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Preferential infectivity of entomopathogenic nematodes in an envenomed host

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ABSTRACT

Entomopathogenic nematodes and parasitoid wasps are used as biological control agents for management of insect pests such as the Indian meal moth, *Plodia interpunctella*. The parasitoid wasp *Habrobracon hebetor* injects a paralytic venom into *P. interpunctella* larvae before laying eggs. A previous study reported that the entomopathogenic nematode *Heterorhabditis indica* preferentially infects *P. interpunctella* that have been envenomed by *H. hebetor* while results in this study showed a similar preference by the entomopathogenic nematode, *Steinernema glaseri*. We therefore tested four hypotheses for why nematode infection rates are higher in envenomed hosts: (1) elevated CO_2 emission from envenomed hosts attracts nematodes, (2) paralysis prevents hosts from escaping nematodes, (3) volatile chemicals emitted from envenomed hosts attract nematodes and increase infection, and (4) reduced immune defenses in envenomed hosts increase nematode survival. Results showed that envenomed *P. interpunctella* larvae emitted lower amounts of CO_2 than non-envenomed larvae. Physical immobilization of *P. interpunctella* larvae did not increase infection rates by *S. glaseri* but did increase infection rates by *H. indica*. Emissions from envenomed hosts were collected and analyzed by thermal desorption gas chromatography/mass spectrometry. The most abundant compound, 3-methyl-3-buten-1-ol, was found to be an effective cue for *S. glaseri* attraction and infection but was not an effective stimulus for *H. indica*. Envenomed *P. interpunctella* exhibited a stronger immune response toward nematodes than non-envenomed hosts. Altogether, we conclude that different mechanisms underlie preferential infection in the two nematode species: host immobilization for *H. indica* and chemical cues for *S. glaseri*.

1. Introduction

Preferential infection within a target host species occurs across various parasite groups and is mediated by various cues and processes (Christe et al., 2003; Tschirren et al., 2007). For example, choices of mosquitoes vectors for host species have been hypothesized to rely on the nutritional quality of blood meals and the host immune response (Lyimo and Ferguson, 2009). Dweck et al. (2010) reported that fruit flies (*Drosophila melanogaster* Meigen) prefer citrus hosts based on specific chemicals emitted from the fermenting fruit surface. In this study we explored mechanisms for preferential infection in two species of entomopathogenic (insect parasitic) nematodes.

Entomopathogenic nematodes in the genera *Steinernema* and *Heterorhabditis* are obligate parasites of insects in nature and have been explored as biological control agents for the management of various insect pests (Shapiro-Ilan et al., 2018). The nematodes kill the hosts with the aid of their symbiotic bacteria, *Xenorhabdus* spp. and *Photorhabdus* spp. associated with steinernematids and heterorhabditids, respectively (Lewis and Clarke, 2012). The nematodes infect the host insect as the infective juvenile (IJ), which is the only free living stage in the life cycle. The IJs invade the host through natural body openings (spiracles, mouth and anus) or occasionally through the insect cuticle (Shapiro-Ilan et al., 2018). After penetrating the host's hemocoel, the IJs release their symbiotic bacterium, which assists in overcoming the host immune response and causing rapid host death (with 24 to 48 h p.i.). The nematodes may reproduce within the host cadaver for one to three generations. Once the host nutrients are depleted, the nematodes exit the host as IJs and disperse to search for new hosts.

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Infection of hosts by entomopathogenic nematodes follows four processes namely, location of host habitat, host finding, host recognition and host acceptance (Lewis and Clarke, 2012; Shapiro-Ilan et al., 2018). Host finding behavior for entomopathogenic nematodes depends on the species and varies along a continuum of foraging strategies that ranges from ambushing, where the nematodes are primarily stationary and wait for a host to pass before infecting it, to cruising, where the nematodes actively seek the host (Lewis et al., 1993; Lewis and Clarke, 2012). Cruisers have been determined to be most effective at infecting stationary hosts belowground, while ambushers can be most successful targeting mobile insects on the soil surface (Lewis and Clarke, 2012).

Irrespective of the foraging strategy, all entomopathogenic nematodes seek out hosts using sensory cues which could be chemosensory, thermosensory, hygrosensory or mechanosensory (Campbell and Lewis, 2002; Shapiro-Ilan et al., 2018). Chemosensory cues, particularly those relating to volatiles from host or host habitat, are perceived by olfaction which is a necessary factor in host location and identification by entomopathogenic nematodes (Dillman et al. 2012). Among the volatiles from the hosts that entomopathogenic nematodes respond to are carbon dioxide, organic volatiles from hosts, and kairomones released by potential hosts or as a result of insect damage to host plants (Grewal et al., 1996; Lewis and Clarke, 2012). Dillman et al. (2012) noted that IJs use CO_2 for host location while host-specific volatiles are used for host identification. IJs of *Heterorhabditis* spp. are known to respond very strongly to CO_2 due to their BAG neurons (neurons that terminate in bag-shaped structures) that regulate responses to CO_2 (Hallem et al., 2011; Lewis and Clarke, 2012). Furthermore, the role of host-specific volatiles in host identification by entomopathogenic nematodes has been elucidated, and several organic compounds that are emitted by invertebrate hosts that elicit olfactory responses to entomopathogenic nematodes such as *Steinernema carpocapsae* (Weiser), *Steinernema scapterisci* Nguyen & Smart, *Steinernema glaseri* (Steiner), *Steinernema riobrave* Cabanillas, Poinar & Raulston, and *Heterorhabditis bacteriophora* Poinar have been identified and characterized (Dillman et al., 2012). Most of the isolated host-specific odorants attracted IJs either when tested individually or with a mixture of neutral host-derived odorants, or in the presence of other olfactory cues (Dillman et al., 2012).

Entomopathogenic nematodes also undergo complex levels of host acceptance in determining the suitability of a host. For example, Lewis et al. (1996) observed that behavioral responses of *S. carpocapsae* IJs to various host species correlated with the number of IJs produced per milligram of host tissue. Additionally, the nematodes can determine the stage of an existing infection and become less likely to join an infection as resources dwindle and mating opportunities diminish (Fushing et al., 2008; Shapiro-Ilan et al., 2018).

Beyond host selection, several other factors influence successful infection by entomopathogenic nematodes including host immune response, population effects and interactions with other organisms (Shapiro-Ilan et al., 2018). The immune response to nematodes includes both humoral and cellular responses (Strand, 2008; Eleftherianos et al., 2010). A reduction in the host immune response increases nematode infection success (Lewis and Clarke, 2012). Entomopathogenic movement and infection patterns have been found to be linked to group behavior (Fushing et al., 2008; Shapiro-Ilan et al., 2014; Ruan et al., 2017). Host selection and the success rate of entomopathogenic infection can be hindered or enhanced by co-infection by other insect pathogens (such as viruses, bacteria or other nematodes) or hymenopteran parasitoids (Koppenhöfer et al., 2005).

