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Lack of susceptibility of soil-inhabiting *Platyprepia virginalis* caterpillars, a native arctiid, to entomopathogenic nematodes in nature

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Abstract

Entomopathogenic nematodes (EPNs) can kill and regulate populations of soil-inhabiting insects, but studies evaluating these interactions in native ecosystems are rare. The objective of this study was to examine the effects of EPNs on a non-agricultural caterpillar, *Platyprepia virginalis* (Boisduval) (Lepidoptera: Arctiidae), under natural conditions. *Platyprepia virginalis* caterpillars live in litter on the soil surface feeding beneath bush lupine during summer, autumn, and winter. Initial laboratory assays revealed that the caterpillars were vulnerable to at least two species of EPNs with which they co-occur in the coastal prairie in northern California (USA). In contrast to laboratory assays, caterpillars survived exposure to prairie soil containing EPNs under natural conditions in field assays. To better understand the divergence between laboratory and field results for this native caterpillar, we used sentinel insects [*Galleria mellonella* L. (Lepidoptera: Pyralidae)] to identify particular locations where EPNs were present in the field. *Platyprepia virginalis* caterpillars were caged at these sites but again showed no evidence of susceptibility to EPNs. *Platyprepia virginalis* caterpillars reduce their exposure to EPNs by spending their time in and above the litter rather than contacting the soil when given the choice in nature. We conclude that *P. virginalis* is unlikely to serve as a reservoir for EPNs and that nematodes are unlikely to be important mortality factors for *P. virginalis* in this natural system.

Introduction

Entomopathogenic nematodes (EPNs) are ubiquitous in soil ecosystems where they kill and consume a diversity of soil-dwelling insects (Kaya & Gaugler, 1993; Hominick et al., 1996). They often occur in great abundance in moist soils, and infective juveniles (IJs) are attracted to the waste products or other cues associated with their insect hosts (Schmidt & All, 1979; Lewis et al., 1995; Hui & Webster, 2000). They enter their host's body and release symbiotic bacteria, which kill the host within a few days (Ciche et al., 2006). The nematodes then feed on the bacteria and host tissues, reach maturity, mate, and produce the next generations of IJs. Because EPNs are capable of killing soil insects, many of which are considered important agricultural pests, there has been great interest in using them to provide biological control in agriculture (Gaugler et al., 1997; Fenton et al., 2001). However, biocontrol attempts have met with mixed success and workers have concluded that pathogenicity is highly context dependent (Georgis et al., 2006); laboratory results often fail to translate into similar effects in the field (Gaugler et al., 1997). Far less is

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known about the efficacy of EPNs in natural systems, and they have rarely been considered in attempts to understand the population dynamics of insects that are not of economic importance.

In 1975, Bedding & Akhurst reported a sampling technique to screen sites for EPNs that relied on exposing waxworm larvae [*Galleria mellonella* L. (Lepidoptera: Pyralidae)] to soil samples. In nature, waxworms are obligate parasites of bee hives but they are highly susceptible to EPNs and other parasites that they do not encounter in their protected natural environment. Waxworms have become the standard assay for EPNs such that we now know which nematodes will attack waxworms in a variety of agricultural and some native habitats worldwide. However, we know very little about the actual relationships between EPNs and their natural insect hosts (Lewis et al., 2006). Even when EPNs are extracted from soil samples, their host associations are rarely determined (Kaya & Gaugler, 1993).

One of the few natural systems for which information about EPNs and their native hosts is available involves ghost moth caterpillars [*Hepialus californicus* Boisduval (Lepidoptera: Hepialidae)] at the Bodega Marine Reserve (Strong et al., 1996; Ram et al., 2008a). Young ghost moth caterpillars feed on the exterior of roots of bush lupine [*Lupinus arboreus* Sims (Fabaceae)] and eventually bore into the roots and stems of their lupine hosts. Based on estimates using waxworm traps, EPNs [*Steinernema feltiae* (Filipjev) (Rhabditida: Steinernematidae) and *Heterorhabditis marelatus* Liu & Berry (Rhabditida: Heterorhabditidae)] have occurred historically at all sites sampled on the Reserve (Gruner et al., 2009); at some sites, the incidence of nematodes rarely dropped below 50% over a 13-year period (Ram et al., 2008b). Entomopathogenic nematodes reduce the densities of ghost moths and their damage, increasing the growth, survival, and seed set of lupine bushes (Strong et al., 1996; Preisser, 2003).

