

Brief Report

# What Is More Important to Host-Seeking Entomopathogenic Nematodes, Innate or Learned Preference?

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**Abstract:** Entomopathogenic nematodes (EPNs), small soil-dwelling non-segmented roundworms, are obligate parasites of insects and commonly used in agriculture for biological control of insect pests. For successful reproduction, EPNs must identify, move towards, and successfully infect a suitable insect host in a chemically complex soil environment. EPNs can have innate host insect preferences and can be attracted to semiochemicals associated with that host. They can also develop strong learned preferences for chemical signals associated with the presence of a host, such as herbivory-induced volatiles. We hypothesized that simultaneous manipulation of innate and learned preferences could result in increased biological control services of EPNs in agriculture. Separate cohorts of the EPN *Steinernema diaprepesi* were raised on two insect hosts, *Galleria mellonella* and *Tenebrio molitor*, for multiple generations until the nematodes in a dual-choice olfactometer exhibited preference for the host they were reared on. Subsequently, the two strains of nematodes were imprinted on three plant-produced terpenoids of agricultural significance: pregeijerene,  $\beta$ -caryophyllene, and  $\alpha$ -pinene. After exposure to one of the plant compounds, the behavior of the EPNs was assayed in an olfactometer where the two host insects were presented with and without the plant compounds. We found that plant volatile exposure increased the infection rate of the nematodes, and some host–compound combinations proved to be attractive, but other combinations appeared to become repellent. These results indicate that learned preference is neither subordinate nor superior to innate preference, and that infection efficiency can vary with compound exposure and insect host.

**Keywords:** behavior; chemical ecology; infection; management; insect pest



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## 1. Introduction

The ability of nematodes, including entomopathogenic nematodes (EPNs), to form association between odors that indicate the presence of food has been well documented in the scientific literature [1,2]. This association allows for broad behavioral plasticity when it comes to survival in a changing and ephemeral chemical landscape. After exiting a consumed host, EPNs in the free-living stage, referred to as the infective juvenile (IJ) stage, need to either enter a resting phase or find a suitable host to infect [3]. To locate host insects, IJs rely on cues such as insect-produced odors [4,5], CO<sub>2</sub> [6], and nematode-produced pheromones or kairomones [7]. These cues can be utilized individually or in combination to enhance host finding [8]. EPNs can also utilize plant-produced compounds to find hosts, especially herbivore-induced plant defense compounds [9–11]. These compounds can, in conjunction with other cues, be reliable indicators of the presence of a host insect. While many of these compounds may be inherently attractive or repellent, associative learning has also been documented in many species of nematodes [1,12,13]. This associative learning of the odors in the environment can be critical for survival of EPNs.

In addition to the behavioral plasticity presented by learned preferences based on odor exposure in the soil environment, EPNs demonstrate preferences for hosts, especially the host in which they were produced [14]. Furthermore, selective rearing of nematodes on a single host species for multiple generations can result in increased preference for

that insect [15], potentially increasing the biocontrol services of these selectively reared nematodes. The selection of the proper nematode species that will seek and infect a particular target pest is critically important to agriculture. For example, the selection of a nematode species that uses an ambush-style foraging strategy will not be effective against a sessile insect pest, as both the insect and the nematode will exhibit little movement. Innate preference of different species of EPNs for different hosts is therefore an important behavior to consider when implementing a biological control program [4].

The importance of herbivory-induced compounds as learned cues for EPNs [3,11,12] and the importance of host cues [15–17] are well documented in the literature. Willet et al. [12] demonstrated that learned cues when combined with a preferred as well as non-preferred host, in both cases, result in increased infection rates, and that compound exposure only increased EPN infection when that compound was present during bioassays. However, all nematodes used in that study had been reared on *Galleria mellonella* (L.), and thus the importance of host-produced signals was not addressed. Furthermore, *G. mellonella* is commonly utilized as an insect host in laboratory settings, and EPN-infected *G. mellonella* cadavers are even used in field settings [18–20]. Since EPNs reared in *G. mellonella* are utilized for pest control, this preference has to a great extent overshadowed the potential importance of combined host-related and environmental signals for maximized efficiency of EPNs. Therefore, this study was designed to specifically investigate the interactions between learned and innate cues with the help of established procedures for nematode learning of common plant volatiles, utilizing *S. diaprepesi* reared on *G. mellonella* as well as on *Tenebrio molitor* (L.). By developing strategies based on the most important host-finding cues used by nematodes, determining the hierarchical importance of innate versus learned attraction could result in enhanced EPN-based biocontrol services in agriculture.

