2-buten-1-ol, 3,3-dimethylallyl alcohol, and 3-methyl-2-butenyl alcohol) and acetyl methyl carbinol (AMC) (also known as 3-Hydroxy-2-butanone or acetoin). The methods that we employed have been previously used with much success in identifying volatile organic compounds (VOCs) that elicit responses from various nematode species[10,11]. However, not much work has been done to identify volatile compounds associated with the progression of an infection. This is the first report of these identified VOCs that occur in relation to nematode-infected insects. These odors were identified at infection time points associated with both IJ attraction and repulsion. Because of their appearance in early time points and gradual increase in relative abundance over time, we hypothesized that the IJs might respond to these odorants in a dose-dependent manner.

AMC has been mentioned in the literature in different contexts, some of which relate to its potential importance in EPN infections. It was identified as a volatile produced by *Zophobas morio* larvae[10] and as a metabolic byproduct of the mammalian-parasitic nematode *Ascaris lumbrioides* under anaerobic conditions[28]. It has even been found in association with *Serratia spp.* bacteria

isolated from the plant-parasitic nematode *Bursaphelenchus xylophilus* (the pine wilt nematode)[29].

Prenol has been mentioned in association with walnut twig beetles (*Pityophthrorus juglandis*)[30] and rectal gland secretions produced by certain male fruit flies (*Bactrocera visenda*)[31]. Prenol is an isoprenoid, formed through the mevalonate pathway used to create isoprene[32,33]. The mevalonate pathway and the formation of isoprene are linked with insect juvenile hormone production[34,35] and the formation of insect pigments[36]. Additionally, isoprene may be a chemical constituent of insect epicuticle layers[37]. In addition, prenol has been found in association with cyanobacteria biofilms, and in this context, it was shown that *C. elegans* adults were attracted to prenol (approximate CI value=0.41)[38]. Although prenol is mentioned in a wide array of circumstances, it was not previously known to be associated with nematode-infected insects. Interestingly, a recent study has found a very similar organic compound by the name of methyl 3-methyl-2-butenoate (MMB) which closely resembles prenol in structure. MMB was found in association with a nematophagus fungus, which produces this odorant to attract adult hermaphrodite *C. elegans*, for the purpose of entrapping the nematodes and using them as a food source[39]. Additionally, the study revealed that the AWC neuron plays a large role in detection of MMB, and that multiple receptors may be activated by this odorant[39]. It is possible that prenol may be triggering similar neural circuitry, but further study is needed to verify if

this is true and explore if MMB has similar effects as prenol on the behavior of EPN IJs.

Despite all the previous mentions of both prenol and AMC, the exact source of these odorants in the context of EPN infections is unknown. It is conceivable that these odors could be produced by the EPNs themselves, the symbiotic bacteria that they carry, or as a byproduct of the decay of the insect cadaver. Further research is necessary to determine the origin of these compounds.

Our results showed that the response from IJs towards AMC was not as intense as the responses to prenol (Fig. 2.5C, D). Additionally, the lack of statistically significant differences in behavioral responses–by *S. glaseri* or *S. riobrave*–towards varying concentration of AMC, suggest that AMC may not be an important cue on its own (despite being associated with time points post-infection). However, it may be an important behavioral cue in combination with other cadaver-associated odors. It is possible that some cadaver-associated odors that interact with AMC or prenol are not highly volatile and therefore were not detected in the current study with SPME and GC-MS.

Conversely, prenol was highly repulsive to EPN IJs, while AMC was mildly repulsive at the highest concentration only. The behavioral responses of *S. glaseri* and *S. riobrave*, coupled to the association of prenol with a late time point

in the infection suggests that prenol may be acting as an informative cue for free-living IJs that encounter an infected host. We speculate that prenol could act as a deterrent signal to free-living IJs and a dispersal cue for IJs within the decaying, resource-depleted insect cadaver, however further experimental evidence is needed to verify this hypothesis. Furthermore, we observed distinctly divergent behaviors between our EPNs and free-living nematodes such as *C. elegans* and *L. texanum* dauers, as well as the *D. melanogaster* larvae. We speculate that the behaviors observed are based on the niches that each organism occupies, but further study is needed. Additionally, the attraction of *D. melanogaster* larvae to prenol raises the question of whether prenol or other EPN-associated odors may be used not only to inform EPN IJs, but also potentially attract new hosts to depleted, EPN-filled cadavers. This remains to be explored. Additionally, the ability of prenol to overwhelm EPN IJ attraction to uninfected host odors, denotes a hierarchy of cues used to evaluate potential hosts. We speculate that prenol might be able to overcome other volatile cues that are generally associated with uninfected/healthy hosts, such as CO_2.

Conclusions

In this work, we have elaborated on the behaviors EPNs exhibit towards uninfected and infected hosts. This work has built upon prior evidence that EPNs exhibit different behavior towards naïve and infected hosts[13], while evaluating possible mechanistic reasons as to how this occurs. Our work shows that

behavioral responses (i.e., directional movement) of EPNs towards infected hosts are species-specific, and are, at least in part, mediated by volatile cues associated with the infected host. Among the volatiles identified from infected hosts, we found that prenol is strongly repulsive to the EPN species we tested, while it is attractive to organisms of different ecological niches (*C. elegans* and *L. texanum* dauers as well as *D. melanogaster* larvae). Prenol is an odorant we found associated with EPN infection and it reaches higher concentrations in late-stage infections. This indicates that prenol might be playing a multi-faceted role in both intra- and inter-specific scenarios, causing deterrence and dispersal for IJs, and attraction of potential hosts available in the vicinity. It may also be a food signal for bacterivorous nematodes such as *C. elegans*. Further investigation looking at a wider array of nematode species and potential hosts may shed light on the ecological relevance of prenol.

Methods

Nematode Culturing

S. carpocapsae was from inbred strain All, *S. glaseri* was from inbred strain NC, and *S. feltiae* was from inbred strain SN[40]. *S. riobrave* was from inbred strain TX-355[41]. EPN species were propagated as previously described[10,11,42]. Last-instar *Galleria mellonella* were infected with approximately 30 nematodes per host in a 6cm petri dish and incubated at room temperature (Approximately 23˚ C). After 7-10 days infected and deceased hosts were placed on white traps, which were

incubated at room temperature. White traps were incubated for 7-10 days. IJs were collected from white traps, rinsed 3 times in tap water, and were placed in cell culture flasks. Collected IJs were kept at room temperature and used within two weeks of being collected (maximum age of 21 days post emergence). IJs were often used within a few days of being collected, with most assays using IJs that were 17 days post emergence or younger (used within 10 days of being collected). However, a few experiments were done using older IJs (14-21 days post emergence).

Levipalatum texanum[20] was isolated from *Scapteriscus borellii* mole crickets obtained through field sampling. Isolation of *L. texanum* was done using methods previously described and used to isolate *Pristionchus pacificus* from insects[43]. Briefly, mole crickets were sampled from Rio Hondo golf course in Downey, CA[11]. The mole crickets were cut in half longitudinally and laid on 2% agar plates. Nematodes observed on the plates after 1 week were isolated and cultured on nematode growth media plates (NGM) seeded with E. coli OP50, in the same manner as *C. elegans* (described below)[44]. Species identification was done by sequencing the 18S rRNA gene and identifying the closest match in GenBank. The 18S sequence we generated was nearly identical to the 18S sequence from *L. texanum* EJR-2014, RS5280, accession number KJ877221 We used primers RH5401 and RH5402 to amplify and sequence the 5' end and primers VL26345 and VL26346 to amplify and sequence the 3' end of the small ribosomal

subunit[45,46]. Our sequences have been deposited in GenBank with accession numbers MF149117 and MF149118. *L. texanum* dauers were obtained using the same process used to obtain *C. elegans dauers* described below. We note that this is the first report of *L. texanum* coming from mole crickets, as they were originally isolated and described from scarab beetles.

C. elegans (strain N2) were cultured as previously described[44] on NGM plates that had been seeded with OP50. Dauers were obtained as described above. Nematodes were transferred to NGM plates with a thin lawn of OP50. Plates were left undisturbed for 10-14 days to allow the food supply to be depleted. These starved plates were then evaluated around 10-14 days after being seeded with adults. Dauer larvae were collected and rinsed 3 times in tap water before being stored in cell culture flasks at room temperature (approximately 23°C) for storage and used within two weeks.

Behavioral Response of IJs to Insect odors

Chemotaxis media plates were prepared as previously described[47] and allowed to sit at room temperature for a minimum of 12 hours before use. Chemotaxis assays were performed as previously described[10,11,48]. 50ml Hamilton gas-tight syringes were used. The test syringes were loaded with 5 *G. mellonella* larvae (uninfected or at various stages of infection), and control syringes left empty. The infected larvae used, were infected with conspecifics (e.g. *S. glaseri* IJs behavior

was evaluated in response cadavers infected with *S. glaseri* IJs). The syringes were loaded into a KD Scientific pump (Model: KDS 220, Catalog number 78-0220NLSU).

Petri dish lids (from 100 mm plates) were modified as described. On either side of each lid two 10 mm holes were drilled approximately 10mm in from the edge. Nalgene PVC tubing (1/8'' diameter) was plugged into these holes to connect the plate lid to the syringes. This allowed air from the syringes to be deposited over the scoring circles of the scoring template, attached to the bottom of each chemotaxis mediate plate. A pellet of approximately 250 IJs was deposited onto the center of the plate, and the plate oriented under the modified lid. The plate was oriented under the ports in the lid through which air from the syringes was being delivered. The plates were set on a vibration-reducing platform during the duration of the assay. Assays ran for approximately 1 hour (at a rate of 0.5 ml per minute). The assays were then scored using the scoring template placed on the bottom of each plate. A minimum of 3 experiments was done for each time point, and each experiment consisted of 9 technical replicates (minimum).

Chemotaxis index (CI) values were calculated[10,11]. Briefly, we counted the number of nematodes inside each scoring circle on either side of the plate, over which odors from the test/host syringe or control syringe were being delivered. CI was calculated using the following equation: CI= # in host circle - # in control circle / Sum of all individuals in both circles.

Participation was calculated by counting the number of nematodes that moved directionally by 1cm. Nematodes that did not move more than 1 cm out of the middle were scored as remaining in the middle section.

Statistical analyses were done using GraphPad PRISM software package. Chemotaxis index statistical analyses for responses to hosts over the course of the infection, was done using unpaired, ordinary, one-way ANOVA, along with Tukey's (multiple comparisons) post-test (recommended by GraphPad PRISM). For age assays the collection of response to uninfected, 16 dpi and 200mM prenol) was evaluated using unpaired two-way ANOVA with Sidak's multiple comparisons posttest (as recommended by GraphPad PRISM). The age assay responses–of variable age, young and old–*S. glaseri* IJs to 16 dpi cadaver volatiles were additionally analyzed in isolation with one another with unpaired, ordinary one-way ANOVA with Tukey's (multiple comparisons) post-test (as recommended by GraphPad PRISM).

Participation behavior was evaluated by statistically analyzing the data points within each group of "Host", "Middle", and "Control". Analysis was done using unpaired, ordinary, one-way ANOVA (with recommended Tukey's (multiple comparisons) post-test) to evaluate statistical differences between time points within each group. It should be noted that the statistical analysis for participation does not represent differences between the groups of Host, middle and Control.

Gas-chromatography and mass-spectrometry analysis

GCMS analysis procedure was based on Villaverde et. al 2007[49]. Insects were incubated for 1 hour at room temperature in GCMS vials (KaptClean Clear 27.5 mm by 95 mm, Part number GLA00797). The headspace volatiles were sampled for 30 minutes at 25°C using the solid phase microextraction fiber (65 µm PDMS/DVB fiber (Supelco Catalog number 57359-U)) exposed into the headspace of the sample vial through the septum cap. The chemicals sampled by the fiber were injected in Agilent Technologies 7890A gas chromatograph equipped with a DB-5 column (30 m × 0.32 mm inner diameter, Agilent Technologies) in splitless mode, with the temperature program: 50°C for 5 minutes, 5°C /minute increase from 50°C to 250°C, and final hold at 250°C for 10 minutes. The temperatures of the injector and transfer line were 250°C. Helium was used as the carrier gas. Electron impact mass spectra (70 eV) were taken with an Agilent 5975C mass selective detector (Agilent Technologies).

Each different sample type was evaluated 3 separate times (on different days). Parameters for chemical candidates: appear 2 out of 3 runs, with an integration value of 10,000 or higher and a match score of 80% or higher (based on NIST 11 mass spectral library). Automatic peak integration of chromatograms was conducted using Enhanced Chem Station software: MSD Chemstation vE.02.02.1431 from Agilent Technologies. The RTE integrator was used, with the following parameters: Data point sampling was set to 5, Start threshold was set

to 0.002 and stop threshold was set to 0. Output was set to calculate area counts.

For chemical identification, retention times and mass spectral data were compared between the synthetic standards and natural compounds detected from the infected-host samples. As the standards, prenol (under the name of 3-methyl-2-buten-1-ol) was purchased from Acros Organics and Acetyl methyl carbinol (AMC) (under the name Acetoin) was purchased from Tokyo Chemical Industry (TCI America). One microliter of the chemical was placed into a GCMS vial (KaptClean Clear 27.5 mm by 95 mm, Part number GLA00797), and the vial was capped. After allowing the volatilization of the chemical in the vial for 1 hour, the headspace was sampled for 1-2 seconds with the solid phase microextraction fiber (65 µm PDMS/DVB fiber (Supelco Catalog number: 57359-U)).

Behavioral Response of IJs to Chemical odorants

Chemical-response chemotaxis assays were also done as previously described[10,11]. To either side of a chemotaxis plate (within the scoring circles) 2 µL of 1M sodium azide was added, along with 5 µL of chemical to the test side and 5 µL of diluent to the control. A pellet of approximately 250 nematodes was applied to the center of the plate and assays were allowed to run for 1 hour in the dark on a vibration-reducing platform. Hybrid assays combined use of syringe pump and chemical chemotaxis set-up.

Chemical odors and sodium azide were prepared as follows:

1M AMC was prepared by dissolving 0.1762 grams in ultra-pure water (autoclaved, distilled, milli-Q filtered water). Ultra-pure water was used to make the serial dilution AMC. AMC was made and stored in glass vials (5/8^{ths} dram). Vials were wrapped in foil to prevent light exposure and were maintained in a plastic tip box kept at 4° C while in storage to prevent degradation. Prepared chemical was used within 3 weeks.

2M prenol was prepared by mixing 203 µL of 99.9% pure prenol with 797 µL of 100% ethanol. Ethanol was used to make a serial dilution series of prenol. Prenol was made and stored in glass vials (5/8^{ths} dram) in a plastic box to limit light exposure. Prenol was kept at room temperature and used within 3 weeks.

1M sodium azide was prepared by dissolving 0.06501 grams of crystalline sodium azide in 1 mL of ultra-pure water. Sodium azide was prepared and stored in 2.5 mL plastic tubes at room temperature and used within 3 weeks.

Behavioral response of *Drosophila melanogaster* to 200 mM prenol

Chemotaxis assays with *Drosophila melanogaster* larvae were modified from previously described assays[50-52]. Briefly, two PCR tube caps were placed on either side of a 1% agarose plate, in the center of the 2 cm scoring circles (template applied to the bottom of the plate was the same as those used in nematode behavior assays). We added 10 µL of 200 mM prenol diluted in

paraffin oil to the PCR tube cap on the test side, while 10 µL of paraffin oil was added to the cap on the control side. The PCR cap ensures that the larvae are detecting prenol strictly as a volatile cue. Fruit flies can sense chemical cues both through gustation and olfaction, unlike IJs, which have sealed buccal cavities, and perceive both volatiles and soluble chemical cues through their amphids[53] .

The plate was placed in a cardboard freezer box and left undisturbed for 5 minutes before being removed and a picture taken for scoring. The same template (as was used in nematode behavior assays) was used for scoring the larvae. Larvae were scored for a chemotaxis index (response index) as previously described in Monte et. al. 1989[50]. In addition, we also collected data on participation to accurately represent the behavior of the *D*. *melanogaster* larvae.

Hybrid Assays: Chemical-response Assays combined with Uninfected-host odors

Hybrid assays combined elements of the chemical response assay described above as well as the gas-tight syringes used in the host volatile assays. The syringes were used to deliver the volatiles from uninfected waxworms as described above. We applied prenol to the plate as previously described in the *Behavioral response of IJs to chemical odors* section above. 5µL of prenol (Diluted in ultra-pure water to 2M) and ultra-pure water were added to the test

and control sides of the plates respectively–within the scoring circles along with sodium azide as previously described. Approximately 250 IJs were applied to the center and the plate was placed under the modified lid as described in the *Behavioral response of IJs to Insect odors* section above. The assay ran for approximately 1 hour on an anti-vibration platform before being scored. Statistical analysis was done using GraphPad PRISM software package. We used a paired, two-tailed t-test for the analysis.

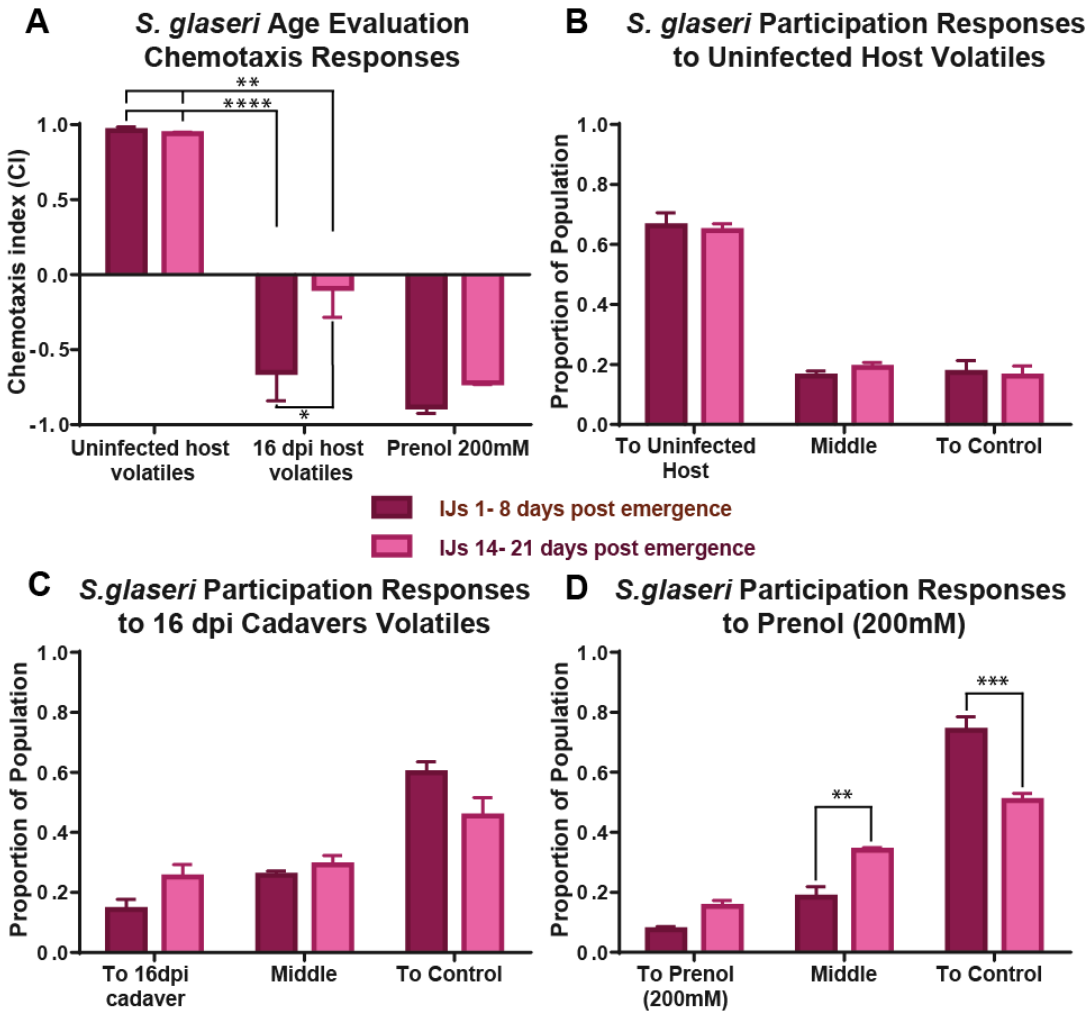
Age Assays

Age assays testing insect odor were done as described above (in *Behavioral Response of IJs to Insect odors*) and assays using prenol were done as described above in *Behavioral Response of IJs to Chemical odorants*. IJs were cultured similarly as described above in *Nematode Culturing*, however culturing methods were changed slightly in the following ways: Infection incubation time was limited to 7 days, and white trap incubation was also limited to 7 days. IJs were washed as described before–in 3 washes of tap water and stored in tap water at room temperature in cell culture flasks. *Young* IJs (1-8 days post emergence) were tested approximately 24 hours post collection, while *old* IJs (14-21 days post emergence) were tested 14 days post collection.

