Conspecific pheromone extracts enhance entomopathogenic infectivity

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Abstract

Entomopathogenic nematodes (EPNs) provide economic control of various insect pests. However, field efficacy can be inconsistent. The ability of a nematode to find and infect (invade) a host insect is critical to successful pathogenesis. Thus, behaviors including dispersal and infectivity play important roles in improving efficacy. Previously, we discovered that EPN-infected host substances enhance nematode dispersal. Later we found that a mixture of pheromones in the infected host induced dispersal and improved EPN efficacy. In this study, we determined if dispersal-inducing pheromone extracts also increase nematode infectivity (the propensity to invade a host insect). Two nematode species, *Steinernema carpocapsae* and *Steinernema feltiae*, and two insect hosts, *Galleria mellonella* and *Diaprepes abbreviatus*, were tested. We discovered that conspecific dispersal pheromone extracts of each EPN species enhanced infectivity. These results indicate that the utility of dispersal pheromone extracts for enhancing EPN activity and biocontrol efficacy is improved not only due to increased nematode movement, but also due to increased host infection.

Keywords

Biological control, Entomopathogenic nematode, Infectivity, Pheromone, *Steinernema*.

Entomopathogenic nematodes (EPNs) in the genera *Heterorhabditis* and *Steinernema* are potent biocontrol agents that are used to control a wide variety of economically important insect pests (Shapiro-Ilan et al., 2017, 2018). The nematodes occur naturally in the soil and kill arthropod hosts with the aid of symbiotic bacteria (*Xenorhabdus* spp. bacteria are associated with steinernematid nematodes and *Photorhabdus* spp. bacteria are associated with heterorhabditid nematodes). Despite the commercial success of EPNs as biological control agents, field efficacy is often variable, and therefore research toward improvement is needed (Shapiro-Ilan et al., 2017). Methods to enhance biocontrol efficacy in EPNs include strain improvement as well as improving nematode production, formulation and application technology (Shapiro-Ilan et al., 2012, 2017).

Clearly, to cause insect mortality, and thereby reduce pest populations, the nematodes must move to the host and successfully infect (invade) it. Therefore, two aspects of EPN biology that contribute significantly to biocontrol efficacy include nematode dispersal and infectivity. Prior research indicates that certain substances within EPN-infected hosts enhance nematode dispersal (Shapiro and Glazer, 1996). Furthermore, EPN-infected host substances enhance nematode infectivity, i.e. the propensity to invade the host (Shapiro and Lewis, 1999). Dispersal-inducing compounds in steinernematid nematodes were later described as specific ascaroside pheromones (Kaplan et al., 2012). Presumably due to these dispersal pheromones, crude macerate of EPN-infected hosts was shown to enhance EPN dispersal in a soil profile (Wu et al., 2018). In agreement with these findings, dispersal pheromone extracts from host cadavers enhanced movement of *Steinernema carpocapsae* (Weiser) and *S. feltiae* (Filipjev) in soil columns, and in greenhouse trials they enhanced efficacy

(Oliveira-Hofman et al., 2019). Prior to our research here, it was not known whether EPN dispersal pheromones can enhance other nematode behaviors that would contribute to biocontrol success, such as infectivity.

Our objective was to determine if conspecific dispersal pheromones increase infectivity of *S. carpocapsae* and *S. feltiae*; accordingly, we tested ascaroside containing pheromone extracts. We chose the two nematode species because the functionality of ascarosides to induce dispersal in these two nematodes has been clearly demonstrated (Kaplan et al., 2012; Oliveira-Hofman et al., 2019). Moreover, the test encompasses two foraging strategies. *S. carpocapsae* is an ambusher (tending to use a sit-and-wait strategy), whereas *S. feltiae* has an intermediate foraging strategy (encompassing aspects of ambushers as well as cruisers that actively seek their host) (Lewis, 2002). Both nematodes are commercially available (Shapiro-Ilan et al., 2017) and thus have relevance to current biocontrol efforts.

In addition to including two nematode species, our study used two different host insects: the greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) and the citrus root weevil, *Diaprepes abbreviatus* (L.) (Curculionidae: Coleoptera). *G. mellonella* is a highly susceptible model host that is used as a model insect used in routine laboratory assays or commercial production of EPNs (Shapiro-Ilan et al., 2012). *D. abbreviatus* is a major pest of citrus that has been targeted extensively with EPNs on a commercial level (McCoy et al., 2007).