## 2. Materials and Methods

### 2.1. Nematode Species and Rearing

Experiments were conducted using *Steinernema diaprepesi* Nguyen and Duncan IJs. Populations of *S. diaprepesi* used in this research originated in Florida citrus groves and were isolated and identified at the University of Florida Citrus Research and Education Center, Lake Alfred, FL, USA. Rearing of the nematodes was conducted at the Center of Medical, Agricultural, and Veterinary Entomology, Gainesville, FL, USA, under laboratory conditions. Cultures of *S. diaprepesi* were propagated using late instars of *G. mellonella* and *T. molitor*. The *S. diaprepesi* used in the experiments had been reared continuously on *G. mellonella* in a laboratory setting for several years and consequently have a strong *G. mellonella* preference. A lineage with preference to infect *T. molitor* was achieved through selective propagation (15 rounds of target host infection) of *S. diaprepesi* to *T. molitor* [15,21]. This selective propagation resulted in two lineages of *S. diaprepesi* that in dual choice tests exhibited a strong preference to infect the insect they were reared on. These lineages were termed *S. diaprepesi* | *mellonella* and *S. diaprepesi* | *molitor*.

For the bioassays, freshly emerged IJs from cadavers of the two host insects were collected using White traps and stored in 100 mL of deionized water in culture flasks at 14 °C for up to two weeks or until used in research trails. Infective juveniles stored in the flasks were not cleaned or desensitized from the in vivo culture to maintain their natural exposure and behavior when exposed to compounds emerging from the insect cadaver [22]. Preliminary trials indicated that desensitization of IJs resulted in reduced behavioral activity and significant no-response trials.

### 2.2. Chemical Compounds and Exposure

To study learned preference in the two lineages of nematodes, IJs of both lineages were exposed to  $\alpha$ -pinene,  $\beta$ -caryophyllene, and pregeijerene. These three compounds were selected due to inherent different levels of attraction that have previously been characterized for *S. diaprepesi* [3]. Past experimentation has demonstrated that  $\alpha$ -pinene is initially repellent to *S. diaprepesi* IJs, while  $\beta$ -caryophyllene is neutral, and pregeijerene is

inherently attractive. However, exposure results in IJ attraction to all compounds [3,12,23]. Compound exposure was achieved through the placement of approximately 200 IJs into 10 mL scintillation vials containing 8 µL of the treatment compounds in 1 mL of water [3]. Densities of IJs were determined by counting the number of individuals in 30 µL of solution 10 times. The corresponding volume that would result in 200 IJs being added was therefore added to the scintillation vial. IJs were left exposed for 48 h before being pipetted into the olfactometer. Control IJs received the same procedure but were not exposed to the treatment compounds.

### 2.3. Insect CO<sub>2</sub> Production

EPNs are well documented to be strongly attracted to CO<sub>2</sub>. To ensure equal production of this highly attractive compound between the two insect species, a study was conducted to determine the respiration rate of the *G. mellonella* and *T. molitor* larvae. A LI-6400XT (Li-COR Biosciences, Lincoln, NE 68504, United States) portable Photosynthesis System equipped with the LI 6400-89 insect respiration kit (part #:9964-053) was used to measure the respiration rate (µg CO<sub>2</sub> g Insect<sup>-1</sup> min<sup>-1</sup>) for five *G. mellonella* and *T. molitor*. Each larva was placed in a glass chamber (22.86 cm long and 2.86 cm outside diameter, with a glass frit inlet and a glass joint outlet with a single port) for sampling. The chamber was covered with a black cloth and the respiration rate monitored for stability (a wait of approximately 15 min) before data collection began. The µg CO<sub>2</sub> produced by each insect was recorded for 30 min. After the respiration sampling was carried out, the larvae were weighed and recorded to standardize respiration rates by insect weight.