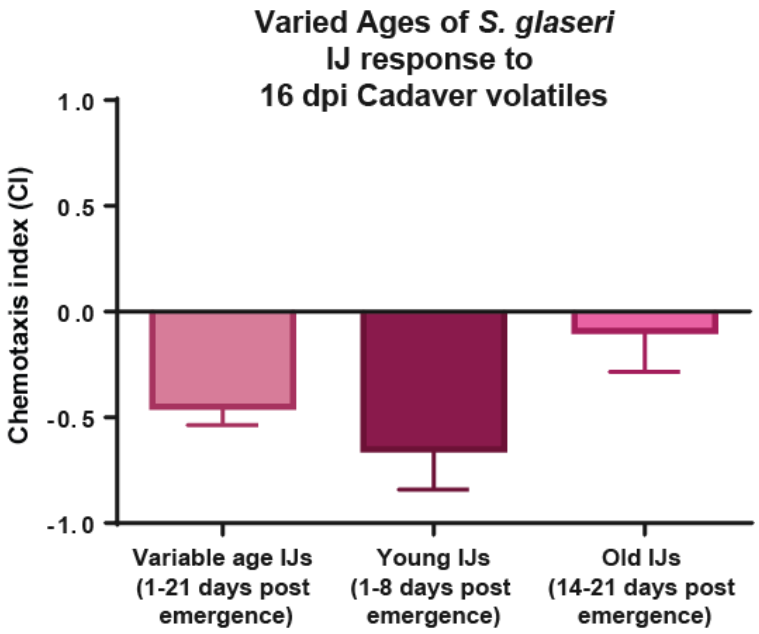
Statistical analyses were done using GraphPad PRISM software package. For CI and participation values in S1 and S3 unpaired two-way ANOVA was used with Sidak's (multiple comparisons) post-test (recommended by PRISM). Statistical

analysis of variable age IJs, young IJs and Old IJs (for *S. glaseri* IJ response to 16 dpi cadaver volatiles in S2) was done using unpaired, ordinary one-way ANOVA with Tukey's (multiple comparisons) post-test (recommended by PRISM).

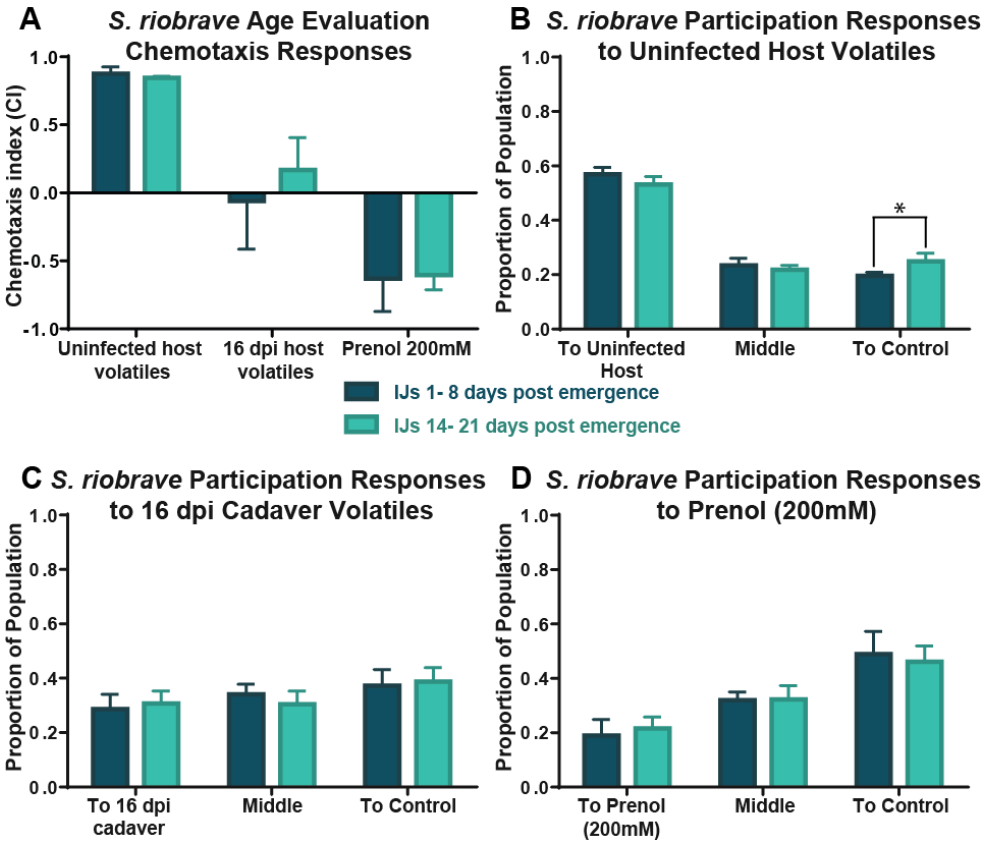
Supplementary Information and Figures



Supplemental figure S2.1 Age Assay results for S. glaseri. (A) Chemotaxis indices for the age assays for responses to uninfected, 16 dpi cadavers infected with S. glaseri and 200mM prenol. There was statistical significance between the two age groups for 16 dpi cadaver volatiles only. For responses to uninfected host volatiles and response to 200mM prenol there was no significant differences in behavioral. **(B)** Participation results for responses to uninfected host volatiles showed no significant differences in behavior between IJ age groups. **(C)** Participation results for IJs responding to 16dpi cadaver volatiles showed no significant differences in behavior between IJ age groups. **(D)** Participation results for IJs responding to prenol. Results indicated a significant increase in portion of older IJs that remained in the middle, as well as a significantly reduction in proportion of IJs that traveled towards the control. Statistical analysis- for both CI and participation results- was done using unpaired two-way ANOVA. Error bars represent SEM.



Supplemental Figure S2.2: Age Assay results for *S. glaseri* response to 16 dpi by variable age, young and old IJs. For experiments done to test *S. glaseri* responses to host volatiles throughout the course of infection the age range of IJs was between 1-21 days post-emergence. Chemotaxis indices above are a comparison of IJ responses to 16 dpi cadavers from variable ages (1-21 days post emergence), young IJs (1-8 days post emergence) and old IJs (14-21 days post emergence). Statistical analysis was done using unpaired, ordinary, one-way ANOVA (with Tukey's multiple comparisons post-test). No significant difference was found between the Variable age CI and the CI values from either young or old IJs. Additionally the statistical analysis revealed there is no significant difference between young and old IJ responses to 16 dpi cadaver volatiles. Error bars represent SEM.

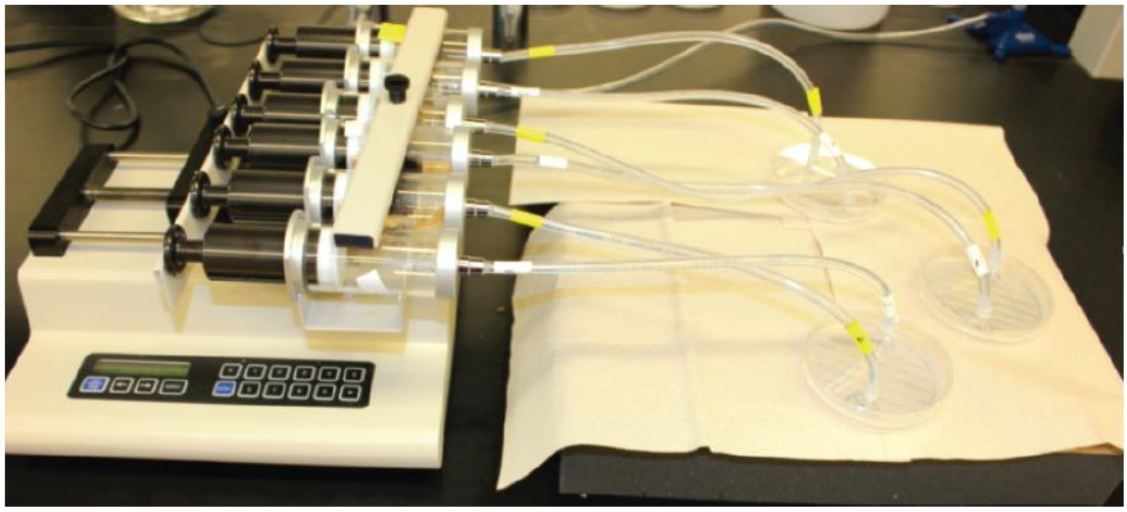


Supplemental Figure S2.3: Age Assay results for *S. riobrave*. (A) Chemotaxis indices for the age assays for responses to uninfected, 16 dpi cadavers infected with *S. riobrave* and 200mM prenol. There was no statistical significance between the two age groups for any of the categories tested. **(B)** Participation results for responses to uninfected hosts. For the proportion of the populations that traveled towards the host or remained in the middle there was no statistical significance. The portion heading away from the uninfected host (to control) we observed an increase for the older IJs. **(C)** Participation results for IJs responding to 16dpi cadaver volatiles showed no significant differences in behavior between IJ age groups. **(D)** Participation results for IJs responding to prenol we saw no significant differences in behavior between age groups. Statistical analysis- for both CI and participation results- was done using unpaired two-way ANOVA. Error bars represent SEM.

A

$$CI = \frac{\text{\# of nematodes in Host circle - \# of nematodes in Control Circle}}{\text{\# of nematodes in Host circle + \# of nematodes in Control Circle}}$$

B



Supplemental Figure S2.4 Host volatiles assay set up. A) The equation used to calculate chemotaxis index. **B)** A photograph of the syringe-pump system set up with syringes loaded with uninfected *Galleria mellonella*. PVC tubing is attached to the end of the syringe and delivers air from the syringe to the chemotaxis plates. Yellow tape marks tubes connected to syringes with hosts, while white tape denotes tubes attached to blank (control) syringes.

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CHAPTER 3

Investigating Dispersal and Repulsion in Entomopathogenic Nematodes

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Abstract

Chemosensory cues are crucial for entomopathogenic nematodes (EPNs)—a guild of insect-killing parasitic nematodes that are used as biological control agents against a variety of agricultural pests. Dispersal is an essential element of the EPN life cycle, where newly developed infective juveniles (IJs) emerge and migrate away from a resource-depleted insect cadaver in order to search for new hosts. Emergence and dispersal are complex processes that involve biotic and abiotic factors, however, the elements that result in EPN dispersal behaviors have not been well-studied. Prenol is a volatile odor associated with EPN-infected, resource-depleted insect cadavers; and it has been speculated that this odor may play a role in dispersal behavior in EPNs. This hypothesis was tested by evaluating the behavioral responses of five different species of EPNs to prenol both as a distal-chemotactic cue and as a dispersal cue. The results indicated that prenol acts as a repulsive agent for all five species tested, while only some species respond to prenol as a dispersal cue.

Introduction

Entomopathogenic nematodes are insect parasitic worms that are leveraged as biological control agents against a variety of pests[1]. Within the EPN life cycle infective juveniles seek out and infect adults before developing into adults which within the confines of their insect host. When resources are depleted from the cadaver the lack of food along with other cues (including small molecules and

pheromones) trigger the development of a modified third stage larva - the infective juvenile (IJ) stage[2-5] which disperses away from the depleted cadaver to search for a new host to colonize, in which it will resume development. These IJs are developmentally arrested, non-feeding and are more tolerant to environmental stress[5-7]. The IJs are the only stage which undergoes the single-time events of emergence and dispersal from the depleted cadaver to find, invade, kill and colonize a new insect host to continue their life cycle. These insect-killing capabilities are why EPNs are used in agriculture as a method of biological pest control[1] and are used as a model system to study host-parasite interactions.

The events of emergence and subsequent dispersal from the natal cadaver not only serves to help EPNs avoid or limit kin competition and inbreeding, but also to drive the newly-developed EPN IJs into the external environment where they will locate new suitable hosts in which they can resume their development[8-10]. It has been shown that ascarosides (nematode pheromones) can stimulate dispersal behavior in nematodes, triggering juveniles to move quickly out of a central location[11]. However, studies on the dispersal capabilities of other small molecules, and the general behavior EPN dispersal remains understudied.

Prenol (3-methul-2-buten-1-ol) is an odor associated with EPN-infected *Galleria mellonella* and has been shown to elicit repulsion from two EPN species: *Steinernema glaseri* and *Steinernema riobrave*.[12]. Our study has built upon this research, investigating additional EPN species; including *Steinernema feltiae*, *Steinernema carpocapsae* and *Heterorhabditis indica*. Moreover, this study has focused on investigating the effects of post-collection age on both chemotaxis responses and dispersal responses to prenol in an effort to differentiate between these behaviors

Despite the differences between dispersal and repulsion behavior, distinguish between these behaviors in EPNs remains under-studied. Dispersal and chemotaxis are both natural events and appear to have some behavioral overlap but are set apart by context. As the insect cadaver becomes depleted of resources a variety of environmental cues, including the absence of food, trigger IJ development[2-5]. Under these conditions there is usually a high density of nematodes within the cadaver and a variety of odors and soluble chemicals that might drive emergence and dispersal, which occur under specific circumstances. Chemotaxis on the other hand is a more general term referring to an organism's response to chemical-based stimuli such as odors or soluble compounds, and is akin to other kinds of taxis such as responses to temperature (thermotaxis), or even responses to magnetic fields (Magnetotaxis)[13-15]. Although dispersal may be behavior that falls under the category of chemotaxis, we propose that

dispersal is a more specific response which may be influenced by various pressures and previous experiences (such as exposure to the odors or soluble compounds found in the natal cadaver). In this study the term “chemotaxis” refers to the nematodes’ response to distal chemotactic cues. Here we address the effect of time post-collection (after the IJs have been removed from exposure to the cadaver’s odors and soluble chemicals) on the IJ response to prenol—as both a distal-chemotactic cue, and a dispersal (direct-contact) cue.

Results

Repulsion to Prenol is Conserved Among EPNs

We evaluated five species of EPNs, including two that had previously been shown to be repelled by prenol at varying doses (*S. glaseri* and *S. riobrave*)[12]. The results revealed that other *Steinernema* species (*Steinernema feltiae* *Steinernema carpocapsae* and even a species in the genera *Heterorhabditis* (*Heterorhabditis indica)* were strongly repelled by doses of 200mM or higher, and that among species responses were comparable for most doses (Figure 1). The response of the five species were also evaluated at varying post-collection ages, at 4 hours, 1 day and 7 days (post-collection) (Figure 2). The results showed that two of the four species (*S. riobrave* and *S. feltiae*) exhibited significantly higher repulsion from prenol at 1 day and 7 days compared to the 4- hour post-collection time point (Figure 2A, B). However, *S. glaseri* and *S. carpocapsae* did not exhibit any significant behavioral shifts in relation to their post-collection age

(Figure 2C,D). *H. indica* exhibited a unique response among the EPNs tested in that repulsion was significantly reduced after 24 hours from being collected (Figure 2E).