Lupinus arboreus is the dominant plant in grassland habitats at the Bodega Marine Reserve, and bushes are attacked by many different herbivores (Strong et al., 1995; Maron, 1998) including several that spend part of their life cycles in or on the soil. Early instars of *Platyprepia virginalis* (Boisduval) (Lepidoptera: Arctiidae) feed on both lupine litter and living leaves from June to March (English-Loeb et al., 1993; Karban & English-Loeb, 1997). During this time, they live in the litter which covers the soil in most places beneath lupine bushes. Later instars become more mobile and more polyphagous, feeding on the leaves of many forb species including lupine (Karban et al., 2010). Some caterpillars can be found at the study site in every year, although abundances varied between 0.004 ± 0.004 and 2.72 ± 0.43 caterpillars m^{-2} of lupine in annual surveys taken every March since 1986 (Karban & de Valpine, 2010). Attempts to understand the dynamics of this population have met with limited success as the usual candidates – food availability, unfavorable weather, and attacks by above-ground natural enemies – have all had relatively small explanatory power (R Karban, unpubl.). This suggests that factors that have not yet been considered may be more important. As caterpillars spend much of their early development (instars 1–4) in proximity with the soil, we asked whether they were susceptible to EPNs and whether these parasites might be important sources of natural mortality for *P. virginalis* in the field.

We conducted laboratory and field experiments to answer three specific questions about a native insect and associated nematodes: (1) can EPNs infect and kill *P. virginalis* caterpillars under laboratory conditions, (2) do EPNs typically infect *P. virginalis* in the field, and (3) do EPNs exert significant mortality on *P. virginalis* under natural field conditions?

Materials and methods

Laboratory assays with *Platyprepia virginalis*

We first assessed the potential for EPNs to kill *P. virginalis* caterpillars in the laboratory by exposing caterpillars to soil saturated with high densities of nematodes isolated from the study site on the Bodega Marine Reserve. Plastic deli containers (11 cm diameter, 540 ml; Solo Cup, Highland Park, IL, USA) were stocked with ca. 3 cm of soil collected from the reserve, coarse-sifted, pasteurized, and moistened to 20% H_2O content by mass. We inoculated deli containers with a 1-ml solution containing one of three treatment levels: 1 000 IJs of *H. marelatus*, 1 000 IJs of *S. feltiae*, or a control of distilled water. Thirty-six *P. virginalis* caterpillars, collected on 26 April 2006 as third or fourth instars, were added singly with a few pasteurized lupine leaves to the treated cups for 12 replicates of each treatment. Treatments were maintained at ambient temperature on a laboratory bench for 12 days, and caterpillar survival in the three treatments was compared using an R × C G-test of independence (Sokal & Rohlf, 1969). These high densities of nematodes were used to determine whether EPNs were capable of killing *P. virginalis* caterpillars under the most favorable circumstances; these circumstances could occur in nature as caterpillars and EPNs are very patchy, and *H. californicus* cadavers at the study site can release more than 500 000 IJs (Preisser et al., 2006).

Because high densities of EPNs were capable of killing *P. virginalis* caterpillars, we conducted a second laboratory experiment that exposed caterpillars to a range of densities of an isolate of *S. feltiae* from the field site to evaluate the critical density required for infection. Second instar

caterpillars from a laboratory colony were placed on filter paper disks in sealed 24-well plates which had been inoculated with six densities of IJs: 0, 1, 10, 100, 500, and 1 000. Four wells in each of five plates were assigned to each density treatment. Each of these density treatments was replicated 20 times, except for the 1 000 nematode treatment which had only 14 replicates because of limited numbers of nematodes. Four of the caterpillars escaped from their wells and were not included in the analyses. Caterpillars were kept in an incubator at 25 °C, and rates of mortality were estimated after 3 days. The experiment was terminated after 3 days before starvation became a mortality factor as caterpillars had no food in the well plates. Caterpillar survival after 3 days in the density treatments was compared with an R × C G-test of independence. A sample of dead cadavers was placed in White traps to confirm their infection with EPNs (White, 1927).