The Indian meal moth, *Plodia interpunctella,* is a worldwide pest species of stored products including grain and other cereals. The ectoparasitoid *Habrobracon hebetor* (Say) (Hymenoptera: Braconidae) is a natural enemy of *P. interpunctella* that parasitizes the larval stage by first injecting a venom, which induces permanent paralysis. Females then lay a variable number of eggs on the cuticle (Warsi et al, 2018). Wasp eggs hatch 2–3 days after being laid and the resulting larvae develop by consuming the host. The heterorhabditid nematode, *H. indica* Poinar, Karunakar & David, also parasitizes *P. interpunctella* larvae.

A previous study reported that *H. hebetor* and *H. indica* increased mortality of *P. interpunctella* (Lepidoptera: Pyralidae) in an additive or synergistic manner above levels observed when *P. interpunctella* was attacked by either species alone (Mbata and Shapiro-Ilan, 2010). While adult female parasitoids were unable to distinguish between nematode-infected and uninfected *P. interpunctella* larvae, *H. indica* preferentially infected *P. interpunctella* larvae that had been envenomed and paralyzed by *H. hebetor* compared with control larvae that had not been envenomed by *H. hebetor* (Mbata and Shapiro-Ilan, 2010). In contrast, the reproductive yield of nematodes in envenomed and control hosts did not differ (Mbata and Shapiro-Ilan, 2010).

In this study we explored four potential hypotheses for preferential infection of envenomed hosts: (1) elevated CO_2 levels in envenomed hosts attract the entomopathogenic nematodes, (2) envenomed hosts are immobilized and thus easier targets for the nematodes, (3) close range volatile chemicals emitted by the envenomed hosts are more attractive to entomopathogenic nematodes, (4) envenomed hosts have reduced immune responses which enhance IJ infectivity. In laboratory studies, these hypotheses were explored for *H. indica* as well as another nematode, *Steinernema glaseri* (Steiner) for which we demonstrated preferential infection of envenomed *P. interpunctella.*

2. Materials and methods

2.1. Rearing of P. interpunctella

The culture of *P. interpunctella* was originally obtained from United States Department of Agriculture-Agricultural Research Service (USDA-ARS) Grain Marketing and Research Laboratory, Manhattan, Kansas, USA, in 2001 and had been reared for several generations at the Department of Biology, Fort Valley State University, USA, prior to the commencement of this study. Larvae were reared on a diet of cornmeal, chick laying mash, chick starter mash, oats and glycerin (volumetric mixture at 4:2:2:2:1) in an environmental chamber set at 28 ± 1.5 °C, 70 ± 5% relative humidity (RH) and a photoperiod of 16:8 (light (L):dark (D)) as previously outlined (Mbata, 1987). All experiments were conducted using last (fifth) instar *P. interpunctella* larvae.

2.2. Rearing of H. hebetor

Habrobracon hebetor was obtained in 2001 from a colony maintained at the Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater, OK, USA. *Habrobracon hebetor* were reared by transferring 10 adult females to 1 L rearing jars containing ~50 last instars of *P. interpunctella* at the wandering stage. The jars containing both the moth larvae and the wasps had their tops screened with filter paper and were placed in an environmental chamber maintained at 28 ± 1.5 °C, 70 ± 5% RH and a photoperiod of 16:8 (L:D). Females paralyzed and laid eggs on host larvae, which was followed by hatching and development of wasp offspring into adults that eclosed ~7 days after egg laying. Adult wasps were collected and held in small plastic cages.

2.3. Nematode cultures

Heterorhabditis indica (HOM1 strain), and *S. glaseri* (VS strain) were reared at ~25 °C in last instar greater wax moth, *Galleria mellonella* (L.), according to procedures described by Woodring and Kaya (1988). The larvae of *G. mellonella* were obtained from Webster's Waxie Ranch (Webster, WI, USA). Nematodes were stored at 13 °C for 15 days or less before being used for experiments. The cultures of *H. indica* and *S. glaseri* used in experiments were obtained from the USDA-ARS International Culture Collection in Byron, Georgia, USA.

2.4. Differential infection of envenomed and control P. interpunctella by H. indica and S. glaseri

In Mbata and Shapiro-Ilan (2010), preferential infection of *P. interpunctella* larvae that had been envenomed by *H. hebetor* was observed using 4000 *H. indica* IJs. In the current study, we assessed preferential infection across different IJ application rates for both *H. indica and S. glaseri*. The methods were based on Mbata and Shapiro-Ilan (2010). In brief, a batch of 400 last instar *P. interpunctella* were first exposed to 20 *H. hebetor* adult females for 24 h. This resulted in wasps paralyzing all larvae. Any eggs on envenomed larvae laid by wasps were removed using a paintbrush. We then placed one envenomed and one control (non-envenomed) *P. interpunctella* larva together in a 60 mm Petri dish lined with filter paper (Whatman No. 1). Prior to inoculation of the *P. interpunctella* larvae, IJs were centrifuged at 582*g* and subsequently diluted to the desired concentration. The rates of IJ application were 200, 400, 600, 1200 and 4000 per dish. IJs (*H. indica or S. glaseri*) were added to the dish in 0.35 ml of tap water. The *P. interpunctella* larvae were removed from the dish 46 h later, rinsed in distilled water to remove any IJs adhering to their surface and frozen. The number of IJs invading in each *P. interpunctella* larva was then determined by dissection and counting the IJs present using a dissecting microscope (Kaya and Stock, 1997). The timing of incubation was based on prior studies that focused on assessing the number of entomopathogenic nematodes invading a host (Caroli et al., 1996). For each IJ application rate, 10 replicate dishes were prepared, and the experiment was repeated once (two trials).