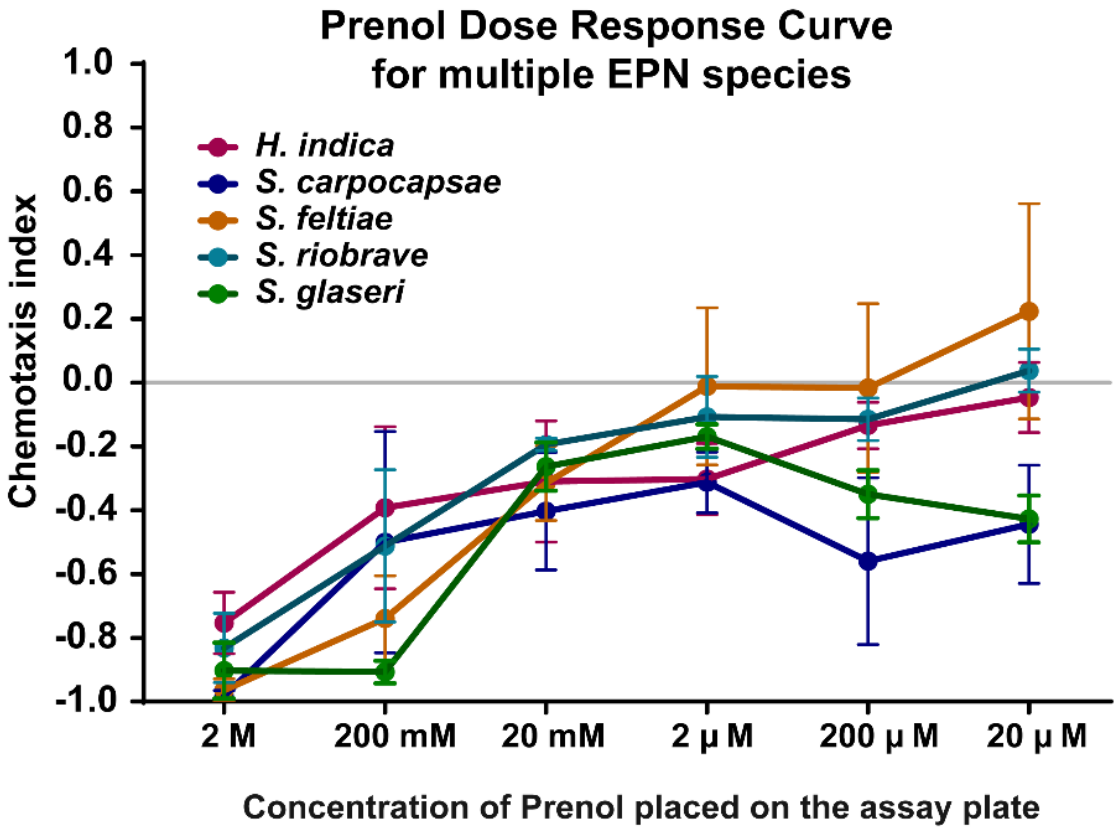


Figure 3.1: Dose response curve results for multiple EPN species *(4-hour post collection IJs). Results of a standard chemotaxis assay where a chemotaxis index (y-axis) near +1 indicates high attraction and a score near -1 indicates high repulsion, with a score near 0 indicating no preference. The concentrations of prenol along the x-axis refer to the concertation that was put on the plate (agar). All species were strongly repelled by 2M and by 200mM, while at 20mM repulsion was mitigated for most species (ranging between -0.2 and -0.45). Below 20mM prenol the responses varied from species to species, between a mostly neutral response to slightly repelled.*

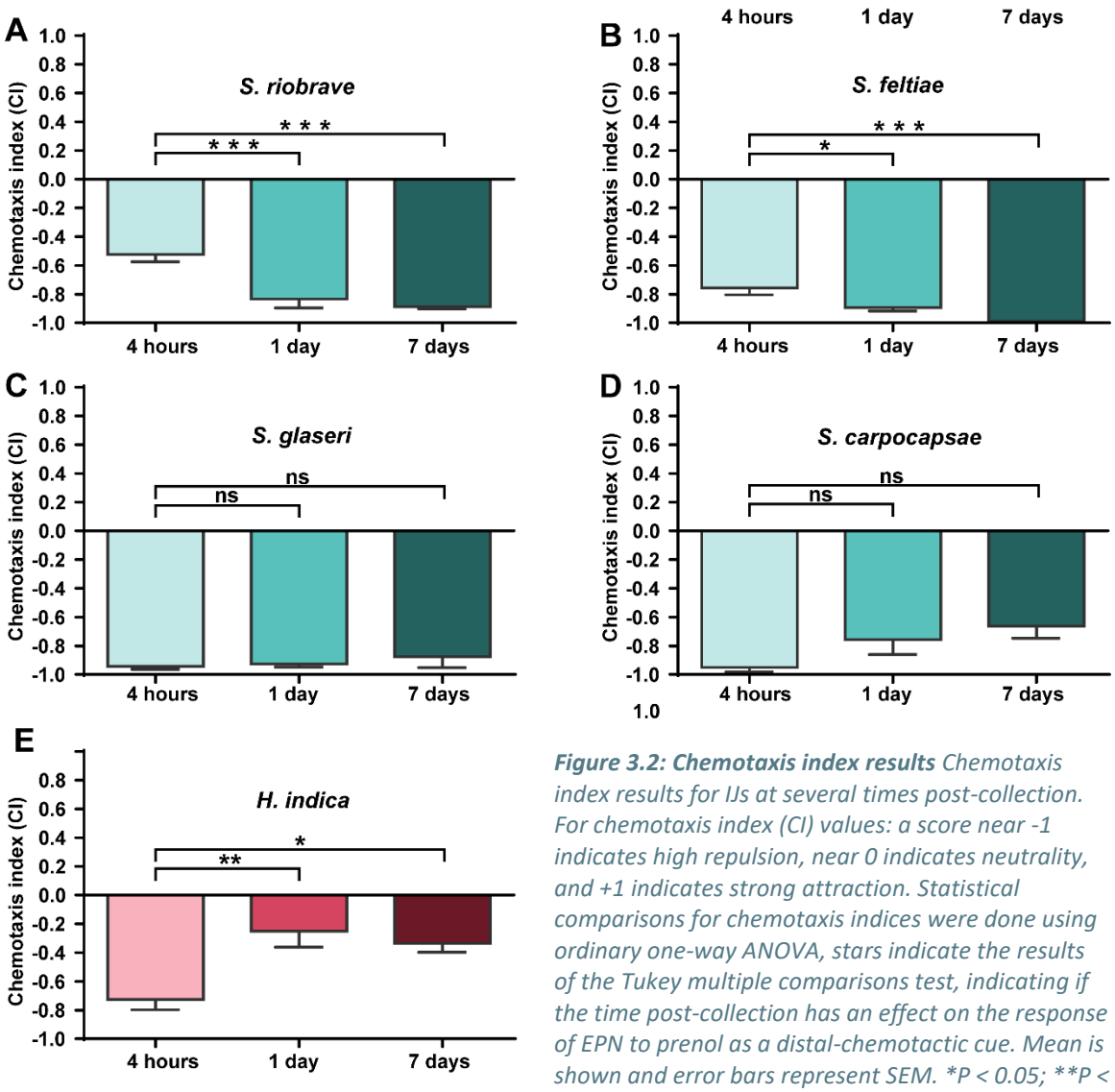


Figure 3.2: Chemotaxis index results *Chemotaxis index results for IJs at several times post-collection. For chemotaxis index (CI) values: a score near -1 indicates high repulsion, near 0 indicates neutrality, and +1 indicates strong attraction. Statistical comparisons for chemotaxis indices were done using ordinary one-way ANOVA, stars indicate the results of the Tukey multiple comparisons test, indicating if the time post-collection has an effect on the response of EPN to prenol as a distal-chemotactic cue. Mean is shown and error bars represent SEM. *P < 0.05; **P < 0.01; ***P < 0.001.*

Dispersal Speed is Consistent Across Time Post-Collection

We evaluated effect of post-collection age on motility, using a 2-zone dispersal assay which we constructed based on previous work[11,16] (Figure 3A). The data revealed that post-collection age had no significant effects on the motility of each species (Figure 3B) and also provided information for approximately how long a dispersal assay should be for each species to allow for approximately 10% of the

population to cross the boundary of zone 1 into zone 2. This information also yielded information on appropriate timing measures to be used in the following dispersal assays: 60 minutes for *S. carpocapsae*, 14 minutes for *S. riobrave* and *S. feltiae*, 9 minutes for *S. glaseri* (for logistical reasons a slightly longer amount of time was chosen) and 20 minutes for *H. indica*.

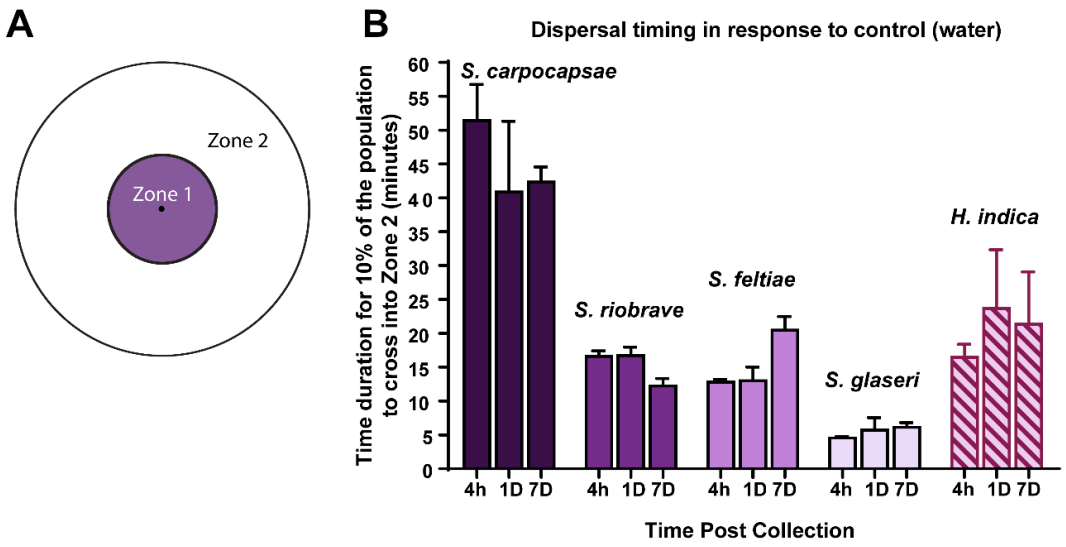


*Figure 3.3: **Timing assay results**. A. Template for timing and dispersal assays, black dot in the center of zone 1 represents initial placement of nematodes. B. Results of timing assays for each species and time post-collection. Along the X-axis the time post-collection is listed: 4 hours (4h), 1 day (1D) and 7 day (7D). Evaluations for each species and time point post-collection were done with photography (see methods) to determine how long it took for the IJs to cross completely over into zone 2 from zone 1 (shown in panel A). The RM two-way ANOVA indicated that there were no statistically significant differences between each species (cross-species comparison can be seen in Supplementary Fig. S3.3).*

Prenol Acts as a Dispersal Cue for Some EPNs

To evaluate the effects of prenol on dispersal behavior among the five species we used the dispersal assay template previously used for evaluating motility and timing of the IJs (Figure 3A). Both prenol and a control (water) were evaluated for how they affected dispersal from the center of the arena (Figure 4A-E). It was our

hypothesis that younger IJs (4hours-1-day post collection) would experience significantly higher dispersal compared to older (7day post collection) IJ populations (See Figure S1). Such a trend was only seen in two of the five species: *S. feltiae* and *S. glaseri* (Figure B, C). Although S. carpocapsae and *S. riobrave* (Figure 4A, D) had significantly higher dispersal compared to water, the trends do not reflect what we would expect to be indicative of response to a dispersal cue (with diminished respond to prenol in relation to the post-collection age). Interestingly *H. indica* (Figure 4E) did not show a strong response to prenol at any of the post-collection time points, instead, water appeared to elicit an increase in dispersal compared to prenol at earliest post-collection time (4 hours). Additionally, IJs exhibited significantly lower dispersal (to water) by 1day post-collection.

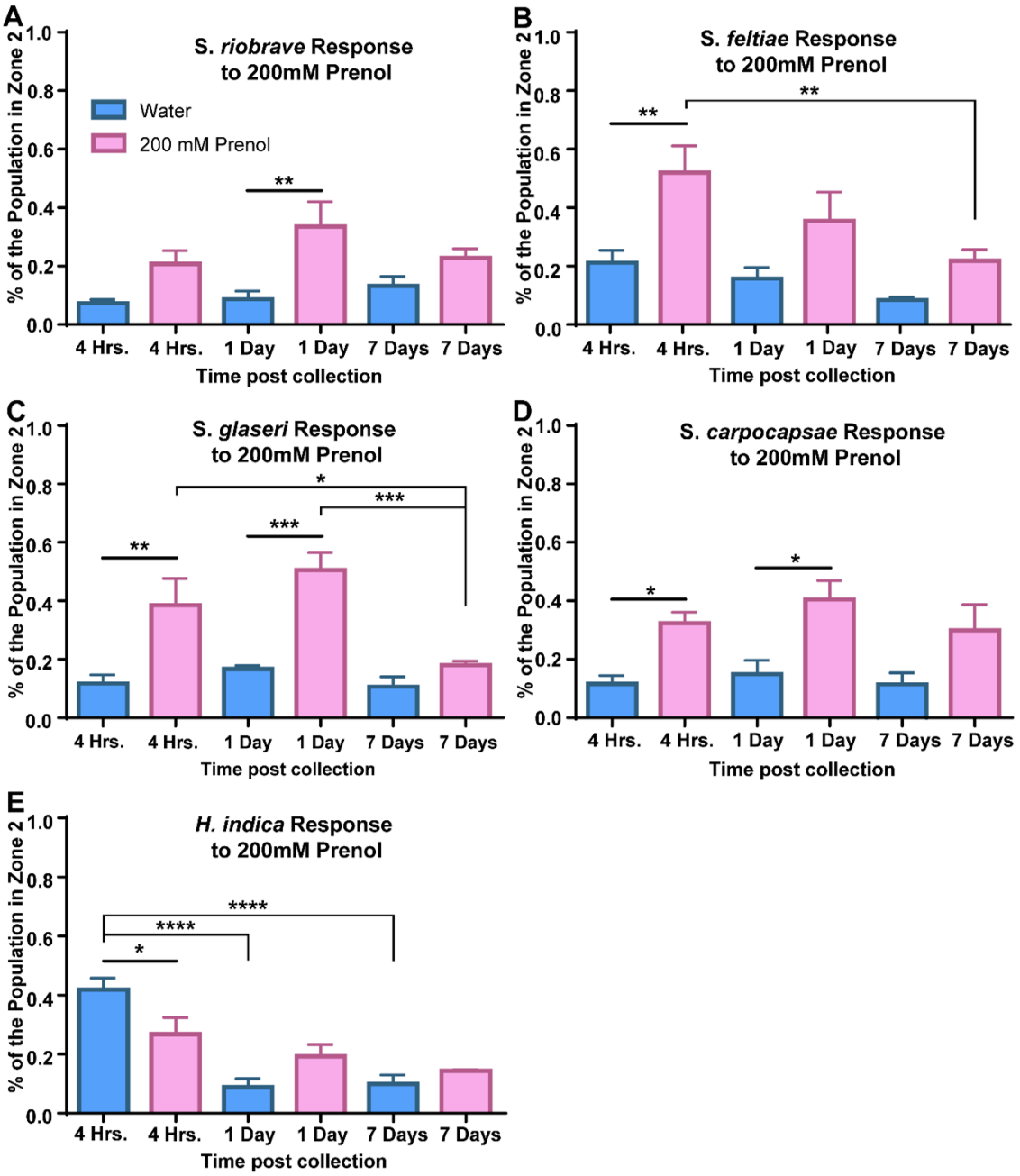


Figure 3.4: Dispersal assay results for different species of EPNs. Responses to water are shown in blue and response to prenol are shown in pink. Assays were run for a different length of time for each species (*S. riobrave, S. feltiae,* and *H. indica* were run for 15 minutes, *S. glaseri* was run for 10 minutes and *S. carpocapsae* was run for 1 hour). After the elapsed time, assays were scored to evaluate what percentage of the population had moved completely into zone 2 (see Fig. 3.3A for breakdown of scoring regions). Statistical analysis used two methods: first was a comparison between water and prenol, done using an RM *two-way ANOVA* with *Sidack's multiple comparisons test*, results shown as a simple bar above prenol and water. A second statistical analysis (using the same parameters as listed above) was used to compare the general responses to prenol over the different time points post-collection.

Discussion

Although much work has been done to evaluate chemotactic responses of EPNs to odors or different sources of odorants[13,15-20], the details regarding dispersal behaviors in response to chemical stimuli has remained relatively understudied, with previous research focusing primarily on ascarosides (nematode pheromones)[11]. The odor prenol had been found in association with *S. glaseri* and *S. riobrave* infected cadavers, and work done in the Baiocchi et. al. 2017 led to speculations that prenol might serve as a dispersal cue. The work here has built upon those findings, revealing that other species in the *Steinernema* genus respond similarly to prenol compared to *S. gaseri* and *S. riobrave,* and that the repulsion behavior is even exhibited by a *Heterorhabditis* species. However, in contrast to the previous study, our work here has primarily focused on the aspect of post-collection age within a specific time frame, allowing us to more closely evaluate on behavioral shifts that occur as a result to time spent away from the natal cadaver (post-collection age). Furthermore, the addition of dispersal assays sought to evaluate each species responses to prenol as a potential dispersal cue, as had been speculated in previous work[12].

Although age can certain cause a variety of behavioral shifts, we observed that motility within the time range that we tested was not significantly affected. However, it was clear that motility between species was a major factor – especially in the case of *S. carpocapsae*, a known ambusher[21]. Since each

species was given an appropriate amount of time for the dispersal assay and motility across the post-collection ages did not shift significantly for any, we propose that the shifts in in behavior seen in the dispersal assay are most likely caused by changes in sensitivity to prenol; indicating the use of prenol as a dispersal cue. Based off Baiocchi et. al. 2017 we had proposed that younger IJs (either 4 hours or 1day post collection) would exhibit significantly higher dispersal than older IJs (as is depicted in Figure S1). This trend was only seen for two of the tested species: *S. glaseri* and *S. feltiae*. Moreover, the behavioral trends exhibited in the dispersal assays by these two species, are not simply a reflection of the CI values displayed in Figure 2. Whereas *S. glaseri* repulsion did not appear to significantly change with respect to post-collection age, the response to prenol as a dispersal cue drops drastically by 7-days post collection. Perhaps even more compellingly, *S. feltiae* IJs exhibit stronger repulsion with increased time post-collection, whereas in the dispersal assays the opposite trend was seen, where the IJs responded most strongly at the 4-hour time point and dispersal dropped significantly by 7-days post collection. These two cases indicate that although prenol may be a repulsive chemotactic cue, that this dispersal assay appropriately serves to evaluate dispersal as a different facet of EPN behavior.

Improving our understanding of EPN behaviors such as dispersal behavior will continue to require more work. However, this study has provided and easy,

quantitative approach to evaluating dispersal behavior, and could potentially be used in future studies for evaluating other odors such as those associated with EPN infections or even ascarosides.

Materials and methods

Nematode Culturing

The following strains were cultured and used for experiments: *S. carpocapsae* (All), *Steinernema feltiae* (Strain SN), *Steinernema glaseri* (Strain NC), *Steinernema riobrave* (strain TX-355), and *Heterorhabditis indica* (Strain Hom-1)[22,23].

To culture the nematode strains used in the experiments, standard procedures were followed as previously described[13,20,24]. A 6 cm petri dish was lined with a 5.5 cm filter paper (Fisher Scientific). Six last instar *Galleria mellonella* larvae (waxworms) were then added to the plate along with approximately 30-50 nematodes per host to undergo the infection. Waxworms were purchased from CritterGrub (Wausau, WI).

The infections were then stored at room temperature (23-25°C) for seven days (for all species except *H. indica* which usually required between 9-11 days of incubation to get a sufficient of amount of IJs) and subsequently white-trapped. Infected waxworms were placed on White traps. The lid of a 35 mm petri dish

was placed in the center of a 10 cm petri dish and was topped with a 7cm size filter paper which was lightly wetted with tap water. The infected and incubated waxworms were then placed on top of the filter paper and arranged in a star-like pattern to provide the newly emerged IJs sufficient room to emerge from the cadaver (only 5 to 6 wax worms were placed on each trap to avoid overcrowding). 6-7mL of tap water was then added to the bottom of the plate to keep the filter paper wet and provide a reservoir for IJs to swim in until collection. Seven days after the infected worms were placed on a White trap, IJs were collected into 15 mL conical tubes. IJs that were to be used in behavioral assays were then rinsed three times in tap water before being stored in VWR 25 mL tissue culture flask at room temperature, in clean tap water, for no more than seven days per the requirements of the study. Densities of the IJ cultures varied between species: For *S. glaseri* - which is larger than the other species of nematodes[25] - the density was kept below 3000 IJs /mL, and most cultures of *S. glaseri* were kept at approximately 2000 IJs/mL. For all other species, the densities were kept between 2,000-5,000 IJs/mL, with most cultures having a density around 3000 IJs/mL. The IJs used for emergence assays were not cleaned as described above in order to prevent the unnecessary loss of IJs, since they were to be quantified but not used for any further behavioral experiments. IJs were kept in the 15 mL conical vials until they could be counted (within seven days).