Field assay in suitable habitat with *Platyprepia virginalis*

We next conducted a similar assay in the field to determine the frequency of mortality to *P. virginalis* caused by EPNs under natural conditions. We haphazardly selected 25 mature lupine bushes on Mussel Point in the Bodega Marine Reserve. This site supports *P. virginalis* caterpillars and has also had a consistently high prevalence of EPNs over the past 15 years (Ram et al., 2008b, DR Strong pers. comm.). Deli containers were modified with fiberglass window screen on the bottom to allow contact and exchange with the soil. Deli containers were filled with either 3 cm of soil or lupine litter from beneath each of 25 bushes and placed under the canopy, one container beneath each bush. The litter and top 1 cm of soil were removed so that the screen bottom of each container was in direct contact with moist soil. One third instar was placed in each container on 4 February 2009. All caterpillars were supplied with a few small branches of bush lupine for food. Our initial intent was to have two treatments – one with only soil which caterpillars would contact and one with litter above the soil. However, this proved difficult, because by adding lupine branches for food, all containers effectively had litter. Caterpillars were retrieved from the field on 11 February and brought into the laboratory in 30-ml plastic cups supplied with lupine foliage. Caterpillars were examined daily for mortality until 23 February. We recorded air temperature and precipitation during the time that caterpillars were out in the field.

Field assays with *Platyprepia virginalis* in sites with entomopathogenic nematodes

Our initial assay with *P. virginalis* showed no evidence that EPNs kill these caterpillars under field conditions. This result could have been caused either by a lack of EPNs, by unfavorable local conditions for infection, by a low preference for *P. virginalis* by EPNs, or because of a behavioral refuge. We conducted additional field assays to assess these possibilities. We first evaluated sites for the presence of EPN IJs by placing out sentinel waxworm caterpillars. Only those specific locations where waxworms were killed by EPNs were used to challenge *P. virginalis* caterpillars. We haphazardly selected 100 sites beneath mature lupine bushes at the top of the hill at Mussel Point and placed two waxworm caterpillars in 15-ml plastic centrifuge tubes with holes drilled in the base of the tubes following methods described by Gruner et al. (2007). The base of each tube was pushed into the soil so that the holes through which nematodes could enter were positioned just below the soil surface. Soil was placed in the bottom of each tube up to holes in the tubes such that the soil in the bottom of the tube was in contact with the top cm of soil in the ground. Both the centrifuge tubes and the deli containers provided the same moist soil contact through which EPNs could move. Tubes with waxworms were placed out on 19 March 2009 and retrieved on 26 March.

Specific field locations (n = 40) verified for the presence of nematodes with waxworm baits were marked with pin flags and used for two consecutive trials with *P. virginalis* caterpillars. On 3 and 10 April, 40 *P. virginalis* caterpillars were deployed at these sites (one caterpillar per site) in deli containers with screen bottoms providing soil contact. As in the previous experiment, litter and the top 1 cm of soil were cleared so that the screen could contact moist soil. Caterpillars were placed on soil in the deli containers and also supplied with lupine leaves for food. After 7 days, *P. virginalis* caterpillars were retrieved from the field and reared in the laboratory in 30-ml containers. We compared the frequency of mortality for waxworm and *P. virginalis* caterpillars using a G-test (Sokal & Rohlf, 1969).

We conducted another field experiment in early winter to compare the susceptibilities of *P. virginalis* caterpillars (second or third instars) and waxworms in deli containers with screen bottoms. Each container was filled with 1 cm of soil and lupine foliage. The deli containers were again placed in the field in November 2010 at 28 locations that had shown positive results for nematode infections during the previous spring. One *P. virginalis* caterpillar and one waxworm were placed in each container (n = 28) on 8 November and surveyed again on 20 November. We compared the frequency of mortality for waxworm and *P. virginalis* caterpillars using a G-test.

The cadavers of caterpillars (waxworms and *P. virginalis*) that died during the course of the experiments were placed on White traps at 25 °C, and infectious agents were allowed to develop and reproduce (White, 1927). To determine whether the nematodes that emerged from

caterpillar cadavers were actually entomopathogenic and not saprophytic, the nematodes that emerged from the cadavers were exposed to healthy waxworms, following Koch's postulates. We exposed eight healthy waxworms to ca. 25 nematodes that were collected from each of the suspected infections in a sealed 24-well plate. After 6 days, we recorded whether those nematodes killed the waxworm. We identified nematode isolates by comparing restriction fragment length polymorphisms (RFLP) of the ribosomal DNA's internal spacer region with the profiles of known isolates and restriction digest patterns generated from GenBank sequences. Variations on this method have been used to successfully identify both steinernematid (Reid et al., 1997) and heterorhabditid nematodes (Stack et al., 2000). We used universal 18S and 28S primers (no. 93, 5′ TTGAACCGGGTAAAAGTCG and no. 94, 5′ TTAGTTT CTTTTCCTCCGCT) designed by Nadler et al. (2000) and the restriction enzymes *Sau3*AI, *Alu*I, and *Dde*I (for more detailed protocols see Hodson, 2010).