Materials and methods

EPNs and pheromones

S. feltiae and *S. carpocapsae* pheromone extracts were obtained as described by Kaplan et al. (2012). Briefly, dispersal pheromones were extracted using 70% methyl alcohol from *S. feltiae* or *S. carpocapsae* consumed host cadaver (Kaplan et al., 2012; Oliveira-Hofman et al., 2019). The nematodes used in all experiments were cultured *in vivo* in last instar of *G. mellonella,* using the White trap method as described by Shapiro-Ilan et al. (2016). The nematodes were then stored in aqueous suspensions in 250 ml tissue culture flasks at 10°C for no longer than three weeks prior to experimentation.

Sensitization of EPNs to pheromones

Prior to experimentation, all nematodes went through a sensitization process to remove any residual pheromones from the *in vivo* cultures. To optimally detect a pheromone response, nematodes need to be sensitized to pheromones by removing them for a period (Srinivasan et al., 2008; Kaplan et al., 2011, 2012; Oliveira-Hofman et al., 2019). Approximately, 10 ml of EPNs (~30,000 IJs) from culture flasks were placed in 15 ml centrifuge tubes and centrifuged at 2,000 rpm (582 g) for 2 min. The supernatant was then discarded, and 10 ml of dH$_2$O was added to each tube. Subsequently, each tube was shaken, and another round of centrifugation followed. This process was repeated for a total of three washes. The final supernatant was discarded and the EPN pellet was resuspended in dH$_2$O. EPNs were again stored in culture flasks at 10°C for 7 days before testing them in infectivity assays.

Infectivity assays

The basic approach to distinguish treatment effects was to expose one half of the nematode infective juveniles (IJs) to pheromone extracts (from their own species) and the other half to tap water only (i.e., the control nematodes); subsequently infectivity of the IJs was assessed. The treated and control nematodes were exposed to last instar *G. mellonella* or 13-week-old *D. abbreviatus* larvae in small arenas that negate dispersal because nematode movement is physically restricted (i.e., 2 ml Eppendorf tubes) (Willett et al., 2018). Specifically, to rule out the possibility that increased infection is due to dispersal leading to increased insect host counter, we reduced the distance between nematodes and the insect host. Thus, all the IJs, in the pheromone treated and control arenas had an equal opportunity to access the insect host and invade. The tubes contained 0.650 grams of oven dried sand. Approximately 1,000 IJs of *S. carpocapsae* or *S. feltiae* were added to each tube in a 0.04 ml volume. The pheromone-treated IJs had been exposed to conspecific pheromone extracts for 20 min prior to the assay, whereas control nematodes were only exposed to water for the same amount of time.

For *G. mellonella*, half the tubes were then incubated for 4 hr at 25°C and the other half exposed in the same manner and incubated for 24 hr at 25°C. For *D. abbreviatus,* all tubes were incubated for 24 hr at 25°C. The 4 hr exposure was not done for *D. abbreviatus* due to lack of insects and because we had already seen similar results between 4 and 24 hr exposure in the *G. mellonella* experiments. After the incubation period, all insects were dissected and the number of invading IJs was recorded (Shapiro and Lewis, 1999; Wu et al., 2018; Willett et al., 2018). There were 20 replicate insects for each nematode

species and exposure interval, and the entire experiment was conducted twice. Treatment effects were assessed by analysis of variance (ANOVA) followed by Tukey's test, and also confirmed by t-test (SAS, 2011). Data were square-root transformed prior to analysis (SAS, 2011); non-transformed means are presented in the figures.

Results

For *G. mellonella*, there were no significant interactions between the trial effect and treatment effect, so trials were combined ($p = 0.4272$ and 0.2694 for *S. carpocapsae* at 4 and 24 hr exposure, respectively and $p = 0.4478$ and 0.4753 for *S. feltiae* at 4 and 24 hr exposure, respectively). The number of *S. carpocapsae* IJs that invaded the host was significantly higher in the pheromone treatment than the control (no pheromone) at 4 hr ($F = 25.47$; df = 1,76; $p < 0.0001$) and 24 hr of exposure ($F = 27.34$; df = 1,76; $p < 0.0001$) (Fig. 1). Similarly, the number of *S. feltiae* IJs that invaded the host was significantly higher in the pheromone treatment than the control at 4 hr ($F = 25.32$; df = 1,74; $p < 0.0001$) and 24 hr of exposure ($F = 53.21$; df = 1,76; $p < 0.0001$) (Fig. 2). Additionally, in all tests for *S. carpocapsae* and