In future experiments it may be advantageous to use a narrower window of time for IJ collection from White traps. In these experiments we allowed IJs to emerge for seven days before collecting all of the IJs in a given White trap, however, emerging IJs could be collected every two days, providing a narrower age range in subsequent behavioral experiments.

Chemicals and their Storage

3-methyl-2-buten-1-ol, more commonly known as prenol, was obtained from Acros Organics. Due to the volatile nature of prenol, the 200 mmol dilutions used for experiments were made bi-weekly and stored in amber glass vials. All aliquots of prenol were stored at room temperature and limited light exposure.

Chemotaxis Assay

Chemotaxis assays were conducted as previously described[12]. A template for scoring (Figure S2) was placed on the bottom of a 10 cm chemotaxis plate, chemotaxis media was made as previously described[26]. To each plate, 2 µL of 1 M sodium azide (a paralytic) was placed on each scoring circle. 5 µL of 200 mM prenol, diluted in Milli Q water, was then applied to one of the scoring circles, and on the opposing scoring circle 5 µL of Milli Q water was applied. IJs were then placed in the center of the chemotaxis plate. A 5 µL pellet, containing approximately 250 IJs was placed in the center of the plate (pellet was prepared by pipetting a calculated volume of IJs based on the density of the collection, IJs

were allowed to settle to the bottom of the 1.5 mL tube before being pipetted as a pellet in approximately 5 µL). Each experimental condition consisted of three plates run simultaneously, stacked such that each plate was turned 120 degrees to account for side bias in the assay. The stack of three plates were placed in an empty freezer box and was left on an anti-vibration platform for the duration of approximately one hour. At the termination of the assay the plates were scored using the following method. The IJs in the scoring circles were counted first giving us our chemotaxis index. The entire population of nematodes on the plate was then counted and scored based on their placement on the template in order to see the participation of the IJs. For all species and conditions 6 experiments were done. For each experiment a total of 3 technical replicates (3 plates run in parallel) were completed, except for *H. indica* experiments where 9 plates were run to help account for observed higher variability in behaviors, and to assist in our initial investigation of this particular genus's behavior in response to prenol.

Dose Response Curve Experiments

For the dose response curve, the IJs were cultured as described above. Within 4 hours of being collected from the White trap, IJs were placed on a chemotaxis plate with a template attached and the methods described in *Chemotaxis Assay* were used to conduct these assays. 3 experiments consisting of 3 plates each were ran for each of the *Steinernema* species. For, *H. indica*,

there was slightly higher variability in the results and thus a 4th experiment was completed.

Timing Assay

Timing assays were conducted in order to determine the duration of the dispersal assays. A template (Fig. 3B) was placed on the bottom of a chemotaxis plate, experimental design was modified after Kaplan et. al. 2012, while the template design was from Castelletto et. al. 2014. The chemotaxis plate media was made in the same fashion as the plates used in the chemotaxis assays[26]. The template design consisted of two concentric circles (the outer one fit just within the rim of the 10cm petri dish bottom). The inner circle had a diameter of 3cm meaning IJs would have to travel approximately 1.5 cm to move outside of the inner zone (denoted as zone 1). Cross-sectional lines were used to assist in counting.

A pellet of 250 IJs was placed in the center of a chemotaxis plate. The excess water from the pellet was allowed to dry and once it had, 2 µl of Milli Q water was placed directly upon the nematodes. The plate was then placed under a camera (Cannon EOS Rebel T5i EOS 700D with attached Macro lens – Cannon EF 100 mm f/2.8L Macro IS USM) and adjusted for clarity. As soon as the first nematodes broke the surface tension of the water the time-lapse photos began. The photo periods for each species were as follows: 30 second intervals were allotted for *S. glaseri, S. riobrave*, and *S. feltiae*. *S*. *carpocapsae*, due to its

known ambusher foraging strategy, was photographed every 60 seconds. The assays lasted a total of 20 minutes for *S*. *glaseri,* 30 minutes for *S*. *feltiae* and *S*. *riobrave,* and 60 minutes for *S*. *carpocapsae.* Ambient lighting from over-head the lights in the room were as it was sufficient for obtaining photos, without exposing the nematodes to excess light or heat from light bulbs. Immediately after the last photo was taken the plates were counted to determine the total number of nematodes on the plate. Image sequences were then analyzed to determine when 9-15% of the population had fully crossed the boundary between zone 1 and zone 2. Timing was recorded and used to establish the duration of the dispersal assays for each species. We would like to note that *S. glaseri* dispersed quickly in our control (Milli Q water) thus we opted for a slightly longer dispersal assay duration which aligned with 9-13% of the population crossing the border. This was done for logistical purposes, as it was far easier to run a 9-minute assay than a 5-minute assay. For timing assays, each experimental condition (each time point, for each species) consisted of a total of 3 experiments, in each experiment3 sequential runs (run one after another since only one camera was available).

Dispersal Assay

In order to determine the dispersal behavior of the IJs when in contact with prenol, a dispersal template (Fig. 3B) was added to the bottom of a chemotaxis plate. A nematode pellet containing approximately 250 IJs was placed in the center of the plate. The pellet was allowed to dry, and then either 2 µL of water or

of prenol (200mM) was applied to the nematodes. Once the applied liquid had soaked into the agar and the nematodes broke the surface tension, the plates were stored in freezer boxes and placed upon anti-vibration platforms for the duration of the assay. The length of the assay varied based on the nematode species. 9 minutes was allotted for *S. glaseri,* 14 minutes was given to *S. riobrave*, and *S. feltiae*, 20 minutes was allotted for *H*. *indica*, and *S*. *carpocapsae* was given 55 minutes to disperse as determined via the timing assays. The plates were prepared individually with approximately 5-minute gaps of time in between each plate to allow enough time for quantification. At the termination of the assay the nematodes were scored. The nematodes that had fully crossed outside of zone 1 and were no longer touching the border were counted first and were classified as being 'dispersed'. The nematodes that were touching the border line were counted and considered to have not fully crossed into zone two of the template. Lastly, the nematodes within the center of the circle were counted, because they remained in the center of the plate they were classified as not exhibiting dispersal behavior. Each experimental condition (each time point, for each species) consisted of 4 experiments, each done with 3 technical replicates (3 plates run in parallel).

For the chemotaxis, timing, and dispersal assays, IJs that had been collected—within 4 hours, 1 day or 7 days—prior to the experiment were used. These timepoints were selected for logistical purposes (in addition, potentially ecologically-relevant changes that might occur with time post-removal/isolation

away from the cadaver) given the time frames of infection incubations, White trap incubations and holding time before the experiment.

For both chemotaxis and dispersal experiments, separate batches of nematodes were used for each replicate (i.e. each 4-hour experiment was done using a batch from a separate infection-white trap batch). For each experiment across the time points we used the batches consistently (i.e. the batch used for our first 4-hour experiment, first 24-hour experiment and first 7-day experiment all came from the same batch of collected nematodes.

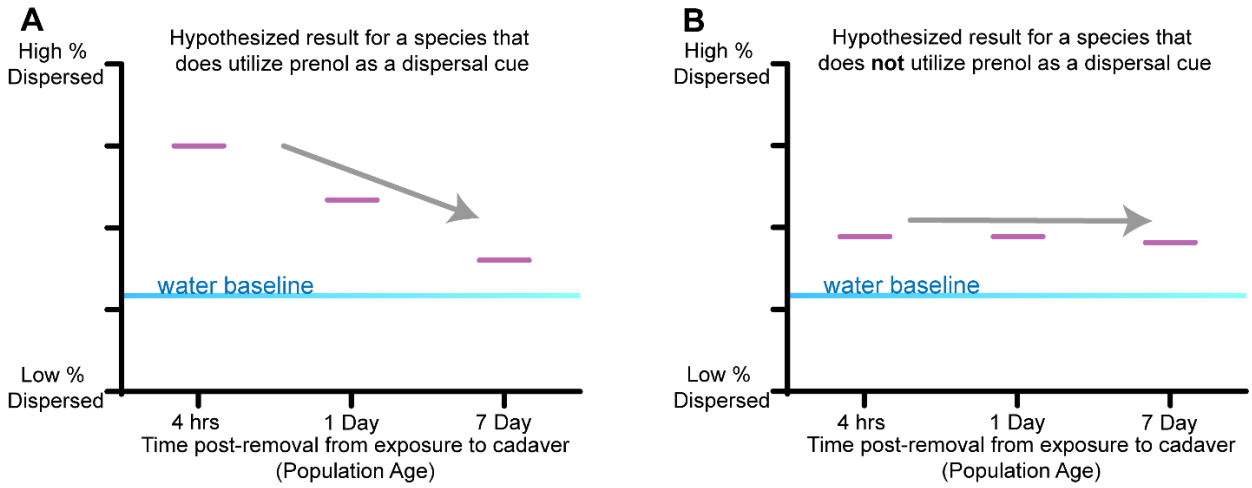
Statistical Analysis

All statistical analyses were conducted using GraphPad Prism software. Analysis of chemotaxis indices utilized an ordinary *one-way ANOVA* and *Tukey's multiple comparisons test*, comparing the means of every column to every other column. For participation values (from chemotaxis assays), a regular *two-way ANOVA* was utilized, with *Tukey's multiple comparisons test*. Analyses compared only within the *test*, *middle*, and *control* sides but not between these regions.

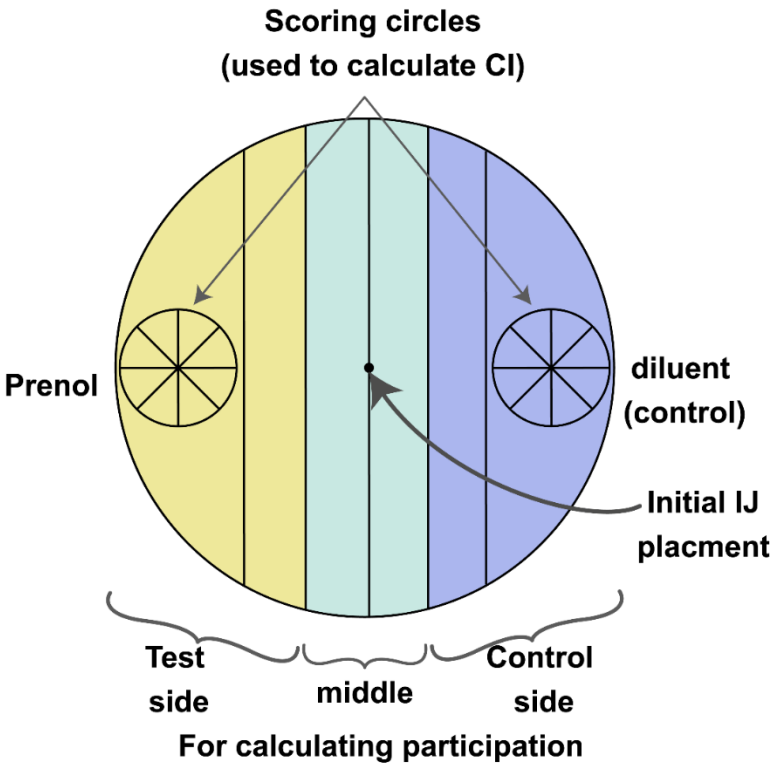
For the timing assays, we used a Repeated Measures (RM) *two-way ANOVA* with *Tukey's multiple comparisons test*. (comparing the behaviors within each species). Additionally, another RM *two-way ANOVA* with *Tukey's multiple comparison test* was run to compare behaviors between species (i.e. comparing the behaviors of species to one another, rather than the time-post collection age within a single species).

Dispersal assays involved an RM *two-way ANOVA* with *Sidack's multiple comparisons test* to evaluate shifts in behavior within each species, and between the time points post-collection.

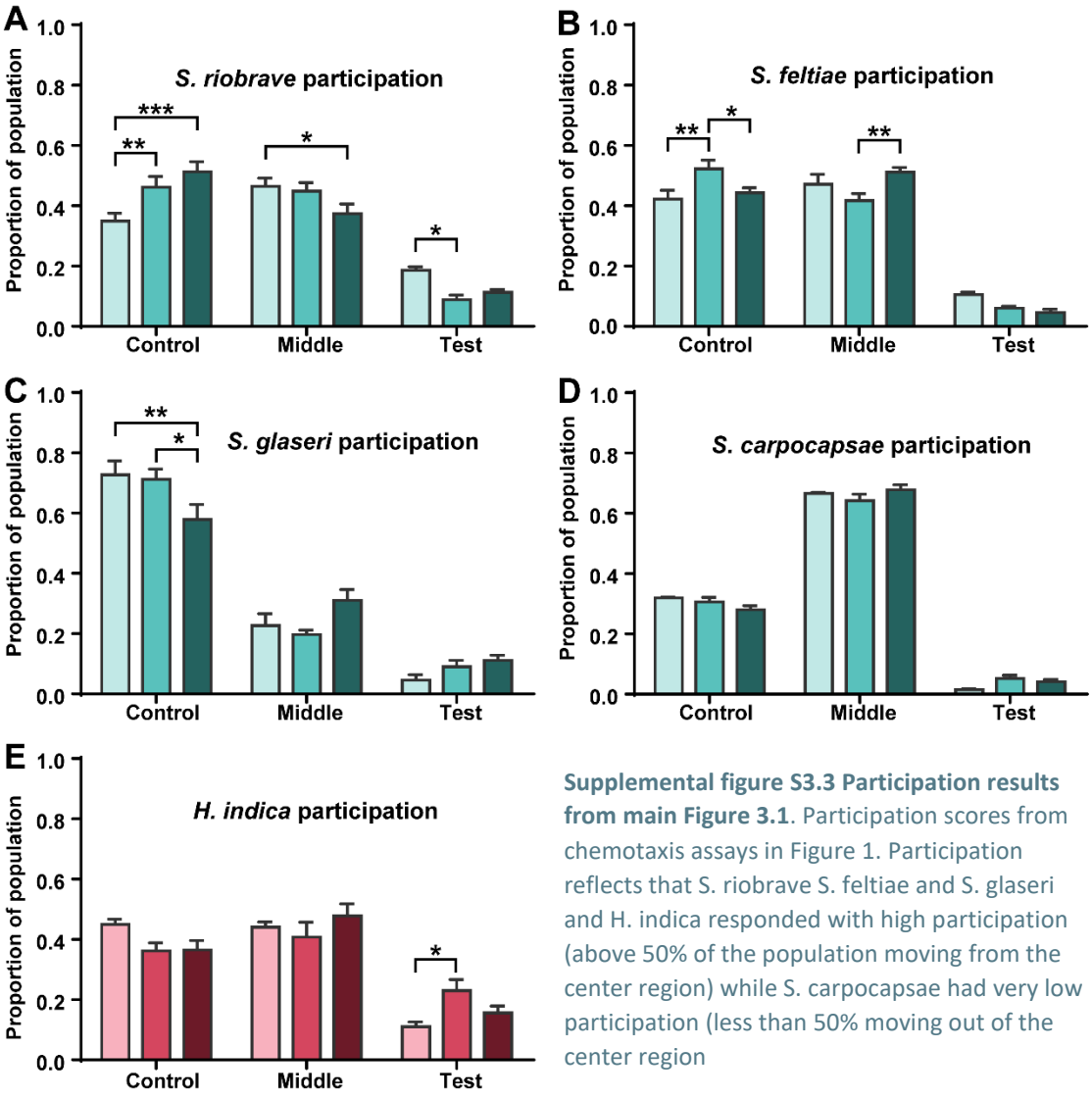
Supplementary Figure legends



Supplemental Figure S3.1 Hypothesized dispersal assay results. Graphical representation of our hypothesis or predicted behavioral trends for A. IJs that do respond to prenol as a dispersal cue B. IJs that do not respond to prenol as a dispersal cue.



Supplemental figure S3.2 Scoring template used for chemotaxis assays. Circles are the scoring sites of the plates and nematodes touching or within the circles are counted and recorded to calculate the chemotaxis index (CI). The participation value is derived by counting the nematodes within each of the three regions of the plate (Test side, middle and control side).



Supplemental figure S3.3 Participation results from main Figure 3.1. Participation scores from chemotaxis assays in Figure 1. Participation reflects that S. riobrave S. feltiae and S. glaseri and H. indica responded with high participation (above 50% of the population moving from the center region) while S. carpocapsae had very low participation (less than 50% moving out of the center region

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CHAPTER 4

Signaling by the AWC Olfactory Neuron is Necessary for Behavioral Response of *C. Elegans* to Prenol, an Odor Associated with Entomopathogenic Nematode-Infected Insects

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Abstract

Chemosensation plays a role in the behaviors and life cycles of numerous organisms, including nematodes. Many guilds of nematodes exist – ranging from the free-living *C. elegans* to various parasitic species such as entomopathogenic nematodes (EPNs) which are parasites of insects. Despite ecological differences, previous research has shown that both EPNs and *C. elegans* respond to prenol (3-methly-2-buten-1-ol), an odor associated with EPN-infections. However, it is unclear how *C. elegans* respond to prenol. By utilizing natural variation and genetic neuron ablation to investigate the response of *C. elegans* to prenol, we found that the AWC neurons is involved in the detection of prenol and that several genes (including: *dcap-1*, *dcap-2*, *clec*-39 and *dod-17*) influence response to this odorant. Furthermore, we identified that the response to prenol is mediated by the canonically proposed pathway which is required for other AWC-sensed attractants. The response to prenol is genetically separable from the response to isoamyl alcohol. These findings reveal specific genes that influence nematode behavior and provide a foundation for future studies to better understand the role of prenol in nematode behavioral ecology.

Author Summary

The ability to detect and respond to environmental cues is critical in the life cycles of many organisms including nematodes. Many nematodes that affect humans rely on olfaction to successfully complete their life cycle, such as those that cause devastating disease (e.g., hookworm *Ancylostoma duodenale* and *Necator americanus*) and nematodes used in biological control such as insect-killing parasites in the genus *Steinernema* and *Hetorhabditis*. While much is known about nematode olfaction and behavior, there is still much to learn about how these microscopic worms translate detection of an invisible odor into a visible phenotype. Using the model nematode *C. elegans* we have identified several genes that influence attraction behaviors in response to an odor (prenol) that was originally identified in association with insect-parasitic nematode infections. Leveraging natural variation in *C. elegans*, genome wide association techniques and other resources were used to uncover genetic components underlying nematode chemotactic behaviors.