We attempted to gain more insight into the difference in susceptibility to soil-dwelling nematodes exhibited by waxworm and *P. virginalis* caterpillars. We placed one waxworm and one *P. virginalis* caterpillar (third instar) in each of 30 deli containers in the field in November 2010. The containers contained 1 cm of lupine litter on top of 2 cm of soil. After 24 h, we recorded whether the caterpillars were found on top of the litter, on top of the soil surface, or down below the soil surface. The distributions of the two caterpillar species in these three habitats was compared using G-tests. We repeated these experiments in February 2011 and recorded the habitat choices of one waxworm and one *P. virginalis* caterpillar in 24 deli containers after 12 h (at 22:00 hours, 'Night') and 26 h (at 12:00 hours, 'Day').

Results

Laboratory assays with *Platyprepia virginalis*

Platyprepia virginalis caterpillars were susceptible to EPNs when placed in containers in the laboratory that contained high densities of IJs of *H. marelatus* or *S. feltiae*. Rates of mortality were higher for caterpillars exposed to these nematodes compared to controls that were exposed to distilled water only, under otherwise similar laboratory conditions ($G = 13.17$, d.f. = 2, $P<0.002$). Only five of 12 caterpillars exposed to 1 000 *H. marelatus* IJs survived, compared to eight of 12 caterpillars exposed to 1 000 *S. feltiae* IJs, and 12 of 12 exposed to water controls.

This result indicated that saturation densities of two locally isolated nematode species (*S. feltiae* and *H. marelatus*) can kill *P. virginalis*. An experiment that tested the pathogenicity over a range of more commonly encountered densities of IJs of locally isolated *S. feltiae* revealed that increasing numbers of IJs increased rates of mortality (Table 1; $G = 39.97$, d.f. = 5, $P<0.001$). Exposure to ca. 100 IJs was sufficient to kill half of the caterpillars. Infective juveniles that emerged from dead insects on White traps confirmed that they were killed by EPNs.

Table 1 Fates of *Platyprepia virginalis* caterpillars exposed to different doses of infective juvenile nematodes (*Steinernema feltiae*) in a laboratory assay

	Control	1	10	100	500	1 000
Died	1	0	3	9	12	6
Survived	19	20	17	10	5	8
Total	20	20	20	19	17	14

Field assay in suitable habitat with *Platyprepia virginalis*

All 25 *P. virginalis* caterpillars remained alive after being placed under lupine bushes selected haphazardly at our field site. We observed no mortality or signs of poor condition during the 19 days of this experiment.

Field assays with *Platyprepia virginalis* in sites with entomopathogenic nematodes

We recorded one or more dead waxworm at 43 of 100 sites where we placed sentinel waxworms. At 40 of the 43 sites, we had evidence for EPN infection and nematodes were recovered in White traps from 58% of the waxworms. We conducted two trials involving *P. virginalis* caterpillars at these sites where waxworms had died and EPNs had been recovered. Of these 40 *P. virginalis* caterpillars, three (7.5%) died during the experiment although none (0 of the 3) produced nematodes in White traps or showed visible signs of infection when dissected.

In the following season, 28 *P. virginalis* caterpillars were placed in deli containers along with waxworms at the same locations that had shown positive results for nematode infection. Two *P. virginalis* caterpillars (7%) and 23 waxworms (93%) died during this 12-day trial. Based on the color of the waxworm cadavers, we suspect that many of the deaths were caused by *S. feltiae*. Waxworms were more likely to die during both trials of the experiment than were *P. virginalis* caterpillars [$G = 19.31$ (2009) and 49.18 (2010), both d.f. = 1, $P<0.001$).