2.5. Effect of differential emission of carbon dioxide

CO_2 emission from envenomed and control *P. interpunctella* larvae was measured as outlined by Shapiro-Ilan et al. (2009). In brief, 200 last instar *P. interpunctella* were exposed to 20 pairs of *H. hebetor* for 24 h as described in Section 2.4. Envenomed and control hosts were infected with *H. indica* at a rate of 4000 IJs per insect (applied in 0.35 ml of suspension to 60 mm Petri dishes with filter paper). The insects were placed in a cage before being moved to the chamber of the CO_2 analyzer. Emission of CO_2 (PPM) was measured 1, 2, 3, and 4 days post-nematode inoculation using a LI-COR CO_2 Analyzer and Portable Photosynthesis System (Model #: Li-6200; Manufacturer – LICOR, Lincoln, Nebraska, USA). Treatments included measuring CO_2 emission from: (i) envenomed larvae infected by *H. indica*, (ii) control larvae (not envenomed) infected by *H. indica*, (iii) envenomed larvae (without nematodes), and (iv) control larvae. Each treatment was independently replicated three times. Each recording of CO_2 emission was obtained with 3–5 *P. interpunctella* larvae but the total mass of larvae per reading was also recorded. The experiment was repeated five times. The analyzer was allowed 5 min to stabilize before recording of emitted CO_2. After recording the emitted CO_2, the insects were removed from the chamber and the analyzer vacated for 5 min before continuing with the next set of insects. The differences in the initial and the final CO_2 values were determined and recorded.

2.6. Effect of host immobilization on host infection by S. glaseri and H. indica

This experiment was aimed at determining the role of host immobilization on infection by *S. glaseri* or *H. indica*. *Plodia interpunctella* larvae were envenomed by *H. hebetor* and any wasp eggs present were removed as described in Section 2.4. Assays were then conducted by placing either one envenomed or one control (non-envenomed) larva in a 14 mL centrifuge tube with a 2.25 cm diameter filter paper disc placed at the bottom of each tube. These tubes were chosen because they taper at the end such that host larval movement was physically restricted when the larva was at the bottom of the tube. Steel wool balls were inserted into each tube after larval placement, which kept control larvae in the bottom of the tubes. For consistency, steel wool balls were also placed into tubes that contained an envenomed larva. Ten centrifuge tubes containing envenomed larvae and 10 centrifuge tubes containing control larvae were used for each nematode species (*H. indica* and *S. glaseri*) and rate of application (200, 400, 600, and 1200 IJs per insect in 100 µl of water); after centrifugation and dilution to desired concentrations, nematodes were pipetted onto the filter paper prior to adding hosts. Centrifuge tubes were capped and placed into an incubator at 25 °C for 48 h. After incubation, *P. interpunctella* larvae were frozen for 24 h and dissected as described above to count the number of IJs present inside each *P. interpunctella*. The experiments were repeated once.

2.7. Effect of 3-methyl-3-buten-1-ol on attraction of nematodes to envenomed P. interpunctella larvae

To detect volatile compounds emitted from envenomed *P. interpunctella* larvae, two larvae envenomed by *H. hebetor* were put in a 4 ml glass vial and a Tenax filled adsorption filter (Alborn, 2018) was lowered into the vial, close to but not touching any of the larvae. Room air was pulled through the filter at 20 ml/min for 3 min. The samples were analyzed using an Agilent 6890N/5975 GC/MS (gas chromatography–mass spectrometry) in electron impact (EI) mode with an ion source temperature of 230 °C. The GC was modified for thermal desorption as described in Alborn (2018) where the splitless injector was used for a 2 min filter desorption at 150 °C. The 30 m 0.25 mm inner diameter (ID), 0.25 µm film thickness Agilent DB5 column was kept at an initial temperature of 30 °C for 3 min, followed by 10 °/min programming to 260 °C and kept at that temperature for 5 min. The same collection and analyses were repeated with non-envenomed larvae. To reveal components specific to envenomed hosts, GC/MS traces of non-envenomed larvae were subtracted from the envenomed larva collections, revealing a primary compound specifically emitted by wasp envenomed *P. interpunctella* larvae, marked in the partial total ion current traces in Fig. 1. The peak of interest was tentatively identified as 3-methyl-3-buten-1-ol by the NIST14 library and the structure was confirmed by analysis of a commercial standard (Sigma Aldrich Co. LLC, St. Louis, MO 63178, USA). The synthetic equivalent of the compound was explored further as a potential stimulus for differential infection.

The following choice experiment was designed to investigate the role of the volatile emitted by *P. interpunctella* envenomed by *H. hebetor* in preferential IJ infectivity. *Habrobracon hebetor* envenomed and non-envenomed *P. interpunctella* larvae were used in this study. Fifty Petri dishes (60 mm diameter) containing differently treated *P. interpunctella* larvae comprising (i) one healthy larva treated with either 0.1, or 1.0, or 10.0 µM 3-methyl-3-buten-1-ol was placed with one non-treated healthy larva in each batch of 10 replicate Petri dishes for each concentration (the treated larvae were dipped in the respective solutions just prior to the assay), (ii) as a negative control that comprised 10 dishes that

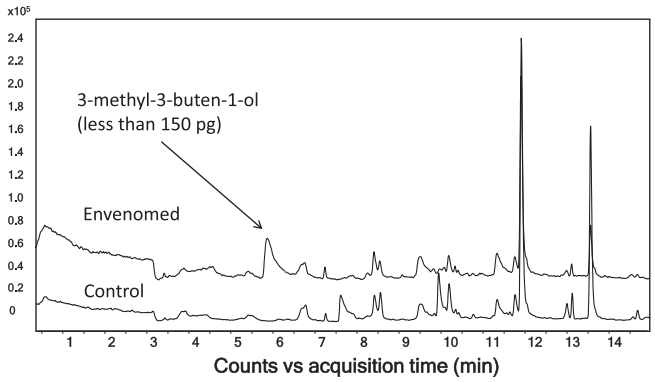

Fig. 1. Thermal desorption (gas chromatography–mass spectrometry) traces of volatiles collected 1 day after *Plodia interpunctella* larvae were envenomed by *Habrobracon hebetor* and of control larvae that had been removed from food for the same time. A chromatographic trace extraction of the control from the envenomed treatment (not shown) revealed a peak identified as 3-methyl-3-buten-1-ol to be specific to the envenomed treatment larvae while no other major differences were detected. Analyses were performed on an in house modified direct injection thermal desorption Agilent 6890 GC combined with a 7953 MS with electron impact ionization. After a 2 min 150 °C desorption/colt trap injection, the column was programmed from 30 °C to 260 °C over 30 min.

contained two healthy larvae each with one larva randomly selected and dipped in distilled water; (iii) 10 positive control dishes with one healthy and one envenomed larva in each. To distinguish host larvae used in the study, dipped or envenomed larvae were marked on the dorsal surface behind the head with a black permanent marker, while all other larvae (non-envenomed and or not dipped in the test solutions or water) were not marked. Initial trials confirmed that marking the larvae did not affect infectivity by the nematodes as there were no significant differences in the infection rates between marked and unmarked host larvae by both *H. indica* ($P = 0.9126$) and *S. glaseri* ($P = 0.9975$) (paired *t*-test, $n = 15$). Prior to inoculation of the *P. interpunctella* larvae with IJs of *S. glaseri* or *H. indica*, the IJs were centrifuged at 582*g*, and thereafter used to inoculate the host larvae at a rate of 1150 IJs/larva. Following inoculation, *P. interpunctella* larvae were incubated for 48 h at 25.0 °C. Upon expiration of the incubation period, the host larvae were dissected and the numbers of IJs in the larvae were noted. The experiments for each nematode species were repeated once.