Introduction

Nematodes use olfactory cues for many purposes including finding mates[1], avoiding danger[2], and locating food sources[3,4]. In a previous study the odorant prenol (*3-methly-2-buten-1*-ol) was identified in association with entomopathogenic nematode (EPN)-parasitized insect cadavers[5]. While the infective juvenile (IJ) stage of these insect-parasitic nematode species are

repelled by prenol, dauers of the free-living nematode *Caenorhabditis elegans* exhibit attraction to this odor[5]. Divergence in behaviors have been observed before between EPNs and *C. elegans*, such as in their responses to odors like farnesol, 2,3-butadione, and others[6,7]. It has also been shown that CO_2 is attractive to both EPN IJs and *C. elegans dauers*[6,7], though it is repulsive to *C. elegans* young adults[8,9], and yet the molecular machinery and neurons involved in the response to CO_2 remain relatively unchanged[7-9].

Although prenol (as well as other olfactory cues) yields opposing behaviors in *C. elegans* versus EPNs, the molecular machinery involved in the detection of and response to it remain unclear. This study was aimed at understanding the mechanism driving attraction to prenol at the neuronal and genetic levels. To identify genes that influence attraction responses to prenol we leveraged the *C. elegans* Natural Diversity Resource (*Ce*NDR)[10]. In addition, we also identified the sensory neurons and related molecular machinery involved in the detection of and response to prenol.

This study has also shown that–like many attractive odors–the AWC neurons and the canonically signal transduction pathway for AWC-sensed attractants is involved in the detection and response to prenol. Additionally, this study revealed multiple genes implicated in the response to prenol, including *dcap-2* (and its paralog *dcap-1*), *clec-39*, as well as *dod-17* (down-stream of daf).

Results

Characterization of the *C. elegans* Response to Prenol

We evaluated the ability of *C. elegans* to detect and respond to prenol by performing a dose response experiment, exposing dauers and adults to different concentrations of prenol. We found that *C. elegans* adults and dauers are attracted to prenol, with dauers responding to concentrations as low as 200 µM while adults responded to concentrations as low as 20 µM (Figure 1). Adults appeared to exhibit much higher participation compared to dauers, especially at lower doses (Supplementary Figure S1); for example, with a starting dose of 2 mM prenol nearly 80% of adults respond to the odor whereas only ~40% of dauers respond.

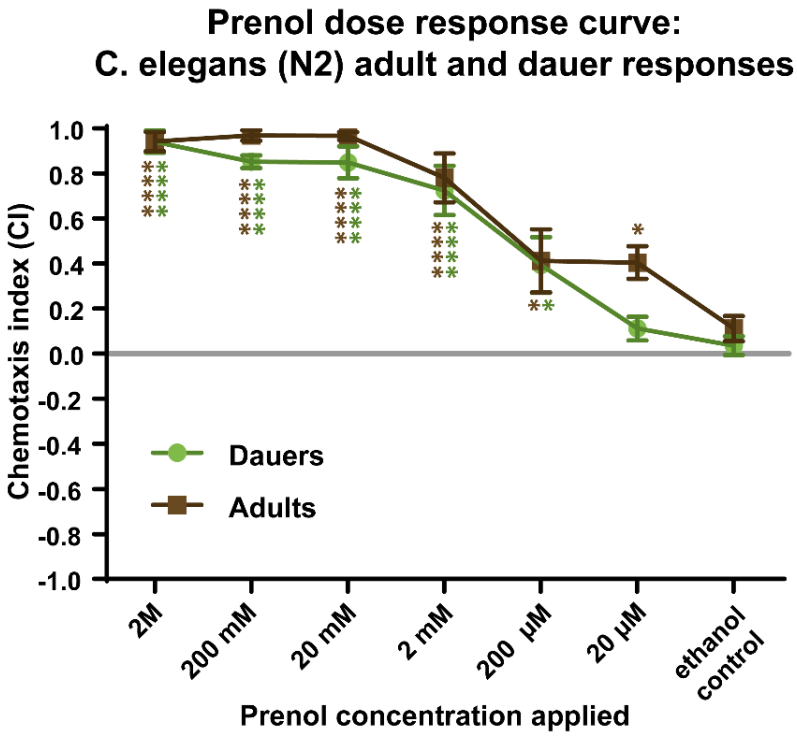
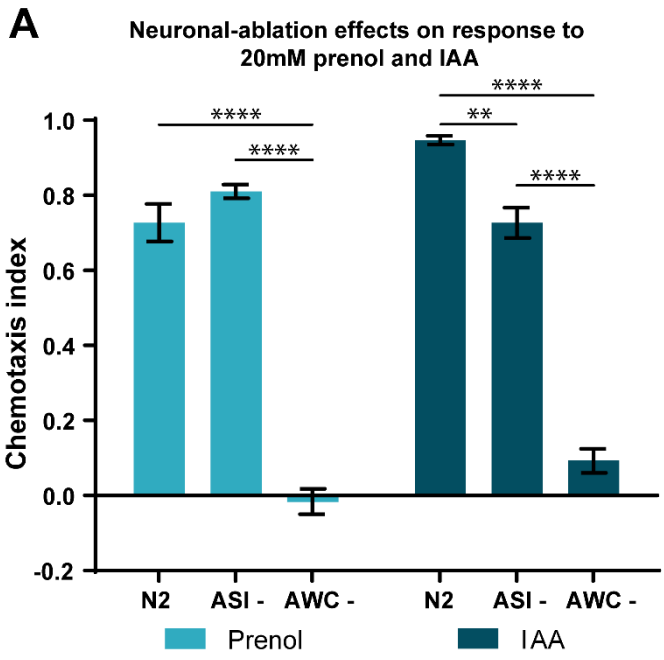


Figure 4.1 Dose response curve for C. elegans dauers and adults*. The concentration of the prenol (in ethanol) dose applied to the test circle of a standard chemotaxis assay. It should be noted that this is the concentration placed on the plate and not necessarily what the nematodes experienced. Error bars indicate SEM, resulting from a One-way Ordinary ANOVA comparing each response to its respective control. Brown *(on left and above data point for 20µM) indicate significant differences between response to dose by adults compared with the ethanol control. Green stars (on right) indicate response to dose by dauers compared with ethanol control. Black stars (between data points for 20µM) indicate comparison between dauer and adult responses to the dose tested. Error bars represent SEM, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001*

AWC Neurons and Canonical AWC Signaling Pathway Mediates Response to Prenol

In the process of identifying the molecular basis of responses to prenol we explored the role of specific neurons. Comparison between N2 (our wildtype control) and strains in which either ASI or AWC neurons had been ablated revealed that the removal of ASI has no notable effects on the response to prenol, while the removal of the AWC neurons completely eliminated attraction to prenol (Figure 2A). As a control, responses to IAA–which is known to be detected by the AWC neurons–were also tested using these strains. Consistent with previous study[11-14], without the AWC neurons, attraction to IAA was drastically reduced, and interestingly the removal of the ASI neurons also led to a statistically significant reduction in attraction to IAA compared to the attraction seen for N2 (Figure 2A), suggesting a contributory role for ASI in the response to IAA.

Having identified the necessity of AWC neurons for detection and response to prenol, several loss-of-function (*lf*) mutants were used to identify the signal-transduction pathway used. We found that detection of prenol likely utilizes the



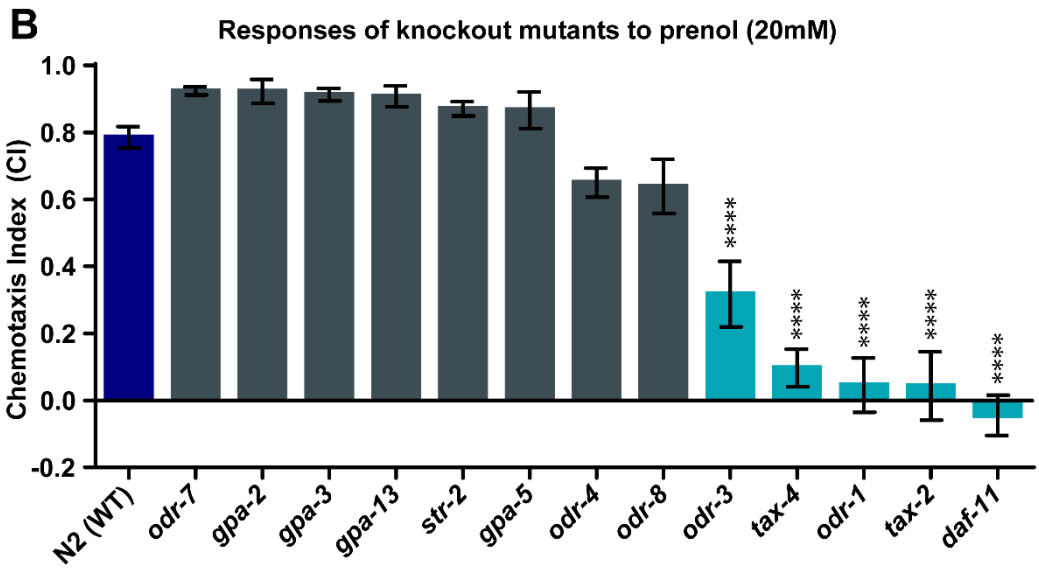


Figure 4.2 Evaluation of neuron-ablated strains and loss of function (lf) mutants *(A) Responses to 20mM IAA and 20mM prenol by genetically-ablated-neuron C. elegans strains. On left is responses to 20mM prenol by N2 (our wild-type control), ASI- (PY7505, (oyls84)) in which the ASI neurons have been genetically ablated), and AWC- (PY7502, (oyls85)) in which the AWC neurons have been genetically ablated. On the right are these same three strains, evaluated for their response to IAA (20mM). Error bars represent SEM. **, p< 0.01; ****, p<0.001. (B) Responses to 20mM prenol by various genetic knockout (lf) mutants—most of which were selected based on the presence in the AWC neurons. All strains were compared to N2 (WT) on far left, highlighted in dark blue. Strains that displayed significantly lower attraction to prenol are highlighted in light blue. Error bars represent SEM. ****, p<0.001. For more information on strain designations for any mutant please see Supplementary TableS4.11 (Resources summary).*

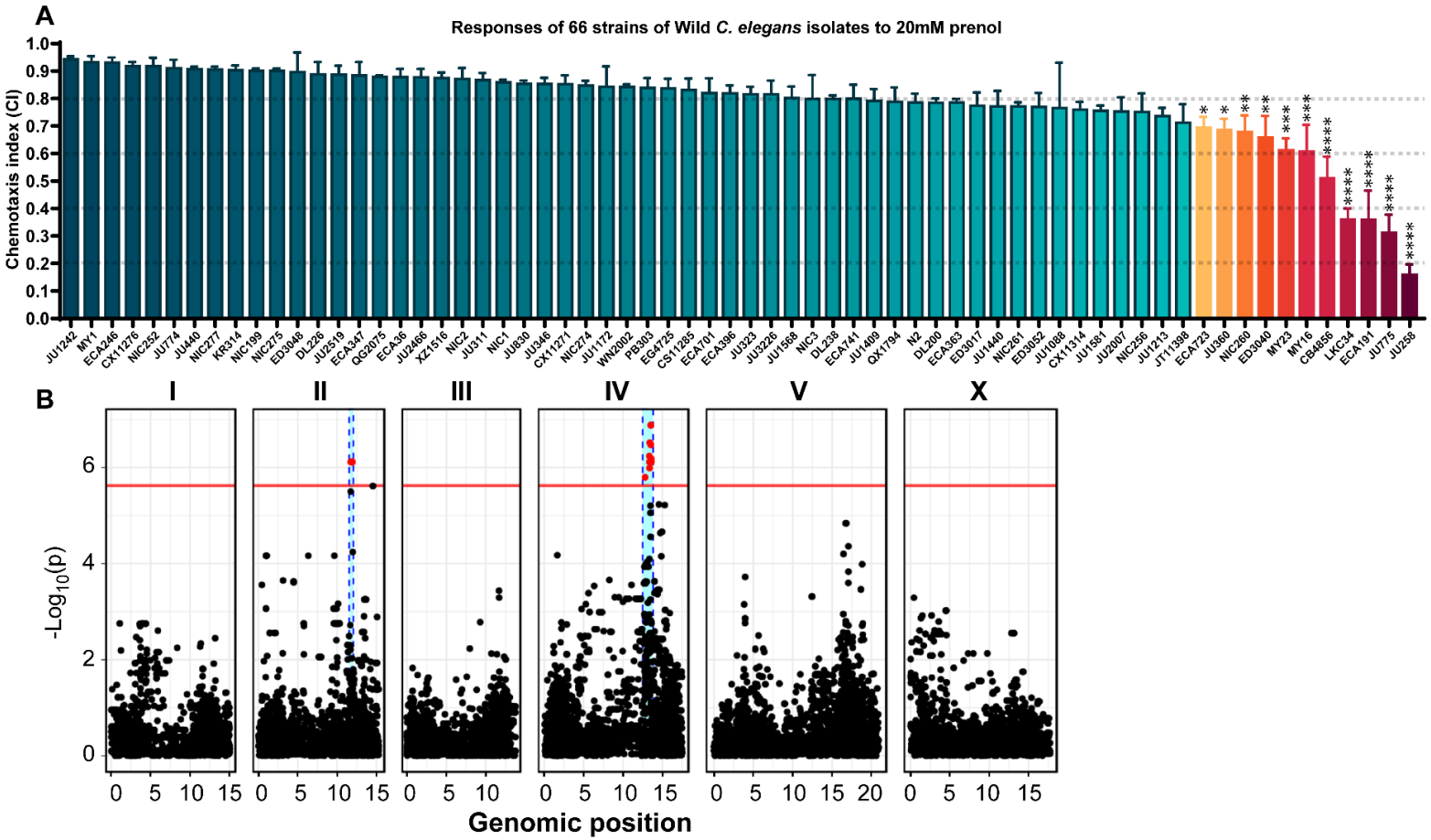
canonical pathway that has been identified for most attractive odors that are sensed by through AWC. The predicted process involves a GPCR (unknown), DAF-11, ODR-1, ODR-3, TAX-2, and TAX-4, among other proteins and various secondary messengers[3]. Loss of function in any one of these signal transduction

genes listed above (*daf-11* (*m47*), *odr-1*(*n1936*), *tax-2*(*p671*), or *tax-4*(*p678*)) results in elimination of attraction response to prenol (Figure 2B). ODR-7 is a nuclear hormone receptor that is required in order for the AWA neurons to express olfactory signaling molecules[15]. We found that *odr-7*(*lf*) had no defect in response to prenol, indicating that the AWA neurons likely do not play a role in the detection of this odor.

Natural Variation Influences Response to Prenol

To further our investigation into the genetic components that either drive or influence attraction to prenol, we utilized the *C. elegans* Natural Diversity Resource (CeNDR), a genome-wide association tool to identify genetic loci associated with various phenotypes. We evaluated 66 natural isolates, 11 of which exhibited significantly reduced attraction to prenol compared to the most attracted natural isolate (Figure 3A). Comparing genetic variation between these strains revealed two quantitative trait loci (QTLs); one on chromosome II and one on chromosome IV (Figure 3B). The QTL on chromosome II spanned 0.543 Mb, while the QTL on chromosome IV spanned just over 1.34 Mb. Within these two QTLs we found 95 high impact genetic variants (variations that would likely result in loss of function such as a frame shift or a premature stop codon), which yielded our list of initial gene candidates. The results also revealed a list of 344 genes with moderate-impact variations, which included missense mutations, splice region variants, as well as in-frame deletions and insertions. Although

missense and splice region mutations have previously been shown to have dramatic effects on gene activity[16,17], we focused on high-impact variations as that resulted in a more manageable list of candidates. Additionally, assessment of linkage disequilibrium indicated that alleles within these two loci may not segregate randomly and thus may be linked with one another (Supplementary Figure S2 panel A). The breakdown of the genes within the QLTs can be found in the Supplementary Tables S3 and S4. A QTL was identified on chromosome V early in our analysis but was eliminated after the inclusion of all 66 strains. This QTL has been included as Supplementary Table S5, since a few genes from this QLT were evaluated further.



C

QTL	Low Impact Varients	Moderate Impact Varients	High Impact Varients
II:11534304-12067524	104	102	27
IV:12490472-13833022	251	241	68

Figure 4.3 Results from CeNDR strains and analysis. *(A) Results of chemotaxis assays evaluating responses to prenol by wild isolates of C. elegans. Most strains displayed strong attraction to prenol with 12 of the 66 strains exhibiting significantly less attraction compared to the top scoring strain (J1242). Error bars represent SEM, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. (B) Manhattan plot based on CI values exhibited by CeNDR strains tested. Along the X-axis the genomic position for each chromosome is displayed and along the Y-axis the levels of significance for the association tested performed by CeNDR is displayed. The red bar set at approximately 5.5 was set by the Bonferroni-corrected value of (-log 0.05/(#SNVs)). The Red dots indicate Single nucleotide variants (SNVs) that display significance values higher than the threshold. The blue highlighted regions within chromosomes II and IV are the quantitative trait loci identified by the analysis, the QTL region is defined by identifying significant single nucleotide variants (SNVs) along with the 50 SNVs before and after the last significant SNV. (C) A breakdown of each QTL displayed in panel B, displaying the location, and number of low, moderate and high impact variants for protein-coding genes within each QTL. For more information on strain designations for any mutant please see Supplementary TableS4.11 (Resources summary).*

Pathways Mediating Attraction to Prenol and IAA are Separable

IAA and prenol have similar chemical structures (Figure 5A) and both elicit attraction of N2 (wildtype control strain) (Figure 5B, C). Among the wild isolates, the highest-scoring strain (JU1242), N2 and the set of strains that exhibited significantly reduced attraction to prenol (CB4856, LKC34, ECA191, JU775, and JU25)(Figure 3A) were evaluated in their responses to IAA (Figure 4) to determine whether natural variation among strains would result in different but specific responses to prenol and IAA. We found that CB4856 and ECA191 displayed reduced attraction to both odors, while LKC34, JU775, and JU258 exhibited much higher attraction to IAA compared to prenol (Figure 4). In addition, CB4856, ECA191, JU258, and JU775 all had reduced attraction to prenol compared to N2, while LKC34 and JU1242 responses were not statistically different compared to N2 (Supplementary figure S6). These data indicate that although there may be overlap in sensing prenol and IAA through

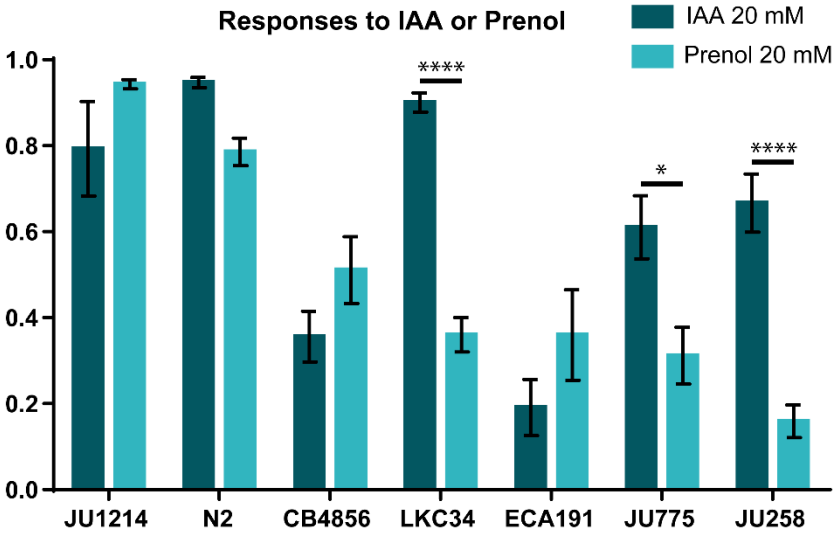


Figure 4.4 Responses of CeNDR strains to IAA (20mM) compared to prenol (20mM). *A comparison between responses to 20mM IAA and 20mM Prenol among indicated strains, including 5 strains that exhibited the most significant reduction in attraction to prenol. Error bars represent SEM. *, p< 0.5; ****, p<0.001.*

the AWC neuron, there are likely genetic components (perhaps within the two identified QTLs) that allow the differentiation of prenol from IAA.