### 2.4. Bioassays for Innate vs. Learned Preference

The effect of innate and learned preference of the nematode strains was tested in a dual-choice olfactometer consisting of a PVC t-joint filled with field collected, filtered and autoclaved sand (Figure S1) at 10% moisture by volume [3]. Each nematode strain was either exposed to chemicals, as described above, or not exposed, to assess the learned preference of the nematodes. At each end of the olfactometer, either a *G. mellonella* or *T. molitor* larva was buried with or without 300 ng of a plant volatile treatment in 10 µL of pentane (Figure S1). After being filled with sand, 200 IJs were applied to the sand at the top of the olfactometer. The nematodes were allowed 48 h to travel through the sand and infect the hosts. Initial trials indicated that 48 h was long enough to allow for location, penetration, and killing of the larvae by the IJs. After 48 h, larvae were dissected, and the numbers of IJs that infected the host were counted. The response variable for the bioassay was number of IJs infecting the larvae, and the control was the response of nonexposed IJs to the larvae with no compound treatment. Previous studies have demonstrated the impact of the volatile compounds without pre-exposure, and therefore this set of experiments was not conducted [3,12,23].

### 2.5. Statistical Analysis

Five replicates were used to assess respiration of the larvae, and the respiration rate was analyzed via student T-test. A total of five replications, over three time points and using three separate cohorts of nematodes, were conducted for each combination of exposed vs. nonexposed IJs and preferred and nonpreferred hosts. Total responding IJs (i.e., IJs infecting either host during the bioassay) were analyzed with an analysis of variance and a post-hoc Tukey test. Exposure of IJs to compounds can result in increased levels of infection; therefore, additional tests were conducted to determine the frequency of choice for the responding individuals. Mean numbers of responding IJs for each treatment combination were subjected to a binomial exact test with a Bonferroni correction for multiple comparisons. Binomial exact tests were compared between the control, which acted as the expected distribution, and the compound treatments, which acted as the observed distribution. All statistical tests were conducted using R, software version 3.5.1 package base (R Core Team, Vienna, Austria).