S. feltiae, t-tests indicated higher infectivity in the pheromone-treated IJs than in control IJs ($t = -4.99$ and -5.18 for *S. carpocapsae* at 4 and 24 hr, respectively, and -4.92 and -7.31 for *S. feltiae* at 4 and 24 hr respectively, df = 78 and $p < 0.0001$ for all four tests except df = 76 for *S. feltiae* at 4 hr).

For *D. abbreviatus*, there was no significant interaction between trial effect and treatment effect, so trials were combined ($p = 0. 9466$ and 0. 7986 for *S. carpocapsae* and *S. feltiae*, respectively). The number of *S. carpocapsae* and *S. feltiae* IJs that invaded the host was significantly higher in the pheromone treatment than the control at 24 hr of exposure ($F = 33.91$; df = 1,76; $p < 0.0001$ for *S. carpocapsae* and $F = 35.28$; df = 1,76; $p < 0.0001$ for *S. feltiae*) (Fig. 3). Additionally, t-tests for *S. carpocapsae* and *S. feltiae* indicated higher infectivity in the pheromone-treated IJs than in control IJs ($t = -5.85$ and -6.01 for *S. carpocapsae* and *S. feltiae,* respectively; df = 78 and $p < 0.0001$ for both tests).

Discussion

Ascaroside pheromones in the nematode *Caenorhabditis elegans* have been found to be highly specific with a single ascaroside, or natural mixtures
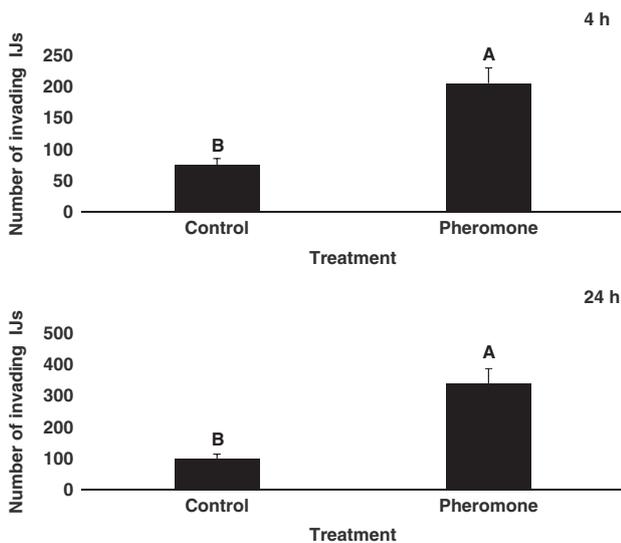

Figure 1: Mean (± SE) number of infective juvenile *Steinernema carpocapsae* (IJs) invading the insect host (*Galleria mellonella*) after 4 or 24 hr exposure with or without dispersal pheromones. Different letters above bars indicated statistical significance (Tukey's test $p \le 0.05$).
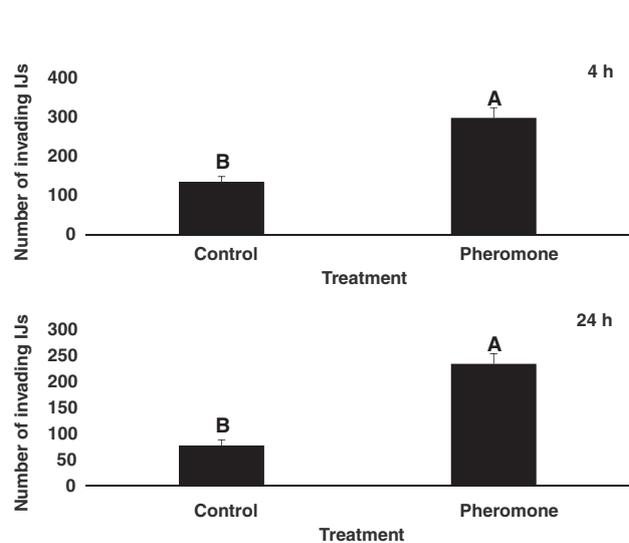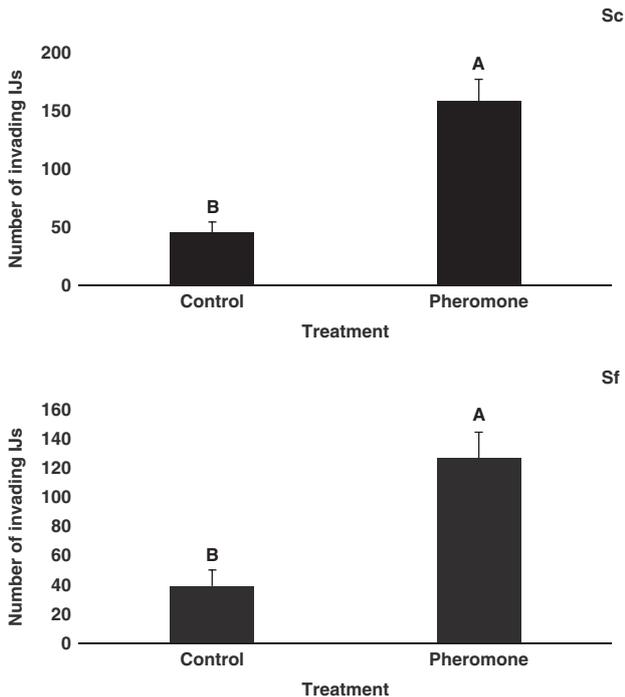