Multiple Genes Influence the Response of *C. Elegans* to Prenol

Of the 95 gene candidates, 23 genes were investigated through evaluation of loss-of-function (*lf*) mutants. We also tested an additional 4 candidate-associated genes (genes which were commonly listed as associated genes for multiple gene candidates identified from *Ce*NDR Results), this included: *pgl-1(bn101)*, *clk-1(qm30)*, *mir-34(gk437)* (which were associated with many of the high-impact candidates identified); and *dcap-1(ok2139)*, a paralogue of *dcap-2* (Figure 5). Among the *lf* mutants tested, three exhibited significantly reduced attraction to prenol compared to the N2 background: *dcap-2* (*ok2029* and *tm2470*), *dod-17* (*ok2387*) and *dcap-1* (*dcap-1*&Y55F3AM.13 (*ok2139*)).

To evaluate the specificity of each gene's influence on the response to prenol, we tested the known *C. elegans* attractant isoamyl alcohol (IAA), which is structurally similar to prenol[11] (Figure 5A, C). We found that *dcap-2*(*lf*) and *dcap-1(lf)* mutants exhibited reduced attraction to both prenol and IAA compared to the N2 wild type (i.e. the reduced attraction is not specific to prenol). Interestingly, *dod-17*(*lf*) mutants–which exhibited reduced attraction to prenol–appeared to have no defect in their attraction to IAA, as responses to IAA were comparable to those of the N2 wild type control.

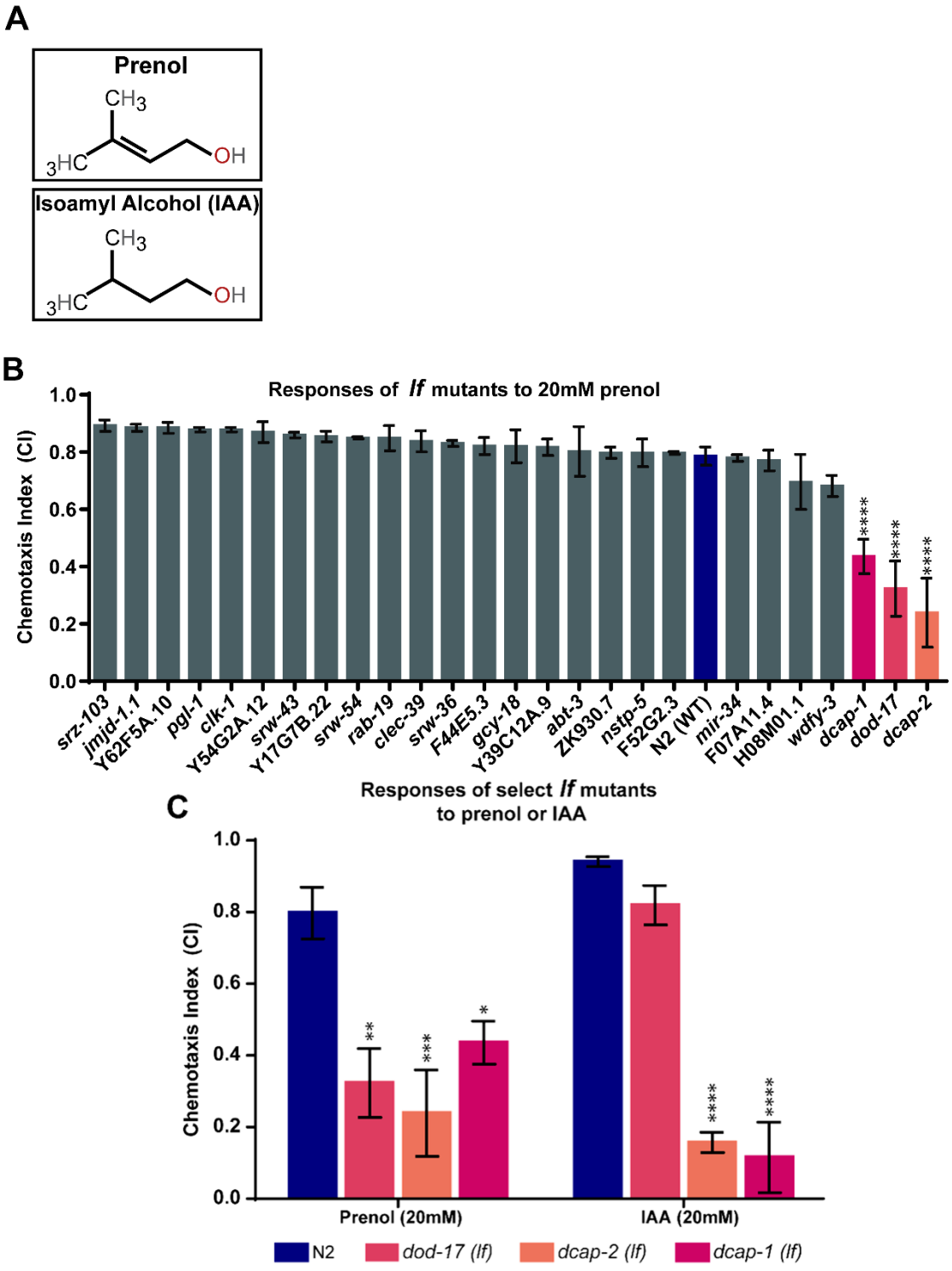

A
Prenol
CH3
3HC
OH
Isoamyl Alcohol (IAA)
CH3
3HC
OH
B
Responses of lf mutants to 20mM prenol
Chemotaxis Index (CI)
1.0
0.8
0.6
0.4
0.2
0.0
srz-103
jmjd-1.1
Y62F5A.10
pgl-1
clk-1
Y54G2A.12
srw-43
Y17G7B.22
srw-54
rab-19
clec-39
srw-36
F44E5.3
gcy-18
Y39C12A.9
abt-3
ZK930.7
nstp-5
F52G2.3
N2 (WT)
mir-34
F07A11.4
H08M01.1
wdfy-3
dcap-1
dod-17
dcap-2
C
Responses of select lf mutants
to prenol or IAA
Chemotaxis Index (CI)
1.0
0.8
0.6
0.4
0.2
0.0
Prenol (20mM)
IAA (20mM)
N2
dod-17 (lf)
dcap-2 (lf)
dcap-1 (lf)

Figure 4.5 Responses of loss of function (lf) mutants to Prenol and Isoamyl alcohol (IAA). (A) Illustration showing prenol and isoamyl alcohol, to show that these two chemical compounds are highly similar in structure; the only difference being the presence of a double bond between the 2nd and 3rd carbon within prenol (which is lacking in the IAA). (B) lf mutants were selected after analysis of CeNDR results. The X-axis displays the name of the gene (or main gene) that was knocked out to create each lf mutant. Comparisons against N2 (blue bar) revealed three low-response lf mutants: dcap-1, dcap-2 and dod-17 (bars of these lf mutants are highlighted in magenta to orange). Error bars represent SEM. ****, $p<0.001$. (C) Analysis of the dod-17, dcap-1 and dcap-2 lf mutants compared to N2 responses to both prenol and IAA. Letters above indicate statistical differences, bars with the same letter are not statistically different. Error bars represent SEM. For more information on strain designations for any mutant please see Supplementary TableS4.11 (Resources summary).

Although *clec-39*(*lf*) did not have a reduced chemotaxis index to prenol (Figure 5), the behavior exhibited by this strain was strikingly different from other mutants we tested. Over 50% of the behavioral assays failed for *clec-39*(*lf*), as the assays did not meet our threshold of 50 nematodes (minimum) moving into one of the four quadrants of the plate during the allotted assay time. This happened enough times to garner our attention, and we decided to quantify and compare the participation of the *clec-39*(*lf*) to the wild type control, N2. Although the chemotaxis index of *clec-39*(*lf*) was comparable to N2 (Figure 6A), they did

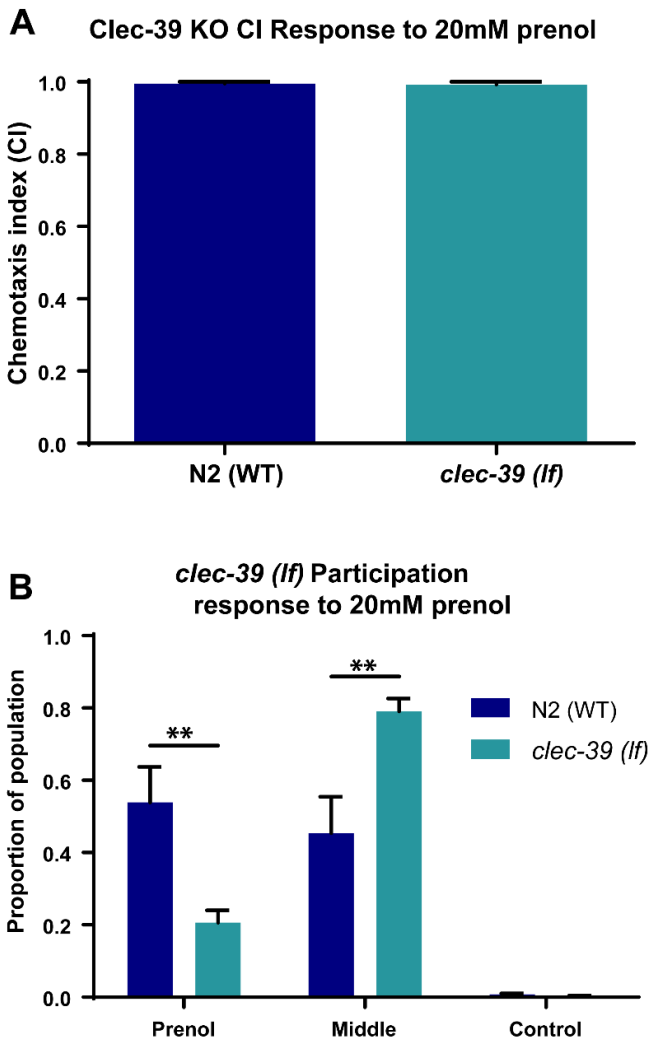


Figure 4.6 Chemotaxis and participation of clec-39 mutants in response to prenol*. (A) Chemotaxis index responses for clec-39 revealed strong attraction to prenol, comparable to N2 responses. Mean is shown and error bars represent SEM. (B) Participation measures reveal that clec-39 knockout mutants exhibit far less participation in response to the attractive odor prenol. Error bars represent SEM, statistical analysis for participation responses was done using an Ordinary Two-way ANOVA with Sidak's multiple comparisons test, comparing N2 to clec-39 mutants within each category of prenol, middle, and control regions. Error bars represent SEM. ** p<0.01.*

not display an uncoordinated phenotype (Unc). However, the *clec-39*(*lf*) mutants exhibited significantly less participation, with nearly 80% of the nematodes remaining in the center of the assay arena compared to <50% of N2 nematodes remaining in the middle. This means that the CI is only representative of about 20% of the population for *clec-39 (lf)* mutants and suggests that *clec-39* affects nematode participation in foraging behavior.

Discussion

Prenol is associated with nematode-infected insects, suggesting that is an ecologically relevant odor and one that may be encountered by soil-dwelling organisms. Prenol elicits strong behavioral responses from a variety of nematodes including EPNs in the genus *Steinernema*, *Levipalatum texanum* (free-living nematode), *C. elegans*, *Pristionchus pacificus* (Supplementary Figure S7), and even *Drosophila melanogaster* larvae[5]. Although the role of prenol in *C. elegans* ecology is unclear, the present study has investigated how *C. elegans* responds to this odorant.

The AWC neurons are involved with the detection and response to numerous attractive odors including isoamyl alcohol (IAA), 2-butanone, and benzaldehyde[11]. Using genetically ablated neuron lines (ASI- (PY7505 *oyIs84*) and AWC- (PY7502 *oyIs85*) (Figure 2A)) and several strains with loss of function in neuron-associated genes (Figure 2B), we determined that the AWC neurons, and several canonical signaling components that are necessary in detecting

other attractive odors are required for detection and response to prenol. We also noted that while removal of the ASI neurons did not affect response to prenol, it did appear to affect response to IAA. Although the ASI neurons are involved in chemotaxis to several complex molecules[18,19], the response to prenol and IAA by ASI-ablated mutants had not been evaluated previously. It was known that the AWC neurons are required for adequate detection and response to IAA, and our data suggest that ASI neurons may contribute to this response[11].

The involvement of *odr-3*, *odr-1*, *tax-2*, *tax-4*, and *daf-11* was also shown through behavioral analyses, supporting previous studies suggesting that these genes (and their resulting proteins) are part of the genetic pathway necessary for AWC-mediated responses to attractive odorants[3]. Both *odr-1* and *daf-11* encode transmembrane guanylyl cyclases, with *odr-1* being involved in discrimination and adaptation[20] while *daf-11* is involved with multiple areas of chemosensation as well as dauer formation and recovery[20,21]. *odr-3* encodes a G-protein alpha subunit, which has been shown to be involved with olfaction and nociception as well as aiding in morphogenesis of olfactory neurons in *C. elegans* [22]. Lastly, *tax-2* and *tax-4* both encode subunits of a cyclic nucleotide gated channel that plays a key role in several aspects of *C. elegans* sensory abilities including chemosensation, thermosensation and even the formation of sensory neurons[23]. Our findings that the AWC neurons as well as the genes and proteins listed above are all involved in the detection of prenol, support the conclusion that prenol likely relies on the same pathway as other odorants, although the proteins

that impart specificity–such as a potential GPCR that might be involved with initial detection of prenol–remain to be determined.

The finding that some natural isolates differed in their responses to prenol versus IAA (Figure 4), indicated that response to these odors are genetically separable, and that there could be a discernable genetic component within the QTLs we identified. Evaluation of candidate genes resulted in identification of four genes that, when knocked-out, appeared to reduce attraction to prenol, none of which had previously been implicated in any chemotaxis behavior. *clec-39* was known to be involved in immune responses to *Serratia marcescens* and although C-type lectins (CLECs) can regulate immune responses both at the physiological level and behavioral level[24], it is not known how or why *clec-39*(*lf*) results in significantly reduced participation in chemotaxis. The connection between *dcap-2* and its paralog *dcap-1* is a bit clearer. Although it was not previously implicated in attraction behavior, it was known that that these two genes are needed for the proper formation of the AWC sensory cilia[25]. Furthermore *clec-39*, *dcap-1*, and *dcap-2* exhibit similar levels of reduced attraction to IAA indicating that their roles in chemosensation are not specific to prenol.

Among the genes identified, *dod-17* stands out as the only gene we identified as having an impact on response to prenol but not IAA (Figure 5C). *dod-17* is expressed mainly in the intestines and is predicted to be involved in innate immunity (see Supplementary Table S8). However, the exact function of *dod*-17 remains unknown. AWC expression data collected in a previous study[26],

also reveals that *dod-17* has lower expression in the AWC neuron compared to whole nematode larvae (though the difference in expression is not statistically significant) (see Supplementary Figure S9). Despite this, it is still clear from the data in this study that *dod-17* can influence response to prenol, though the details of how *dod-17* plays a role in chemosensation is not yet understood and more research is needed.

We have established AWC neuron involvement in detecting prenol and we have identified several genes that affect the response to prenol. We wondered whether examining the transcriptional profile of genes with high-impact genetic variations that we identified would reveal any genes of interest[26] (see Supplementary Figure S9). The transcriptional data revealed that among *dcap-1*, *dcap-2*, *clec*-39, and *dod-17*, none had differential expression between the AWC and whole larvae, with the exception of *dcap-1* which had significantly lower expression in the AWC. In addition, several of the genes we tested that bore no influence on response to prenol also had lower expression in the AWC (see Supplementary Figure S9). However, among the untested high-impact variant gene candidates, three appear to have significantly higher expression in AWC compared to whole larvae: *ttr-51*, *Y45F10D.7*, and *rsd-2*. There were also two other genes with >10-fold higher expression in the AWC neuron but were not found to be statistically significantly differentially expressed; *cul-6* and *F09E8.2*. These may be good candidates for future studies of AWC-mediated behavior.

Information about particular genes–such as those that play critical roles in the AWC neurons–have been leveraged in previous studies to provide a better understanding of the molecular underpinnings of free-living and parasitic nematode behavior. For genes identified in this study, which affect response to prenol, several have orthologues in both EPN species and in nematode parasites of mammals (See supplementary Figure S10). Such information is of value, not just for the improved understanding of *C. elegans* behavioral genetics, but also for understanding what types of genes might be implicated in the detection of olfactory cues in other species of nematodes–such as the EPNs with which the connection between prenol and nematodes was first made. While the molecular genetic pathway underpinning response to prenol is still unclear, the work reported here provides a foundation for future evaluation and efforts to bridge the gap between *C. elegans* biology and its application in EPNs.

Methods

Propagation and Care

Nematode strains were obtained from the C. elegans Natural Diversity Resource (CeNDR), the C. elegans Genetic Center (CGC), the National BioResource project (NBPR) headed by the Mitani lab in Japan, as well as strains created by the Sternberg Lab for this project.

Newly received nematodes were chunked and placed on fresh NGM plates with *Escherichia coli* (OP50) and stored at 21 ± 1°C for several days to allow for sufficient growth before freezing. Once the population was large enough, they were then frozen, and stored at -80°C for long term storage until they were thawed to create a new culture for use[27].