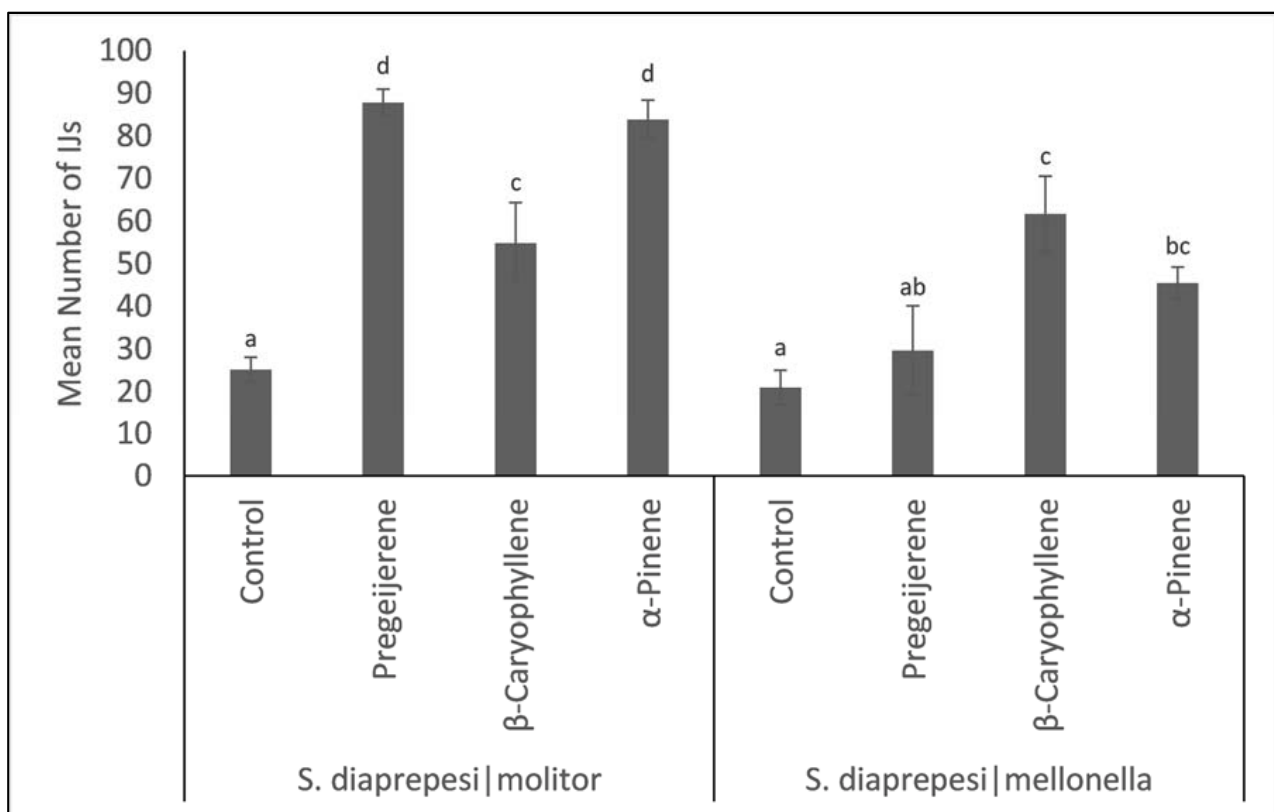
### 3. Results

#### 3.1. Insect CO<sub>2</sub> Production

Trials indicated that larvae of comparable weights did not produce significantly different amounts of CO<sub>2</sub>. Therefore, all subsequent trials utilized larvae of the two species that were of the same weight to minimize the potential confounding factor of one species producing significantly more CO<sub>2</sub> compared to the other. Results indicated that *T. molitor* respired CO<sub>2</sub> at a rate of  $20.4 \pm 3.2 \mu\text{g CO}_2 \text{ g}^{-1} \text{ min}^{-1}$ , while *G. mellonella* respired at a rate of  $18.0 \pm 2.8 \mu\text{g CO}_2 \text{ g}^{-1} \text{ min}^{-1}$  ( $T(5) = 2.77, p = 0.50$ ).