2.8. Effect of immunity on host infection by S. glaseri and H. indica

The primary immune defense in insects against multicellular parasites such as entomopathogenic nematodes is encapsulation. During an encapsulation response, immune cells (hemocytes) bind to the parasite and one another to form a multicellular envelope (Dunphy and Thurston, 1990; Eleftherianos et al., 2010). We therefore conducted three experiments that compared encapsulation of *H. indica* or *S. glaseri* IJs in wasp envenomed versus non-envenomed *P. interpunctella*: (i) a no-choice test with continuous exposure of envenomed and control larvae to nematodes, (ii) a choice test with continuous exposure of envenomed and control larvae to IJs, and (iii) and a no choice test with 6 h of limited exposure of envenomed and control larvae to nematodes. In all experiments, *P. interpunctella* larvae were envenomed by *H. hebetor* 3 days before use. Envenomed and control *P. interpunctella* were then exposed to IJs of *H. indica* or *S. glaseri* as follows. For the no choice test, five envenomed or control larvae were placed per 60 mm Petri dish, followed by addition of 500 IJs in 350 µl of water using either *H. indica* (HOM1) or *S. glaseri* and incubation for 72 h. For the choice test, one envenomed and one control larva were placed in each 60 mm dish followed by addition of 4000 IJs in 350 µl of water and incubation for 72 h. For the 6 h no choice test, Petri dishes were set up identically to the no choice test except 4000 IJs in 350 µl of water were added and incubated for 6 h. Hosts were washed at 6, 24, 48 or 72 h in water to remove any IJs adhering to their surface, followed by dissection. The number and percentage of nematodes in each host were then assigned to the following categories: dead nematodes (with or without hemocytes), total nematodes with hemocytes, and live nematodes with hemocytes (An et al., 2012). All tests were repeated five times. Encapsulated nematodes and dead nematodes have been considered indicative of an overall immune response in previous studies (An et al., 2012); however, we recognize some individuals in the 'dead' category may not all be attributable to host defenses.

2.9. Statistical analyses

In confirmation experiments for preferential infection (Sections 2.4 and 2.5) and choice experiments comparing host larvae treated with volatile chemical (3-methyl-3-buten-1-ol) versus non-treated *P. interpunctella* larvae (Section 2.8), a paired *t*-test was implemented to assess attraction/infection. The impact of immobilization (Section 2.7) was also analyzed by *t*-tests. CO_2 emission data (Section 2.6) were examined by repeated measures analysis (Proc Glimmix, SAS software: version 9.1. SAS Institute, Cary, NC. 2002). Statistically significant treatment differences were further analyzed using a post-hoc Tukey Kramer least significant difference test ($\propto = 0.05$) (SAS software: version 9.1. SAS Institute, Cary, NC. 2002; Steel and Torrie, 1980). Based on residual plots, prior to analysis, numerical data from these experiments Sections 2.4 to 2.8) were square root transformed to minimize variances and to standardize means (Steel and Torrie, 1980). Immune responses toward IJs (Section 2.9) in envenomed versus non-envenomed hosts were analyzed by Wilcoxon signed rank or *t*-tests.

3. Results

3.1. Heterorhabditis indica and S. glaseri IJs preferentially infect envenomed P. interpunctella larvae

Infectivity assays showed that significantly more *H. indica* and *S. glaseri* IJs infected envenomed than control *P. interpunctella* larvae across all application rates we tested (degrees of freedom (df) = 19 $P \leq 0.02$ for all tests) (Fig. 2). The number of IJs per larva also increased with inoculation density with the maximum invasion rate for *H. indica* being at an inoculation density of 4000 IJs per Petri dish and the maximum invasion rate for *S. glaseri* being 1200 IJs per Petri dish (Fig. 2).

3.2. Envenomed P. interpunctella larvae emit less carbon dioxide than control larvae

Bioassays showed that envenomed *P. interpunctella* larvae emitted less CO_2 than control larvae, regardless of whether or not they were infected by *H. indica* IJs ($F = 17.43$; df = 3, 222; $P = 0.0001$) (Fig. 3).

3.3. Immobilization of P. interpunctella differentially affected infection by H. indica and S. glaseri IJs

At all application rates tested, more *S. glaseri* IJs were present in envenomed host larvae compared with immobilized control larvae (df = 38; $P < 0.0001$ for all tests; Fig. 4). In contrast, no differences were detected between treatments for *H. indica* except at an inoculation rate of 600 IJs per host larva (df = 38; $P = 0.0018$) (Fig. 4).

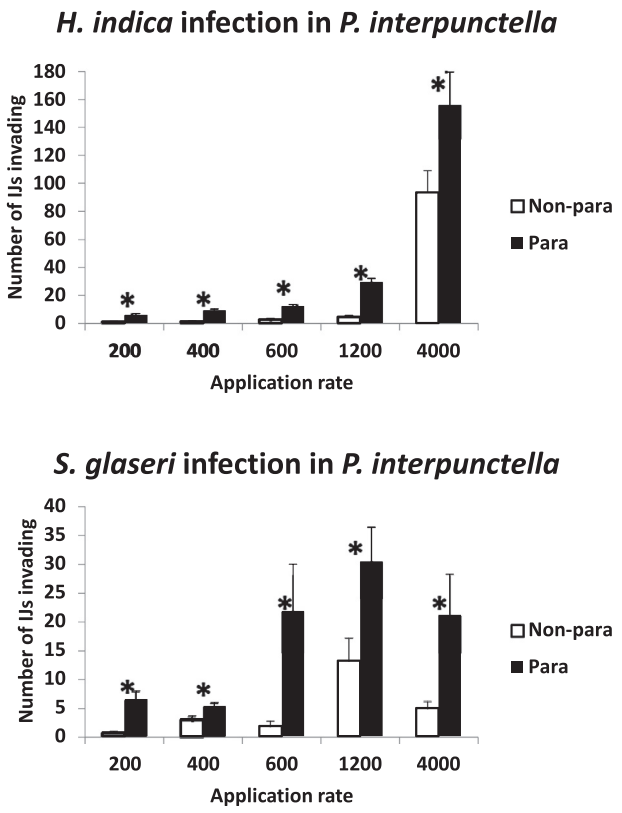

Fig. 2. Number of *Heterorhabditis indica* or *Steinernema glaseri* infective juvenile nematodes (IJs) invading *Plodia interpunctella* larvae after 72 h of exposure to various nematode application rates; *P. interpunctella* envenomed by *Habrobracon hebetor* were compared with non-envenomed larvae. Asterisk indicates significance between envenomed and non-envenomed (paired *t*-test, $\alpha = 0.05$). Error bars indicate S.E.M.