For propagation and care the *C. elegans* strains were maintained as previously described[27,28]. 10cm NGM plates were seeded with approximately 0.2mL of OP50 *Escherichia coli* liquid culture and were incubated overnight at 37°C or for 2 days at room temperature (23 ± 1°C) and were used within 3 days to provide a food source for nematodes in culture. The bacterial liquid stock of OP50 was made and stored at 4°C[28,29] and was remade as needed (usually within 2-3 weeks). To culture the nematodes, 20-30 adults were placed on NGM plates and were stored either at 17°C for continued cultivation, or at 21 ± 1°C for use in experiments or freezing. Nematodes for experimental use were generally bleached within 4-6 days after plating. There were a few exceptions to this time window which we attribute to the genetic modifications in the strains used, might have caused developmental differences in the nematodes. For the *dcap-2* knockout mutants (CGC strain number: RB1641, Mitani (NBRP) allele tm2470) grew slowly and required an additional 2-4 days to propagate before undergoing synchronization via bleaching.

Synchronization via Bleaching and Experimental Preparation

Preparation of a synchronous population of *C. elegans* was done as previously described. Briefly, nematodes were plated onto NGM plates with OP50 as a food source. Populations were then propagated at 20 ± 1°C until a sufficient number of well-fed, gravid adults were obtained. Worms were monitored daily to prevent starvation and were subsequently bleached as described[30] in Porta De La Riva 2012. in order to obtain a synchronous population of young adults. After bleaching nematode eggs were then stored in the 20 ± 1°C incubator overnight to hatch then the nematodes were stored at 20 ± 1°C incubator until the majority of the population were young adults (approximately 2 days and 8 hours for most strains). At this time, they were used for experiments and were cleaned following the procedure described previously[31].

Quadrant Assays

Quadrant assays were used for the majority of evaluations, with the exception of the dose response curve and participation evaluations of *clec-39*.

To examine the behavior elicited by the various C. elegans strains when exposed to various chemicals, a quadrant assay was used. This assay was done as previously described previously [31].

In this assay a quadrant template was attached to the bottom on a 6cm chemotaxis plate. The control (sodium azide) was then applied on the two

quadrants that opposed each other, while the test (prenol in sodium azide) was applied to the remaining two sides. On the plate approximately 200 clean young adults were placed in the center. 3 plates were then stacked and placed in freezer boxes on top of anti-vibration platforms. Each experiment consisted of the three plates (technical replicates run in parallel). These assays were conducted at room temperature for 1 hour, and after the allotted time, the nematodes were counted and scored. 3 plates were used per experiment and 3 experiments (biological replicates) were conducted per strain. The only exception to this was the initial divergent set (Figure 3 and Supplementary Figure S7) in which 6 experiments (biological replicates) were done for all strains. For Figure 3 the 6 experiments were normalized to 3 experiments in order to run statistical analysis. At least 50 nematodes needed to participate (move into one of the quadrants) in the assay in order for it to be entered in the data. This rule applied to all strains except CeNDR strain: CX2386; due to consistent extremely low participation regardless of nematodes on the plate. For this strain we accepted numbers below the threshold of the 50 nematode minimum and ran 6 total experiments (biological replicates) which were normalized to 3 experiments to account for the differences and variation in participation.

Large (10cm) chemotaxis assay

A large chemotaxis assay was used in a few key areas of this study including the initial dose-response curve evaluation and to evaluate participation of *clec-39*.

This assay allowed for evaluation of both chemotaxis as well as participation of the strains that were tested in this manner. The assays were done in the exact same manner as has been previously described[5] by Baiocchi et. al 2017.
A few key differences to note between the large (10 cm) chemotaxis assay and quadrant assay include: the size difference, 2 vs 4 directions for the nematodes to move, and the placement and method of placement of sodium azide. In the quadrant (6 cm) assay sodium azide is the solute, while in the Large chemotaxis (10 cm) assay, 2ul of sodium azide is placed at the top of the scoring circle immediately before the 5 µL of the control or test compounds are added to the assay arena on the scoring circles. test chemical is diluted in milliQ H2O. Aside from these differences both assays were used to measure the olfactory responses of *C. elegans*.

Chemical Preparation

All chemicals were remade approximately every 4 weeks and stored in glass amber vials within an empty freezer at room temperature to limit light exposure.

Sodium azide (paralytic)

Sodium azide (CAS: 26628-22-8) was obtained from Fisher Chemical, (LOT 157679) and is commonly used in chemotaxis experiments[5,11,18]. For quadrant assays, we utilized Sodium Azide (NaN_{3-}) diluted in MQ water served as the diluent for most of our odors. This chemical served to paralyze the nematodes[11] after they had made their behavioral decisions in the assay arena, this served to

minimize the effects of odor adaptation and to increase the accuracy of quantifying the nematodes in the assay. In these 6cm quadrant experiments, Sodium Azide was made at a concentration of 0.5M in MQ water and was also used at a 1M concentration for the large chemotaxis assay.

Prenol

Prenol (3-methyl-2-buten-1-ol) obtained from Acros Organics, LOT A0360271, CAS 556-82-1. Prenol was diluted to a concentration of 20mM in 0.5M Sodium Azide, while in large chemotaxis assays prenol was made at a concentration of 20mM in MQ water.

Isoamyl Alcohol

Isoamyl Alcohol (IAA) (3-methyl-1-butanol; CAS 123-51-3) obtained from Tokyo chemical industry America (TCI America). was used as a positive control. In a 6 cm quadrat experiment, IAA was diluted to a concentration of 20mM in 0.5M Sodium Azide.

Generation of knockout mutants using CRISPR/Cas9

New putative null mutants of candidate genes from this study were generated using a co-conversion CRISPR/Cas9 strategy: the STOP-IN method [32]. Briefly, preassembled Cas9 ribonucleoprotein and short single stranded DNA oligo repair templates were injected to the gonad of the wild-type N2 strain, according to the standard microinjection protocol for *C. elegans* [33]. Desired mutants were identified in following generations by PCR. All alleles were confirmed by Sanger

sequencing. The sequences of guide RNAs and repair oligos, as well as the allele information of the resultant strains, are listed in the Supplementary Table S11- Resources summary.

Statistical analyses

For dose responses (Figure 1A), each data point consists of 3 experiments (biological replicates), each experiment made up of 3 plates run in parallel. Comparisons between responses to dose and responses to the ethanol control were evaluated use GraphPad PRISM software, utilizing and Ordinary Two-way ANOVA with Sidak's correction for multiple comparisons as was recommended by the PRISM software. Statistical analysis of participation was done using an Ordinary Two-way ANOVA, with Sidak's multiple comparisons test.
For evaluation of *C. elegans* strains with specific neurons genetically ablated and their responses to prenol and IAA (Figure 2A), an Ordinary two-way ANOVA was used with Tukey's multiple comparisons test to compared between N2, AWC-, and ASI- within each category of Prenol responses and IAA responses (but not between responses of IAA and prenol). For comparison of loss-of-function mutants for genes related to neuron function (Figure 2B) an Ordinary One-way ANOVA was performed with Sidak's multiple comparisons test.

Statistical analysis of the CeNDR results (Figure 3A), and its components (the divergent set – shown in Supplementary Figure S12) were evaluated using an

Ordinary One-way ANOVA with Sidak's comparisons test. For the full set (of the divergent set, mapping set 1, and alternative strains) shown in Figure 3A, all strains were compared against JU1242 (the top scoring strain). For the Divergent (Supplemental Figure S12), all comparisons were against N2 as a representative of a high-scoring strait. Additionally, in the results and discussion below a comparison to N2 is also mentioned- regarding the full compilation of all strains- Figure 3A; this too was done using an Ordinary One-way ANOVA with Sidak's multiple comparisons test.

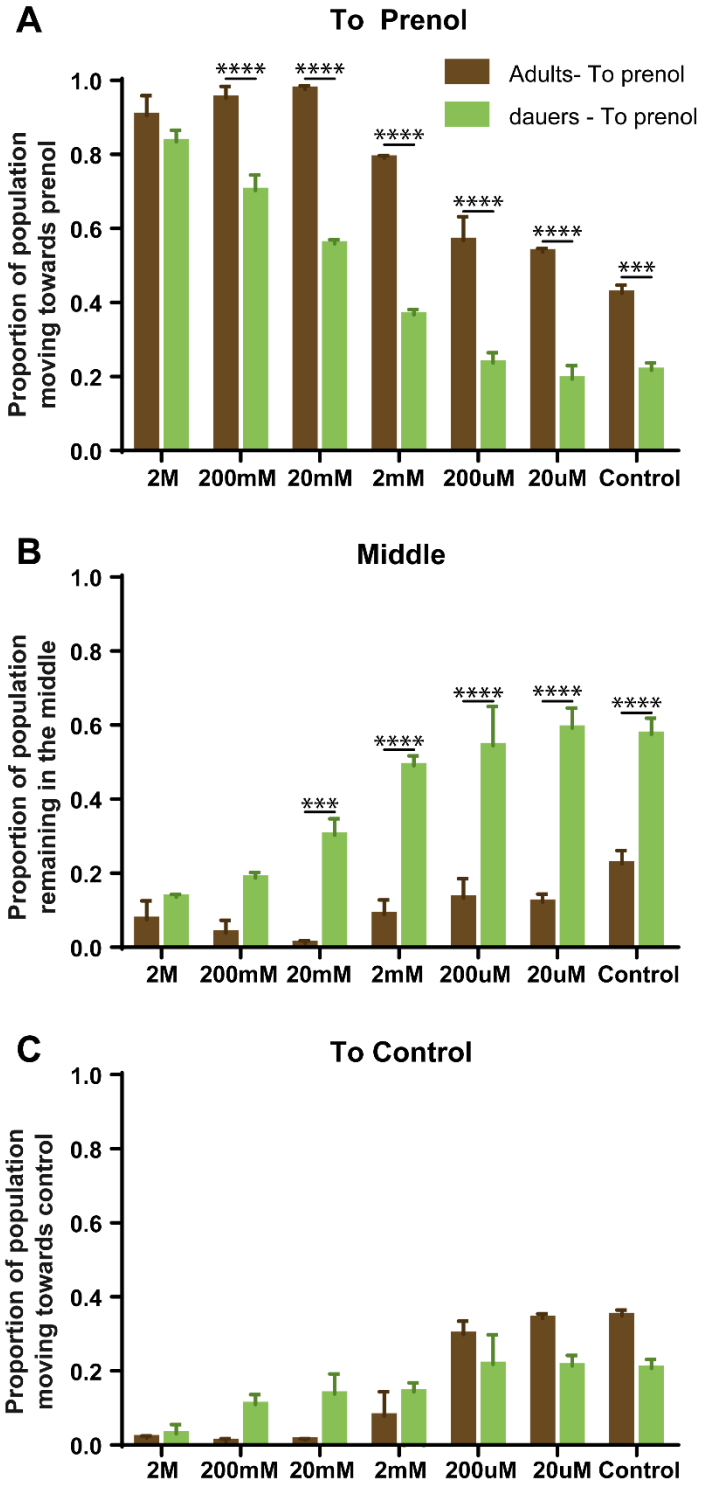
In the prenol vs IAA comparisons shown in Figure 4, the data was analyzed using an Ordinary Two-way ANOVA with Sidak's posttest, comparing between IAA and prenol response for each strain. An ordinary-one-way ANOVA comparison of IAA responses among all the strains was done for supplementary Figure S6.

For evaluation of gene candidates from *Ce*NDR (Figure 5B) the complication responses by loss-of-function mutant strains were analyzed by Ordinary One-way ANOVA with Sidak's post-test, comparing all knockout mutant results against those of N2 (the wild type). The comparisons of *dcap-1*, *dcap-2*, *dod-17* and N2 (Figure 5C) were done using an Ordinary Two-way ANOVA with Sidak's multiple comparisons test comparing the three loss-of-function mutants to N2 within each condition of prenol or IAA but not between the conditions them.

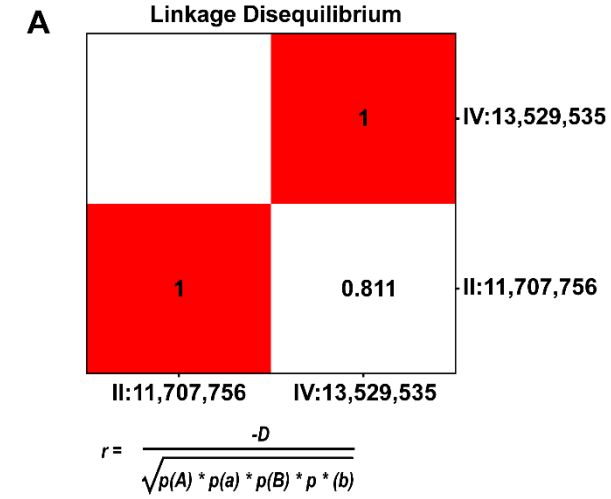
For evaluations of *clec-39* using the large chemotaxis assay, a paired, two-tailed T-test was used to evaluate the chemotaxis indices (Figure 6A), while participation results (Figure 6B) were analyzed using an Ordinary Two-way ANOVA with Sidak's multiple comparisons test was used to evaluate the results.

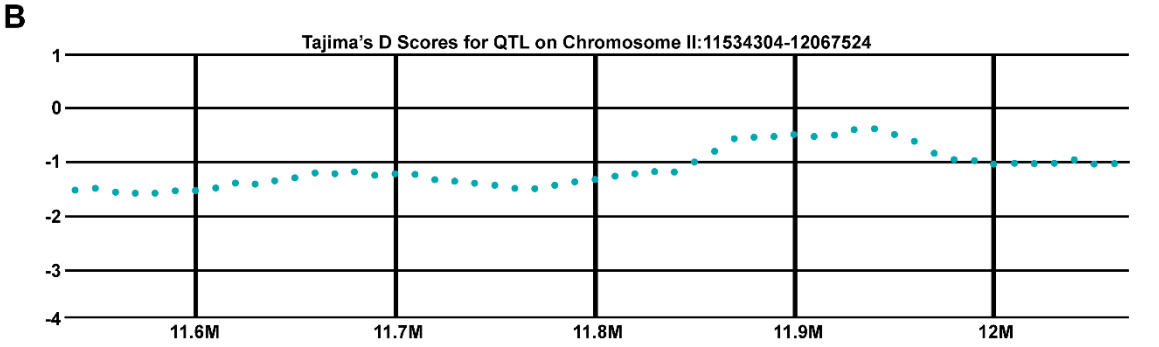
Evaluation of *P. pacificus* vs. *C. elegans* responses to prenol (Supplementary Figure S7) was analyzed using a Two-way Ordinary ANOVA and Sidak's multiple comparisons test. Additionally, supplementary figure S13 is an excerpt highlighting the ALT *Ce*NDR strains that were evaluated. Statistical analysis is based on Figure 3- using an ordinary one-way ANOVA comparing all strains to JU1242.

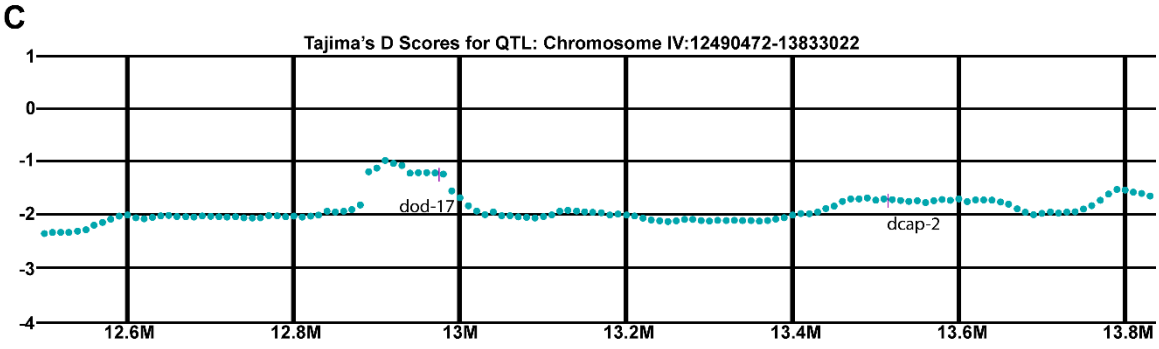
Supplemental Figures and Tables

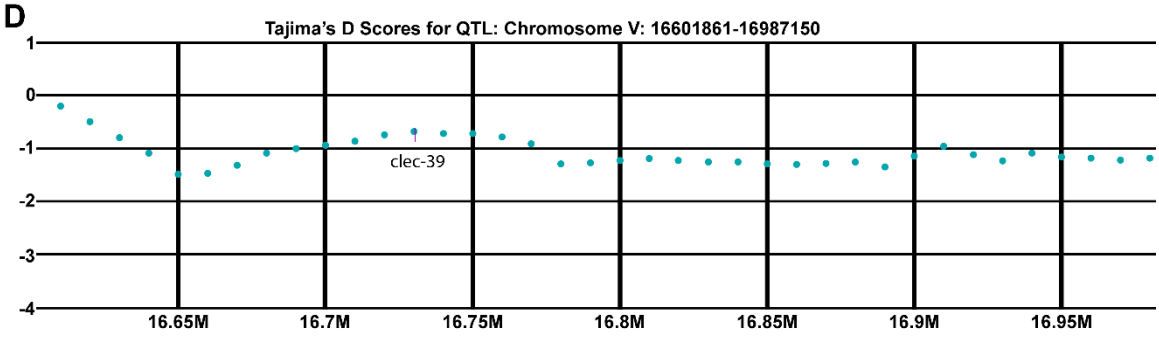


Supplementary Figure 4.1 Participation of C. elegans adults and dauers in prenol chemotaxis assays. *(A) Scores for the proportion of the population that moved towards prenol (B) proportion of the population for adults and dauers that remained in the center and did not move directionally towards or away from prenol. (C) Proportion of the population that moved towards the control region. Dose listed reflect the (5 µL) of each concentration put on the plate and not necessarily what the worms experienced. Error bars represent SEM * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001; statistical analysis for each panel was done using an ordinary using an ordinary 2-way ANOVA with Sidak's multiple comparisons test comparing between adults and dauers for each dose tested.*


A
Linkage Disequilibrium
1
IV:13,529,535
1
0.811
II:11,707,756
II:11,707,756
IV:13,529,535
r = -D / √(p(A) * p(a) * p(B) * p * (b))


B
Tajima's D Scores for QTL on Chromosome II:11534304-12067524
1
0
-1
-2
-3
-4
11.6M
11.7M
11.8M
11.9M
12M


C
Tajima's D Scores for QTL: Chromosome IV:12490472-13833022
1
0
-1
-2
-3
-4
dod-17
dcap-2
12.6M
12.8M
13M
13.2M
13.4M
13.6M
13.8M


D
Tajima's D Scores for QTL: Chromosome V: 16601861-16987150
1
0
-1
-2
-3
-4
clec-39
16.65M
16.7M
16.75M
16.8M
16.85M
16.9M
16.95M