#### 3.2. Bioassays for Innate vs. Learned Preference

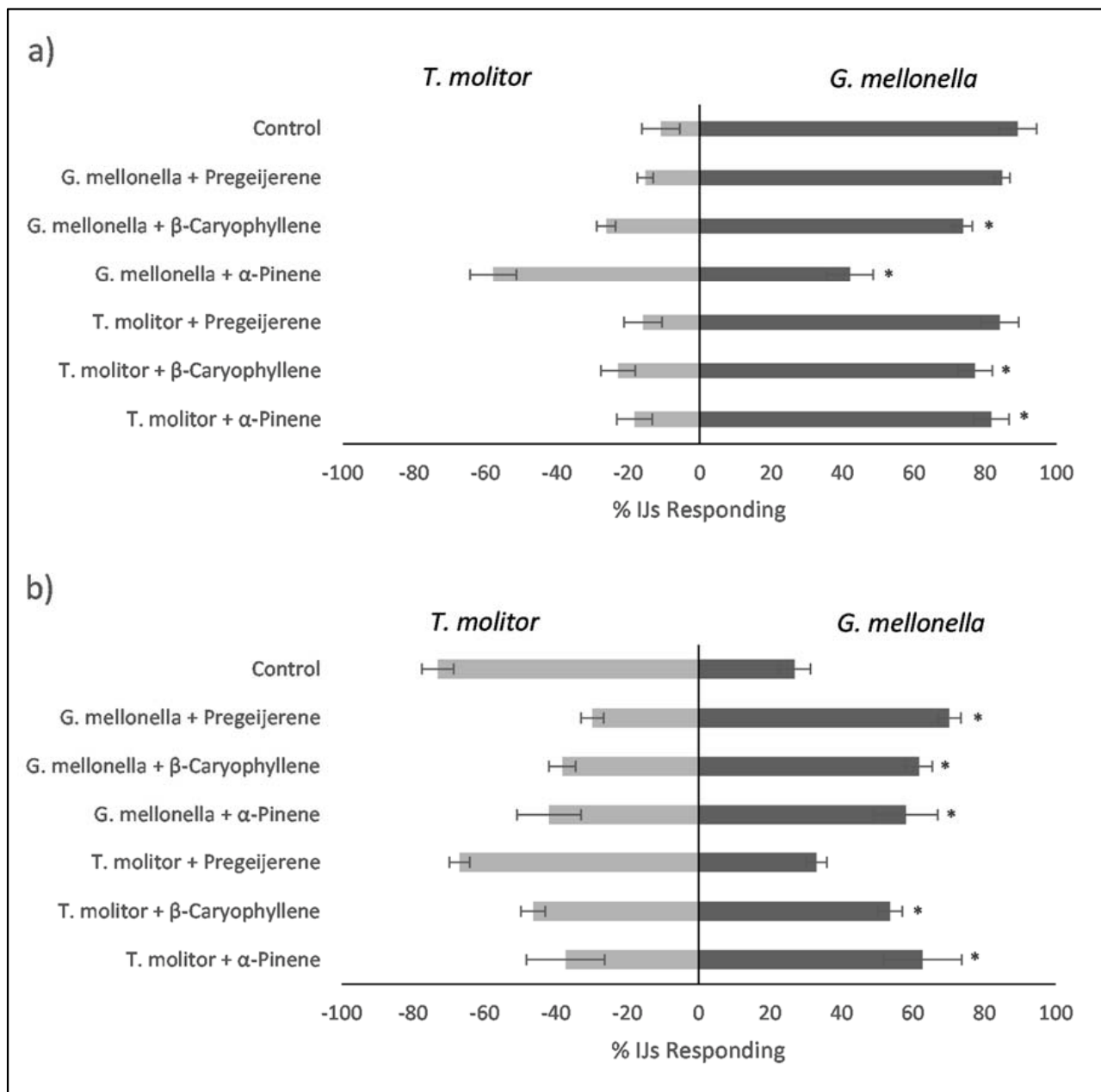
Total infection rates for both strains were impacted by exposure to the volatile compounds (Figure 1), except for *S. diaprepesi* | *mellonella* when exposed to pregeijerene ( $p = 0.72$ ). The exposure of *S. diaprepesi* | *mellonella* IJs to plant volatiles increased the number of responding IJs from an average of 21 for the control to 61.7 when exposed to  $\beta$ -caryophyllene and 45.5 when exposed to  $\alpha$ -pinene ( $p < 0.001$  and  $p < 0.001$ , respectively). The exposure of *S. diaprepesi* | *molitor* IJs to plant volatiles increased the number of responding IJs from an average of 25.2 for the control to 87.8, 54.9, and 83.9 when exposed to pregeijerene,  $\beta$ -caryophyllene, and  $\alpha$ -pinene, respectively ( $p < 0.001, p < 0.001$ , and  $p < 0.001$ , respectively).



**Figure 1.** Mean number  $\pm$  SE of IJs infecting both insect host after exposure to pregeijerene,  $\beta$ -caryophyllene, and  $\alpha$ -pinene. Letters above bar denote significant differences within the two nematode strains.

Behavioral bioassays using the olfactometer indicated mixed results from semiochemical-exposed and unexposed nematodes in the presence of their preferred and nonpreferred host. Prior to exposure, both strains of nematodes demonstrated a strong preference for the host they had been reared on. During the control, 89% of the *S. diaprepesi* | *mellonella* IJs infected the

*G. mellonella* compared to 11% infecting *T. molitor* (Figure 2a). For *S. diaprepesi* | *molitor*, 73% of the responding IJs infected *T. molitor*, while only 27% infected *G. mellonella* (Figure 2b).