Figure 2: Mean (± SE) number of infective juvenile *Steinernema feltiae* (IJs) invading the insect host (*Galleria mellonella*) after 4 or 24 hr exposure with or without dispersal pheromones. Different letters above bars indicated statistical significance (Tukey's test $p \le 0.05$).

Sc

Sf

Figure 3: Mean (± SE) number of *Steinernema carpocapsae* (Sc) or *Steinernema feltiae* (Sf) infective juveniles (IJs) invading the insect host (*Diaprepes abbreviatus*) after 24 hr exposure with or without dispersal pheromones. Different letters above bars indicated statistical significance (Tukey's test $p \leq 0.05$).

of ascarosides, affecting a single behavior (Srinivasan et al., 2008, 2012; Kaplan et al., 2011). This specificity is suggested to be achieved with only small changes to their chemical structures affecting their impact (Srinivasan et al., 2012). However, other biologically active compounds induce multiple behaviors in EPNs. For example, two plant volatiles, pregeijerene and α-pinene, compounds that attract or repel EPNs (respectively) are also found to enhance infectivity (Willett et al., 2018). Our findings showed that the EPN dispersal pheromone mixture, which contains ascarosides, also has at least two functions: stimulating dispersal and infectivity.

In this investigation we focused on dispersal pheromone extracts that have been shown to be mixtures of ascarosides in both *C. elegans* and *S. feltiae* (Kaplan et al., 2012). We tested ascaroside containing pheromone extracts where additional contribution from non-ascaroside components cannot be excluded (and so the contribution of other components can be explored in future studies). Ascaroside pheromones found in biological systems exist as mixtures as reported by numerous studies (Butcher et al., 2007; Srinivasan et al., 2008; Kaplan et al., 2011; Choe et al., 2012). Our findings suggest that future EPN studies should focus on the function of ascaroside pheromone mixtures and their effects on multiple behaviors.

Given that enhanced infectivity was observed in both nematode species and both insect hosts, the results suggest this phenomenon may occur broadly across foraging strategies. Indeed, ascaroside effects on EPN behavior appear to be highly conserved (Kaplan et al., 2012). However, to determine whether our findings can be applied to broad EPN behavior, including cruiser-type foragers among *Steinernema* spp. and *Heterorhabditis* spp., and in larger arenas and field conditions, requires further testing.

In addition to enhancing biocontrol applications for suppression of insect pests, the pheromone extracts can be used to improve EPN infectivity for other purposes. For example, several companies produce EPNs commercially *in vivo*; enhanced infectivity would lead to increased efficiency in *in vivo* production and lower inoculum rates would be required. Moreover, IJs that are stimulated by pheromone exposure may be better able to infect certain insect pests that are resistant to infection due to physiological or physical deterrents (Eidt and Thurston, 1995; Shapiro-Ilan et al., 2017). The potential to improve biocontrol against diverse insects of varying susceptibility will need to be explored further under field conditions.

Acknowledgments

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