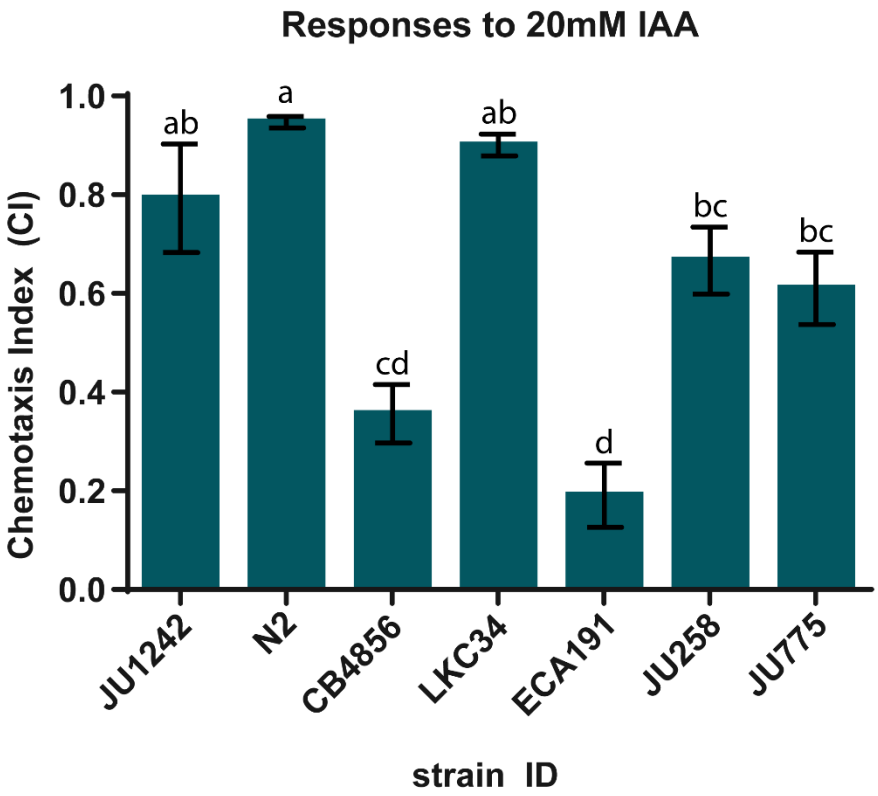
Supplementary Figure 4.2 Linkage disequilibrium and Tajima's D score results from CeNDR *(A) shows the linkage disequilibrium comparing the peak markers for each QLT. CeNDR indicates that an LD value (values from comparisons between peak markers) above 0.8 indicate that the two peak markers may not segregate randomly, and thus might be linked. (B) the Tajima's D score for the QTL on chromosome II. (C) The Tajima's D scores for the QTL on chromosome IV, dod-17 and dcap-2 (the two genes that appeared to influence detection of prenol are marked on the span of chromosome IV, indicating where the Tajima's D-score is for each gene's approximate location. CeNDR indicates that values close to zero suggest that there is no particularly strong selection on the region of the genome, while a high positive value indicates there is balancing selection that may have occurred due to recent population contractions, and that values with a negative score suggest that there are rare variants present that may have arisen during a population shift such as expansion after a bottleneck, or some type of selective sweep.*

Supplementary Tables: S4.3, S4.4 and S4.5 <u>are supplemental excel files attached to dissertation</u>

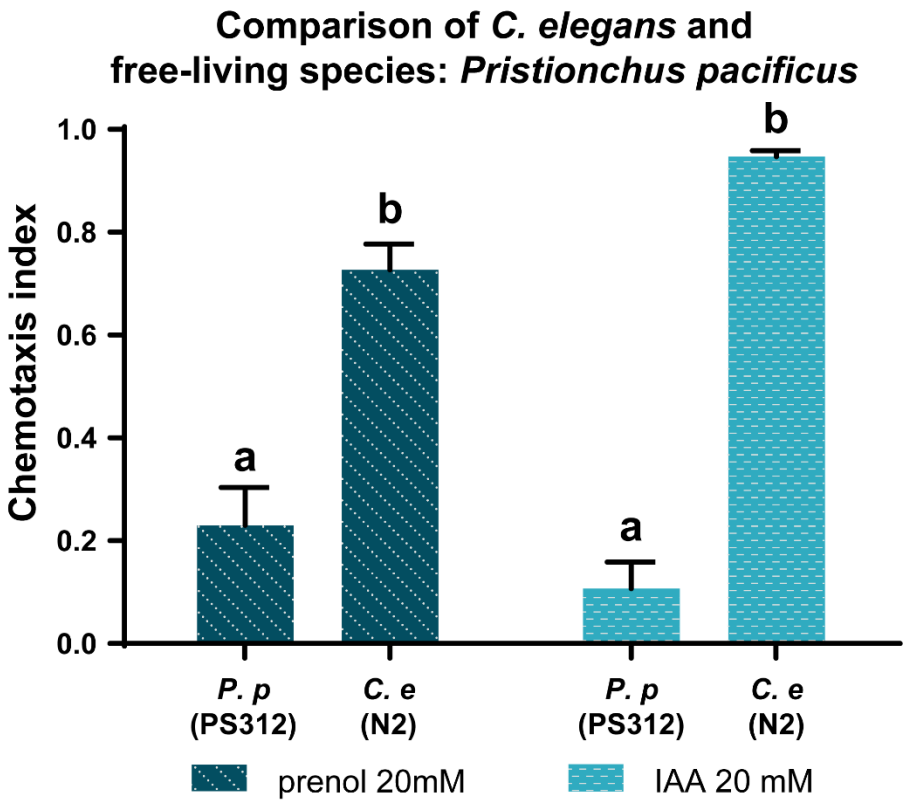
Supplementary Table 4.3 Chromosome II genetic variants *Table displays the CeNDR results for the genetic variants obtained after the mapping analysis. The file contains tabs of high, moderate, and low impact variants as well as those designated as modifier. Additional genetic variants with no impact designation are listed as other.*

Supplementary Table 4.4 Chromosome IV genetic variants *Table displays the CeNDR results for the genetic variants obtained after the mapping analysis. The file contains tabs of high, moderate, and low impact variants as well as those designated as modifier Additional genetic variants with no impact designation are listed as other.*

Supplementary Table 4.5 Chromosome V genetic variants *Table displays the CeNDR results for the genetic variants obtained after a mapping run using only the divergent set and mapping set 1. In this older analysis a third QTL had been identified (and later was eliminated with the use of additional wild isolates data). The file contains tabs of high, moderate, and low impact variants as well as those designated as modifier. Additional genetic variants with no impact designation are listed as other.*



Supplementary Figure 4.6 Statistical comparisons of IAA responses only from Main Figure 4.4 results*. Select wild isolate strains: N2, and most prenol-attracted strain JU1242, as well as the 5 strains that had shown significant reduction in attraction to prenol; were evaluated for response to IAA, as the results show in Figure 4.5. This panel shows the statistical comparisons between the responses to IAA only, showing that only CB4856 and ECA191 have significantly reduced attraction to IAA compared to both N2 and JU1242.*



Supplementary Figure 4.7 C. elegans and Pristionchus pacificus responses to prenol and IAA (20mM). *The figure reflects the response to Pristionchus pacificus (PS312) young adults and C. elegans young adults to prenol (20mM) on left (dark blue), and to IAA (20mM) on right (light blue). Error bars represent SEM. Statistical analysis was done using an ordinary 2-way ANOVA, with Sidak's multiple comparisons test, comparing all conditions and species to each other.*

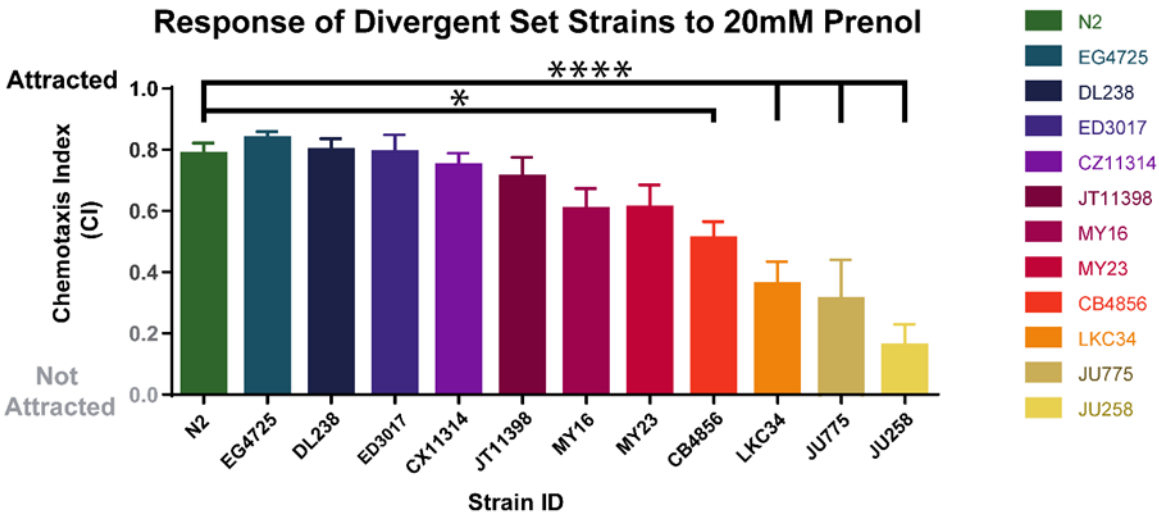
Supplementary Tables: S4.8, S4.9, S4.10 and S4.11 are supplemental excel files attached to dissertation

Supplementary Table 4.8 Simple mine results *for high-impact variants from chromosome II, IV and V. Genes containing designated high impact variants- based on CeNDR mapping results- were listed and cross referenced with information from Wormbase using the Simple mine data mining and batch queries tools. The table lists the gene, additional names, the gene type, a description as well as any expression information on each gene that is available on Wormbase.*

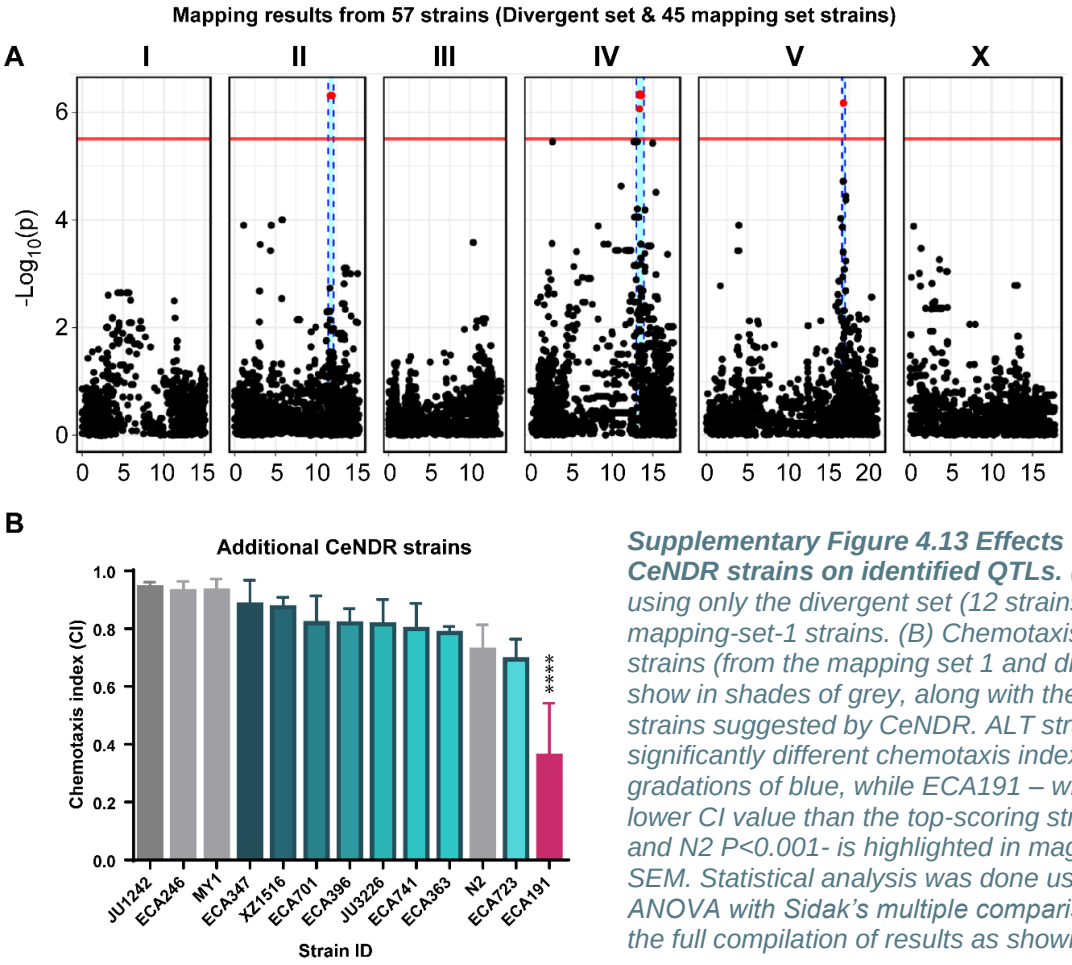
Supplementary Table 4.9: High-impact-variant genes cross referenced with AWC Expression data from Hsueh et. al 2017. *The list of genes that contained high-impact variants (as designated by CeNDR) was evaluated and cross referenced with data from Hsueh et. al. 2017, in which AWC expression data was obtained. This cross referencing was done for genes that were tested and shown to have an effect on prenol response, for tested genes that had no effect on response to prenol as well as the set of untested gene candidates. Highlights in blue designate significantly lower expression, and highlights in yellow indicate significantly higher expression of designated genes in the AWC (as is determined by the expression level ratio, and the statistical analysis score designated in the AWC.vs.larvae_padj column).*

Supplementary Table 4.10 Orthologous genes*The set of lists is a compilation of the genes evaluated- which either had an affect or no effect on responses to prenol- and those that have yet to be evaluated. For each gene the identifier from C. elegans is listed as well as any existing orthologs for Pristionchus pacificus (another free-living specie) and EPN species: Heterorhabditis bacteriophora, Steinernema carpocapsae, Steinernema glaseri, Steinernema feltiae; as well as three human parasitic species of nematodes Strongyloides stercoralis (threadworm), Necator americanus (hookworm) and Trichuris trichura (whipworm). Each ortholog identified is linked with its corresponding informational page on Wormbase Parasite, from which the information was obtained.*

Supplementary Table 4. 11 ***Resources Summary*** *A complication of information regarding STOP-IN gene editing information as well as strain for C. elegans and Escherichia coli used in the experiment are listed. Underlined items are linked to corresponding sources of information.*



Supplementary Figure 4.12 CeNDR Divergent Set responses to 20mM prenol. *Figure shows the initial test results yielded by the divergent set from CeNDR. This is the set of wild isolates first tested to evaluate if the CeNDR resource would serve as a viable way to identify natural variations that affect response to prenol by C. elegans. Error bars represent SEM. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001; statistical analysis for each panel was done using an ordinary using an ordinary one-way ANOVA with Dunnet's multiple comparisons test comparing between N2 and all other strains*



Supplementary Figure 4.13 Effects of additional ALT genotype CeNDR strains on identified QTLs. *(A) shows original results using only the divergent set (12 strains) and 45 out of the 48 mapping-set-1 strains. (B) Chemotaxis results for the 3-top scoring strains (from the mapping set 1 and divergent set) as well as N2- all show in shades of grey, along with the 9 alternative (ALT) genotype strains suggested by CeNDR. ALT strains that did not bear significantly different chemotaxis index (CI) results are shown in gradations of blue, while ECA191 – which yielded a significantly lower CI value than the top-scoring strain (JU1242); **** p<0.001 and N2 P<0.001- is highlighted in magenta. Error bars represent SEM. Statistical analysis was done using an ordinary one-way ANOVA with Sidak's multiple comparisons post-test (derived out of the full compilation of results as shown in Figure 4.2).*

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CHAPTER 5

Conclusions and Final Remarks

Nematodes display great diversity in not only the range of ecological habitats they occupy but also the behaviors they employ to survive[1-5]. Included in the wide array of behaviors nematodes exhibit the responses to various odors play a large role in many aspects of their life cycles. The detection of environmental cues is important for nematodes, assisting in the processes of dispersal[6], locating food, and avoiding unfavorable conditions among other implications[7].

In this study- as shown in Chapter 1- my work had first focused on entomopathogenic nematodes (EPNs) which are parasites that kill insects and are leveraged as biological pest control agents. As was demonstrated by the work in Chapter 1, EPNs can assess host odors through olfaction, and are eventually repelled by conspecific-infected cadavers (at 16 days post infection), when not resources are left in the cadaver. Evaluation of the odor profiles that emanate from either the naive or EPN-infected *Galleria mellonella* revealed several odors- most of which were associated with the 16dpi time point. Of the odors identified, prenol was shown to elicit robust behaviors form EPNs (which exhibit repulsion) and *Caenorhabditis elegans* (which exhibit attraction)

The work displayed in Chapter 3 further explores the potential ecological roles of prenol in the EPN life cycle, the odor was evaluated for its implication in both repulsion and dispersal behaviors. This evaluation revealed that prenol appears

to have age-related effects, include increased dispersal behaviors in younger IJs compared to older IJs for two species of *Steinernema*. It was also identified that moisture may serve as an important cue on its own for *Heterorhabditis indica*, although prenol can elicit repulsion, as well, from this species.

Lastly, the work shown in Chapter 4 demonstrates the use of prenol and the model organism *C. elegans* to identify the molecular underpinnings of responses to prenol. Since *C. elegans* exhibited robust attraction to prenol, we leveraged this behavior along with natural variation and genome wide association techniques (through use of the *C. elegans* Natural Diversity Resource) to identify gene candidates that might be implicated in responses to prenol. Through use of loss-of-function (*lf*) mutants we evaluated the impacts-of several genes-on response to prenol. This work uncovered the involvement of the AWC neuron as well as several genes such as: *dcap-1*, *dcap-2*, *dod-17*, *clec-39*, *daf-11*, *odr-1*, odr-*3*, *tax-2* and *tax-4* as genes that are needed for efficient attraction to prenol.

Despite all the work that has been done, there is still much to explore. Several genes remain untested, several of which were shown (via cross-referencing between CeNDR results and AWC gene expression[8]) to have significantly higher expression in the AWC and are good candidates for continued exploration. Additionally, many of the genes identified as being necessary in detecting prenol have orthologs in EPN and other parasitic nematode species. Although molecular

tools in EPNs remain undeveloped, it is possible to do rescue experiments in *C. elegans*, using the orthologues versions of the genes that were identified. Doing so may shed light on the functionality of these genes in EPNs. Such information might be leveraged in the future as the molecular tools in the EPN system improves, and couple potentially be used to improve EPN abilities to detect and respond to environmental cues.

This work has aimed at bridging the gap between model and non-model nematode systems. In the case of the work demonstrated in this dissertation, we have identified some of the molecular underpinnings in chemotactic responses to prenol- and ecologically relative molecule due to its presence and association with EPN-infected cadavers. Through leveraging *C. elegans* behaviors and tools we not only identified important information but were able to relate the information to EPNs due to the presence of orthologs. Through this work and continued efforts, our understanding of EPN behavior and ecology will continue to improve and eventually lead towards more efficacious application of these biological control agents in the field.

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