**Figure 2.** Two choice tests of percent ( $\pm$  standard error) for (a) *G. mellonella* lineage and (b) *T. molitor* lineage of *S. diaprepesi* IJs responding to and infecting *T. molitor* and *G. mellonella* with and without (control bars) a plant volatile treatment. Asterisks denote significant difference ( $p < 0.05$ ) between the control and corresponding treatment.

When nematode strains were exposed to semiochemicals with the two insect hosts present, responses of the nematodes were mixed. When *S. diaprepesi* | *mellonella* was trained to pregeijerene,  $\beta$ -caryophyllene, or  $\alpha$ -pinene and these compounds were paired with its preferred host *G. mellonella*, 84%, 73%, and 42% of the responding IJs infected *G. mellonella*, compared to the 89% infection for the control ( $p = 0.96$ ;  $p < 0.001$ ,  $p < 0.001$ ) (Figure 2a). When trained *S. diaprepesi* | *mellonella* was given the choice of infecting *G. mellonella* or *T. molitor* with the semiochemical treatment, more IJs infected *T. molitor* (16% for pregeijerene, 23% for  $\beta$ -caryophyllene, and 18% for  $\alpha$ -pinene) compared to the control infection rate of 11%



( $p = 0.24$ ,  $p = 0.001$ , and  $p = 0.006$ , respectively) (Figure 2a). However, in this bioassay, *G. mellonella* was always strongly preferred for infection compared to *T. molitor*.

Similar to *S. diaprepesi* \ *mellonella*, when trained *S. diaprepesi* \ *molitor* was exposed in the bioassay to pregeijerene,  $\beta$ -caryophyllene, or  $\alpha$ -pinene with the two insect hosts present, responses of the nematode strain were mixed, with both attraction and repellency of the semiochemical/host being observed. When *S. diaprepesi* \ *molitor* was trained to pregeijerene,  $\beta$ -caryophyllene, or  $\alpha$ -pinene and these compounds were paired with its preferred host, 67%, 46%, and 37% of the IJs infected *T. molitor* compared to the 73% infection rate for the control ( $p = 0.13$ ;  $p < 0.001$ ;  $p < 0.001$ ) (Figure 2a). When semiochemical-exposed *S. diaprepesi* \ *molitor* were given the choice to infect *G. mellonella* in the presence of the semiochemicals versus *T. molitor*, 30%, 38%, and 41% of the responding IJs infected *T. molitor* compared to the control infection rate of 73% ( $p < 0.001$ ;  $p < 0.001$ ;  $p < 0.001$ ) (Figure 2b).

#### 4. Discussion

This investigation showed that selective rearing of *S. diaprepesi* resulted in host specificity, as shown for the “controls” in Figure 2, but also that the host specificity was not due to significantly different release of CO<sub>2</sub> for the two species. We found that increased levels of infection were observed for all compounds tested, suggesting that exposure to these plant compounds increases or initiates host-seeking behaviors in the exposed IJs. However, the pairing of host specificity and priming on different plant volatiles provided mixed results, with some pairings of host and plant volatiles being highly attractive and others less attractive or even appearing repellent. No clear hierarchical order of the importance of innate or learned preference could be deduced, highlighting the complexity of nematode behavior. In both instances, when the two strains of nematodes were exposed to  $\beta$ -caryophyllene and  $\alpha$ -pinene and these compounds were paired with their preferred host, reduced infection rates of the preferred host were observed. The decreased attraction (relative to the control) of IJs to the  $\beta$ -caryophyllene- and  $\alpha$ -pinene-exposed preferred hosts was unexpected. However, when  $\beta$ -caryophyllene and  $\alpha$ -pinene, which were repellent when combined with the preferred host, were paired with the alternative host, significantly higher rates of infection were achieved. In contrast, pregeijerene did not result in any negative interaction between the treatments but also did not result in any synergistic effects, which has previously been reported [3].

While the slight increase in infection rates observed by *S. diaprepesi* \ *mellonella* when *T. molitor* was paired with a plant volatile treatment was statistically significant, the increased infection rate from the control of 11% to 16–23% for the semiochemical treatments may not be biologically relevant. In this study, the 5–12% increase in infection rates correlated to an additional 2–15 individual IJs infecting the insect. Additional testing would be required to determine the biological relevancy of these increases. In a natural setting, these small percentages may however become more important as it could impact the ability of the nematodes to maintain populations once applied in an agricultural setting.