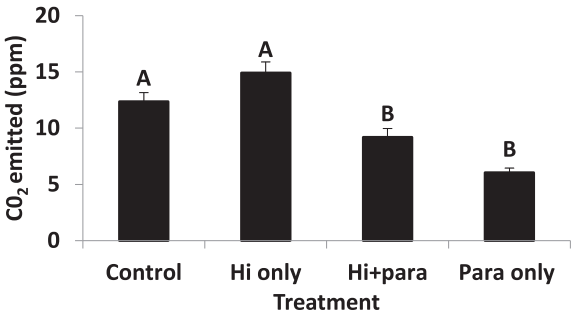

Fig. 3. Carbon dioxide output by *Plodia interpunctella* larvae infected by *Heterorhabditis indica* (Hi) or envenomed by *Habrobracon hebetor* (Para), or a combination of the two biological agents. Control = *P. interpunctella* without *H. indica* or *H. hebetor*. CO_2 was measured over a 4 day period. Different letters above bars indicate statistical significance (Tukey's test, $\alpha = 0.05$). Error bars indicate the S.E.M.

3.4. 3-methyl-3-buten-1-ol promotes infection by S. glaseri but not H. indica

The numbers of *H. indica* and *S. glaseri* IJs responding to non-envenomed *P. interpunctella* larvae dipped in different concentrations of 3-methyl-3-buten-1-ol are given in Fig. 5. At all concentrations, the numbers of *S. glaseri* IJs that infected non-envenomed *P. interpunctella* larvae dipped in solutions of 3-methyl-3-buten-1-ol were significantly higher than those that were not exposed to the chemical ($P = 0.004$, 0.03 and 0.01 for the 0.1, 1.0 and 10 mM solutions, respectively); also the positive control, which consisted

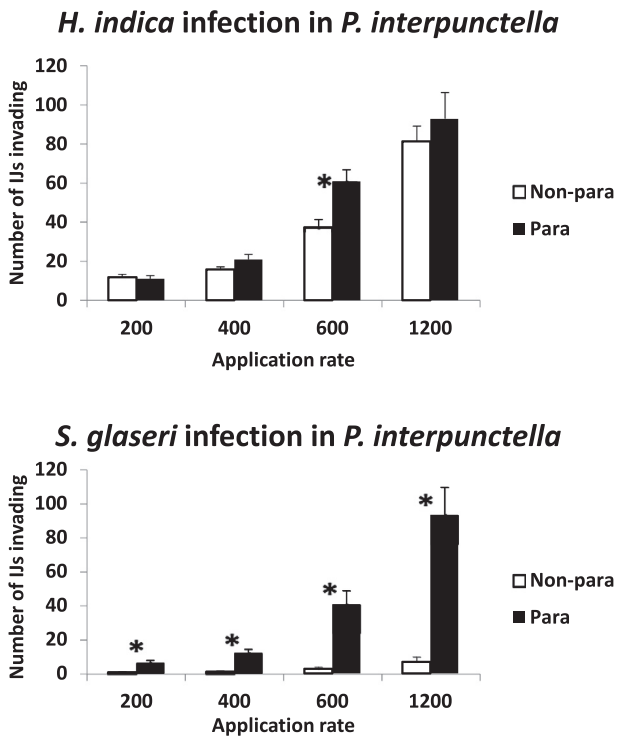

Fig. 4. Number of *Heterorhabditis indica* or *Steinernema glaseri* infective juvenile nematodes (IJs) invading physically immobilized *Plodia interpunctella* larvae after 72 h of exposure to various nematode application rates; *P. interpunctella* envenomed by *Habrobracon hebetor* were compared with non-envenomed larvae. Asterisk indicates significance between envenomed and non-envenomed (paired *t*-test, $\alpha = 0.05$). Error bars indicate the S.E.M.

of wasp envenomed larvae, showed preferential invasion ($P = 0.0002$), whereas the negative control did not ($P = 0.477$) (Fig. 5). The numbers of *H. indica* IJs that infected *P. interpunctella* larvae dipped in the different concentrations of 3-methyl-3-buten-1-ol were not significantly different from those of IJs that invaded the control larvae dipped in water ($P = 0.853$, 0.093 and 0.376 for the 0.1, 1.0 and 10 mM solutions, respectively); only the positive control exhibited preferential infection ($P = 0.0003$), whereas the negative control did not ($P = 0.103$) (Fig. 5).

3.5. Effect of host immunity on infectivity of wasp envenomed and non-envenomed P. interpunctella larvae

Differential immune responses to *H. indica* and *S. glaseri* IJs in envenomed and control *P. interpunctella* larvae under no choice or choice tests and with continuous exposure (up to 72 h) or limited exposure (6 h) to the nematodes are given in Figs. 6–8. Overall, few differences in immune responses for envenomed and control larvae were detected, and in cases where differences were observed there was an increased immune response in the envenomed larvae. For *H. indica* (Figs. 6 and 7), there were six comparisons out of 33 that showed higher levels of immune responses in envenomed compared with control larvae ($P \leq 0.05$ for each of these six comparisons) with each of the significantly different comparisons being detected in no choice tests (Figs. 6 and 7). Differences were also detected in four comparisons in regard to the percentage of dead nematodes, in one comparison in regard to the total percentage of dead nematodes with attached hemocytes, and one comparison of live nematodes with attached hemocytes (Figs. 6 and 7). Note that the data for all of the other choice tests we conducted are not presented because no differences were