It has been suggested that the limited success of mass-reared EPNs when applied in agricultural settings is due to the lack of dispersal and host-finding cues for the IJs before they are applied [24,25]. While some success has been achieved increasing infection rates by exposing IJs to their dispersal pheromone [26], exposure to cheap and readily available plant-produced compounds compared to specialized pheromones may produce similar results. One conclusion of this investigation is that attempts to utilize plant-produced compounds to improve EPNs in the field will need to include thorough testing of the compounds for possible antagonistic interactions. For example, Rasmann et al. [7] identified  $\beta$ -caryophyllene as an important below-ground cue to maintain biocontrol services of EPNs in corn systems, especially for control of *Diabrotica virgifera virgifera*. Rasmann et al. [7] also suggested that the benefits of a compound like  $\beta$ -caryophyllene may depend on the host insect causing the damage. This study strongly indicates the response of the system to the addition of compounds such as  $\beta$ -caryophyllene may depend on the target host

insect in addition to the response of the nematode. Depending on the target pest, it may become more or less attractive in the presence of  $\beta$ -caryophyllene, even if the nematodes had previously infected that host in the absence of  $\beta$ -caryophyllene.

It is unclear why the addition of a volatile with the preferred host would result in a loss of attraction. This interaction was not observed with pregeijerene, the compound that was innately attractive before training of the *S. diaprepesi* IJs. For now, we can only speculate whether this is a technique artifact or of real biological significance. Continued research will be needed to determine the mechanisms that result in the preferred hosts becoming less attractive when paired with a plant volatile. Many herbivore-induced plant compounds have been reported to modulate insect immune systems, and this modulation could be the source of the repellency [27,28]. However, the combination of the nonpreferred host with  $\beta$ -caryophyllene and  $\alpha$ -pinene increased attraction, which does not readily support the hypothesis that interactions with the insect's defensive system reduce attraction.

This present study was conducted utilizing the innate response of the nematodes to the two insect hosts as the control, with manipulations of the volatile treatments. It is presumed that the innate response of the EPNs is controlled through genetics, but these factors were not investigated during this study. Understanding the genetic mechanisms controlling these preferences would be highly beneficial. The learning response of the EPNs is also poorly understood. The learning likely arises from an increase in sensitization of the nematodes to the compounds during exposure, but why initially repellent compounds such as  $\alpha$ -pinene become attractive under certain conditions needs to be further investigated. Additional studies should be conducted utilizing the learned response towards the volatiles as the control and then manipulating the host insects as the treatment. This different approach could result in additional insight into the behavior and infection dynamics of the nematodes. However, due to the limited ability of farmers and pest managers to manipulate insects in the field, we believe the current study design presents results more applicable to field settings, as the manipulation of chemical profiles in the soil can be achieved. Investigations of the findings of this study under field conditions and with agricultural pests will be critical for determining the applications of this research to pest managers. Studies investigating the impact of concentrations in the soil and concentration gradients on nematodes and how the presence of multiple hosts at a location would impact behavior would also be highly valuable to pest managers.

Increasing chemical diversity in the soil may result in broad changes to biocontrol services provided by EPNs. It is plausible that the control of a pest insect may be compromised if new semiochemicals are incorporated into the soil, resulting in the target pest becoming repellent or less attractive towards the EPNs. Still, the use of semiochemical cues to attract and enhance EPNs needs to be further exploited for management of subterranean agricultural pests. This current study supports the growing body of literature showing that semiochemicals, especially those released in response to insect damage, can be used to increase infection rates of EPNs [29–31]. However, this study also identifies underlying negative interactions that could reduce the efficacy of the nematodes if the full mechanism and interactions of the nematodes, insects, and semiochemicals are not thoroughly investigated and understood.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agriculture13091802/s1>, Figure S1: Diagram of olfactometer used for behavioral bioassay.

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