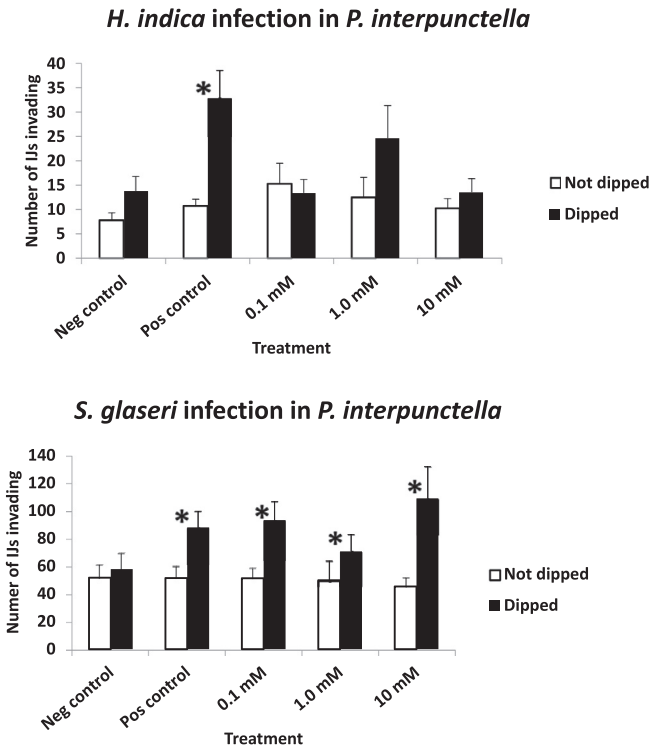

Fig. 5. Number (mean ± S.E.) of *Heterorhabditis indica* or *Steinernema glaseri* IJs that invaded *Plodia interpunctella* larvae dipped versus not dipped into solutions of 3-methyl-3-buten-1-ol at 0.1, 1.0 and 10 mM in a choice test. Negative control = two healthy (not-dipped) *P. interpunctella*. Positive control = one *P. interpunctella* larva envenomed by *Habrobracon hebetor* versus one non-envenomed larva. Asterisk indicates significance within pairs (paired *t*-test, $\alpha = 0.05$).

detected among treatments and results were otherwise similar to no choice tests.

For *S. glaseri*, out of the 36 comparisons made, differential responses were observed between envenomed and non-envenomed hosts in only two comparisons. The differences were detected in the percentage of dead IJs and in percentage of total hemocytes in the choice test comparison at 72 h ($P \leq 0.05$ within each comparison); for both of these instances, lower numbers were observed in non-wasp envenomed hosts relative to envenomed hosts (Fig. 8). In all other comparisons for *S. glaseri,* no differences were observed in immune responses between wasp envenomed and non-envenomed hosts ($P > 0.05$); data for the *S. glaseri* no choice tests are not shown as no differences were detected and results were otherwise similar to choice tests. When the hemocyte response in the two nematodes was compared, *S. glaseri* had a notably lower hemocytes response compared with *H. indica* (Figs. 6–8). Hemocytes were not observed for *S. glaseri* in the no choice tests (data not shown), and were found on live nematodes in the choice test (Fig. 8). In contrast, numerous hemocytes were observed for *H. indica* in the first timeframe (Figs. 6 and 7). Although *S. glaseri* showed zero to low hemocyte activity, there was a high mortality rate of *S. glaseri* IJs at 6 h post-exposure (Fig. 8).

4. Discussion

The IJs of *H. indica* and *S. glaseri* showed preferences for wasp envenomed compared with non-envenomed *P. interpunctella* larvae at all nematode rates investigated, which confirms and expands upon an earlier observation (Mbata and Shapiro-Ilan, 2010). The new observation that IJs of *S. glaseri* also preferred wasp envenomed larvae to non-envenomed larvae may imply that this behavior of IJs may extend across species of the *Heterorhabditis* and the *Steinernema* genera. The venom from *H. hebetor* permanently paralyzes *P. interpunctella* larvae, which enables *H. hebetor* larvae to feed undisturbed (Moreau and Asgari, 2015). Parasitism of lepidopteran larvae by braconid wasps more generally is also known to affect host hemolymph proteins and other aspects of host physiology (Altunas et al., 2010).

CO_2 is among the most important stimuli that alert IJs of the nearness of an insect host (Lewis and Clarke, 2012). Indeed, CO_2 was found to act as an attractant to various entomopathogenic

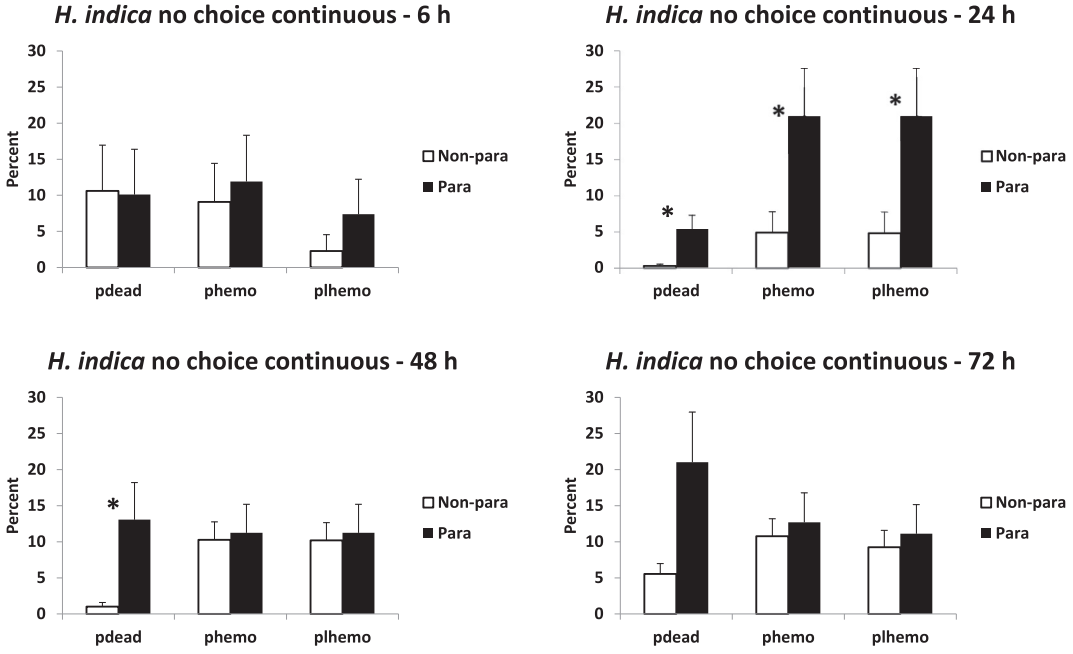

Fig. 6. Immune response to *Heterorhabditis indica* in a no choice test where the nematodes were continuously exposed to *Plodia interpunctella* that were either envenomed by a parasitic wasp, *Habrobracon hebetor* (Para), or not exposed to the parasitoid (non-para). The immune response was measured 6 h, 24 h, 48 h and 72 h post host-nematode exposure based on the percentage of dead nematodes (pdead), total nematodes with hemocytes (phemo), and live nematodes with hemocytes (plhemo). Asterisk indicates significance between para and non-para ($\alpha = 0.05$). Error bars indicate the S.E.M.

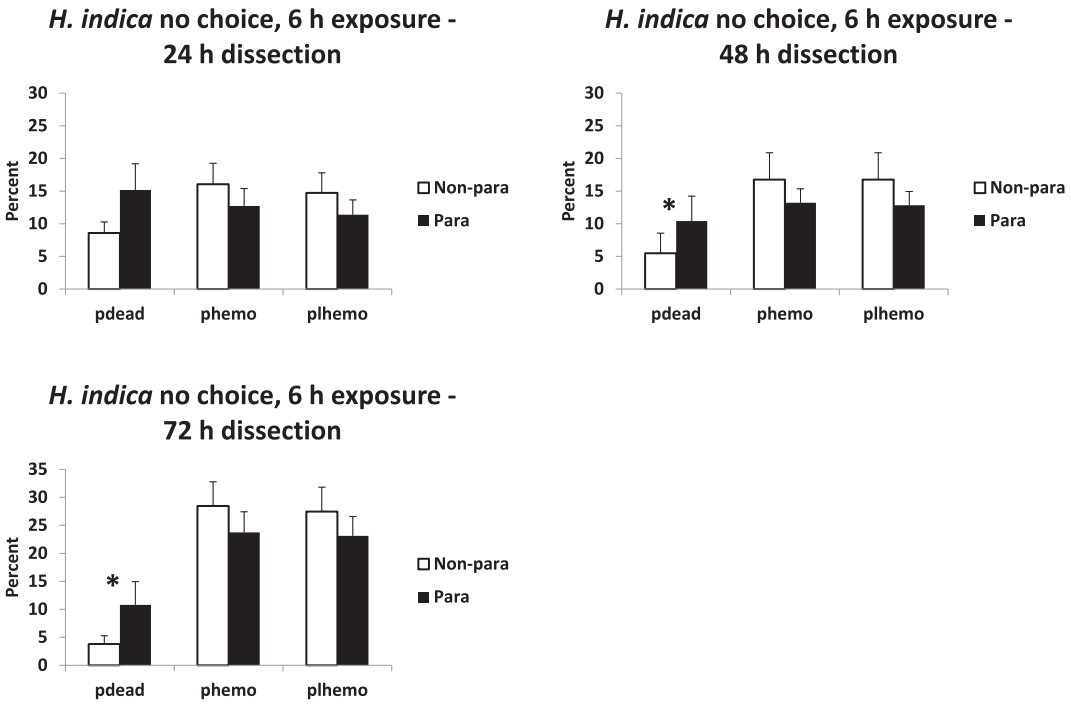

Fig 7. Immune response to *Heterorhabditis indica* in a no choice test where the nematodes were exposed to *Plodia interpunctella* that were either envenomed by a parasitic wasp, *Habrobracon hebetor* (para), or not exposed to the parasitoid (non-para). The hosts were exposed to nematodes for 6 h and then removed from exposure. The immune response was measured after 24 h, 48 h, and 72 h based on the percentage of dead nematodes (pdead), total nematodes with hemocytes (phemo), and live nematodes with hemocytes (plhemo). Asterisk indicates significance between para and non-para ($\alpha = 0.05$). Error bars indicate the S.E.M.

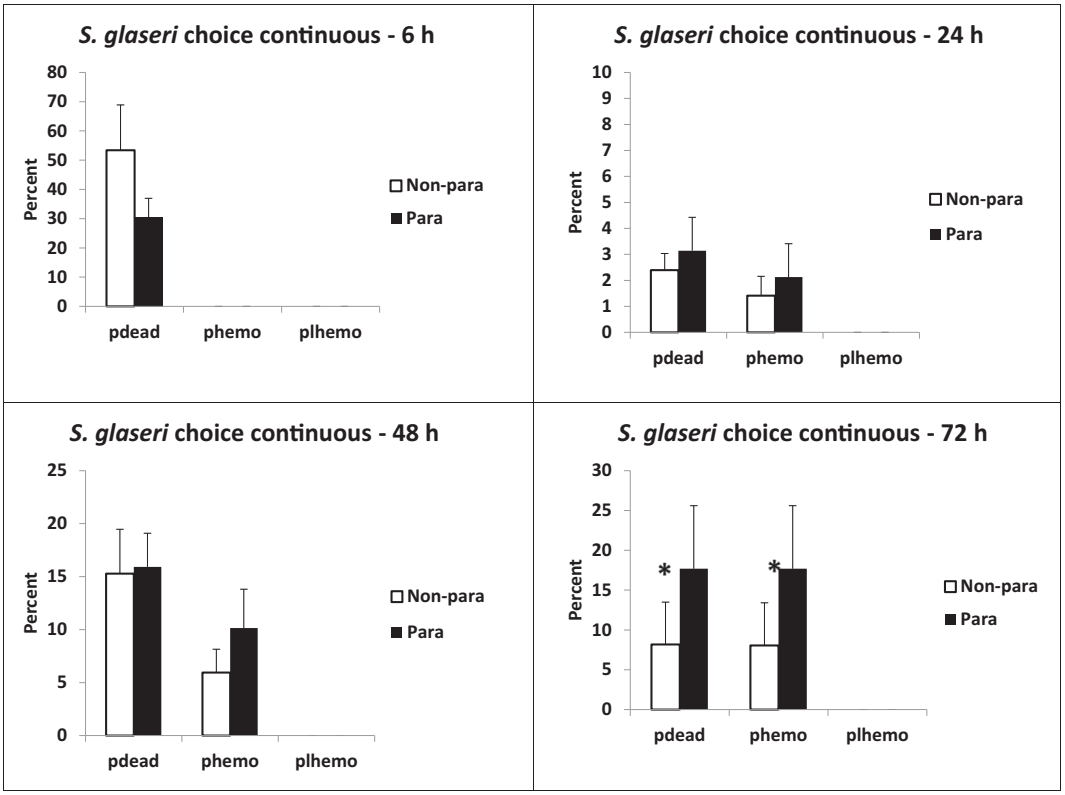

Fig. 8. Immune response to *Steinernema glaseri* in a choice test where the nematodes were continuously exposed to *Plodia interpunctella* that were either envenomed by a parasitic wasp, *Habrobracon hebetor* (para) or not exposed to the parasitoid (non-para). The immune response was measured 6 h, 24 h, 48 h and 72 h post host-nematode exposure based on the percentage of dead nematodes (pdead), total nematodes with hemocytes (phemo), and live nematodes with hemocytes (plhemo). Asterisk indicates significance between para and non-para ($\alpha = 0.05$). Error bars indicate the S.E.M.

nematode species of differing foraging strategies, and the responses were positively correlated to a broad range of CO_2 concentrations (Dillman et al., 2012). To our knowledge CO_2 has never been indicated as a repellent to entomopathogenic nematodes. In this study, *P. interpunctella* larvae envenomed by *H. hebetor* emitted lower levels of CO_2 compared with non-envenomed larvae, both in the control (non-envenomed larvae), and *H. indica*-infected larvae. This result indicates that CO_2 is likely not the cue that elicits preferential infection of wasp-envenomed larvae; if CO_2 was the cue then we would have expected higher CO_2 levels in envenomed larvae relative to non-envenomed. Although we utilized 3–5 insects in each replicate, the approximate total mass was similar among all samples and treatments; moreover, the number of insects used was randomized across replicates and treatments, and therefore we do not expect that insect mass or the number of insects influenced the results. Three proteins in *H. hebetor* venom presynaptically block glutaminergic transmission, which in turn also affects host endocrine and metabolic activity (Slavnova et al., 1987; Pennachino and Strand, 2006; Kryukova et al., 2011). Reduced metabolic activity likely contributes to the lower CO_2 output we observed in envenomed *P. interpunctella* larvae. In addition, paralysis from envenomation may also impair spiracle function since innervation is through lateral nerves arising from segmental ganglia. Impairment of the spiracles will also result in low CO_2 output. The discovery that *H. hebetor* envenomed hosts produce lower CO_2 levels than non-envenomed hosts resulted in our not exploring further the CO_2 hypothesis for increased infection of hosts by *H. indica* or *S. glaseri*.

Insect hosts can defend against entomopathogenic nematode infection through avoidance behavior (Shapiro-Ilan et al., 2018). For example, Japanese beetle larvae (*Popillia japonica* Newman) avoid concentrations of entomopathogenic nematode IJs (Schroeder et al., 1993). Therefore, an immobilized or incapacitated host can become easier prey for entomopathogenic nematodes. Indeed, Koppenhöfer et al. (2000a,b) reported that chemical insecticide-induced sluggishness in white grub hosts (Coleoptera: Scarabaeidae) facilitated entomopathogenic nematode infection. Similarly, we hypothesized that immobilization of *P. interpunctella* hosts due to envenomation by *H. hebetor* could provide a basis for the observed increased nematode infectivity. Immobilization of host larvae was not a factor in the infection preference displayed by *S. glaseri* since this nematode continued to prefer envenomed *P. interpunctella* larvae even when the non-envenomed hosts were immobilized. However, immobilization due to wasp envenomation appears to be a key factor in preferential infectivity for *H. indica* because (in three of the four application rates tested) the nematodes showed no preference for envenomed hosts relative to immobilized healthy (non-envenomed) hosts. A preference for immobilized hosts by *H. indica* supports the broader hypothesis of risk-sensitive foraging (Fushing et al., 2008) in entomopathogenic nematodes in which the nematodes follow a path of least resistance.

Olfaction is an important component of host finding behavior and is critical for nematodes that forage as cruisers such as *H. indica* and *S. glaseri* (Lewis and Clarke, 2012). Thus, we hypothesized that volatile cues emitted by *P. interpunctella* envenomed by *H. hebetor* may contribute to preferential attraction and infection of envenomed hosts. The compound, 3-methyl-3-buten-1-ol, emitted by *P. interpunctella* larvae envenomated by *H. hebetor* was an effective stimulus for *S. glaseri* but not for *H. indica*. Several host-derived odorants have been identified as sources of volatile stimuli for the attraction of IJs to hosts (Dillman et al., 2012). We believe this is the first report of the compound, 3-methyl-3-buten-1-ol, as a volatile that is emitted by envenomed host Lepidoptera larvae and that is attractive to nematode IJs. It is not known why 3-methyl-3-buten-1-ol is effective for *S. glaseri* but not for *H. indica*. Olfaction responses to specific chemical cues are known to vary substantially among entomopathogenic nematode species (Dillman et al., 2012). Thus, it may be that *S. glaseri* is simply attuned to a chemotaxis response for 3-methyl-3-buten-1-ol whereas *H. indica* is not.

A reduced host immune response can be correlated with preferential parasitism (Tschirren et al., 2007; Lyimo and Ferguson, 2009). Studies of several parasitoid species also indicate that venoms can affect host immune defenses (Usherwood and Machili, 1966; Walther and Rathmayer, 1974; Carton and Nappi, 1997; Shelby et al., 2000; Labrosse et al., 2003; Beckage and Gelman, 2004; Andrew et al., 2006). These include prior studies of *H. hebetor* where venom was found to reduce encapsulation, melanization of capsules, and production of reactive oxygen species in *Galleria mellonella* (Kryupkova et al., 2007, 2011). We thus hypothesized that a potentially reduced immune response in envenomed *P. interpunctella* could contribute to the increase in nematode infection we observed relative to non-envenomed hosts. However, contrary to expectations, our results identified an elevation in immune response toward *H. indica* in envenomed *P. interpunctella* compared with non-envenomed larvae. A differential immune response between envenomed and non-envenomed hosts was less pronounced in *S. glaseri* treatments, but results overall also supported higher immune responses in envenomed than non-envenomed *P. interpunctella*. Given these outcomes, we thus conclude that host cellular immune defenses are likely not a key factor in explaining why infection rates by *H. indica* and *S. glaseri* are higher in envenomed than non-envenomed *P. interpunctella.* However, it is possible that alterations in host humoral immune defenses could be affected by envenomation of *P. interpunctella* with potential consequences for infection by nematodes.

In conclusion, our results indicate that host immobilization is a significant factor inducing preferential infection of *H. indica* for *H. hebetor*-envenomed *P. interpunctella*, whereas for *S. glaseri*, a host volatile chemical emitted by the envenomed host is a key factor. Although the two genera of nematodes we studied (*Steinernema* and *Heterorhabditis*) have similar life-cycles and share some other biological components, they diverge significantly in their phylogenetic origins (Stock, 2015). Therefore, it is not surprising that the two nematodes from different genera have some disparate mechanisms of host selection. Our results expand upon current knowledge of the complexities of host preference, particularly when there is more than one parasite group utilizing a single host resource. Our findings also provide a foundation for future research to explore mechanisms of preferential infectivity in two-parasite systems. For example, it will be interesting to determine if preferential infectivity exists in other nematode-wasp combinations among various insect-parasitic nematode and hymenopteran parasitoid genera and species. Trends will provide insight into evolutionary relationships in host-parasite interactions and competition.

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