Each cadaver was individually placed in a White trap, and 58% of the waxworm cadavers produced nematodes although none of *P. virginalis* caterpillars yielded nematodes. Nematodes collected in the White traps from waxworm cadavers were confirmed to be entomopathogenic as they killed the healthy waxworms in all but one case. All recovered nematodes were identified as either *S. feltiae* or

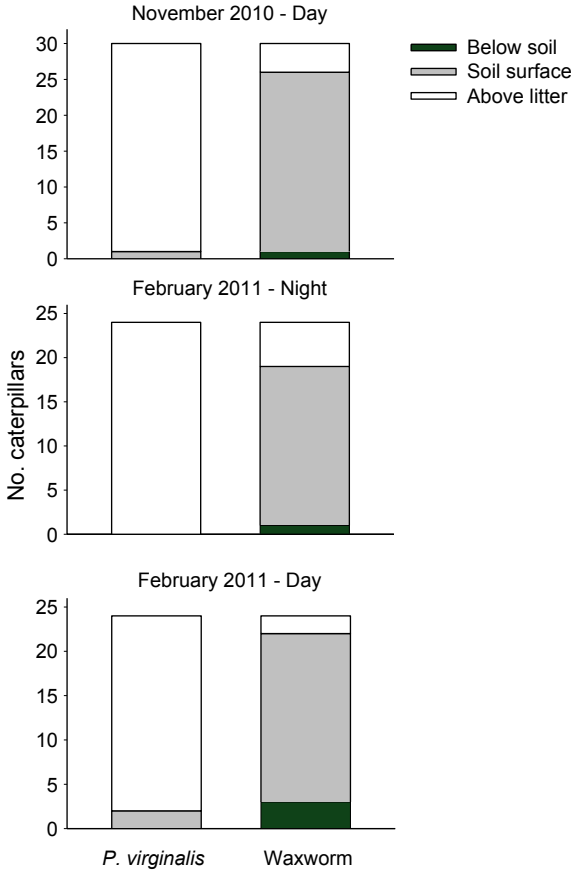

Figure 1 Microhabitats chosen by *Platyprepia virginalis* and waxworm caterpillars (n = 30). *Platyprepia virginalis* caterpillars remained above the litter layer, whereas waxworms penetrated down to contact the soil.

H. marelatus using RFLP. In summary, nematodes were likely to have killed many of the waxworm caterpillars in the field assay although we found no evidence that nematodes killed the dead *P. virginalis* caterpillars.

Although both waxworm and *P. virginalis* caterpillars were associated with the soil environment in our experimental arenas (containers), they chose different microhabitats ($G = 50.32$, d.f. = 2, $P<0.001$; Figure 1, top). *Platyprepia virginalis* caterpillars remained above the soil surface, in or on top of the litter layer. Most waxworms penetrated down through the litter and remained on or below the soil surface. Waxworms were in much more contact with the soil and soil-inhabiting microbes than were *P. virginalis* caterpillars. We found similar results when the experiments were repeated during both day and night [$G = 39.6$ (night) and 37.8 (day), both d.f. = 2, $P<0.001$; Figure 1, middle and bottom, respectively).

Discussion

Waxworms were highly susceptible to EPNs at our study site, Mussel Point. At the study site, EPNs are an important source of mortality for another native caterpillar, *H. californicus* (Strong et al., 1996; Ram et al., 2008a). In contrast, *P. virginalis* caterpillars (instars 2–4) were killed when placed in close proximity with moderate to high densities of EPNs in the laboratory, but showed no evidence of mortality caused by EPNs under more natural field conditions when given the opportunity to escape in the litter. These results have several potential explanations and consequences.

It is difficult to conclude from any negative results that a particular phenomenon does not occur. In this case, our negative field results potentially could arise from low statistical power (Cohen, 1988). However, the fact that waxworms were killed by EPNs at the exact same field locations and containers makes it very unlikely that *P. virginalis* caterpillars are as susceptible to endemic EPNs as are waxworms. We have conducted larger field experiments throughout the year at our study site in which *P. virginalis* caterpillars were placed in deli containers with lupine litter and soil contact (R Karban, unpubl.). Although these experiments were not designed specifically to test for effects of EPNs, we have never observed significant mortality of *P. virginalis* caterpillars in the field when given the option to stay above the soil.

The variability in susceptibility of *P. virginalis* in different environments and situations (laboratory or field, refuge or not) suggests that pathogenicity of EPNs is dependent on the environmental conditions experienced by these organisms. This inference supports the findings and conclusions of many scientists who have attempted to use EPNs for biological control of agricultural pests and come to similar conclusions (Gaugler et al., 1997; Georgis et al., 2006). Investigations at our study site involving hepialid caterpillars, a stem- and root-boring herbivore of *L. arboreus*, have suggested that nematode mobility, survival, and pathogenicity are positively associated with soil moisture (Preisser & Strong, 2004; Preisser et al., 2006). Insufficient soil moisture does not provide a simple explanation for the negative results we obtained for *P. virginalis* caterpillars in field experiments. Rains occurred at the study site during all of our field assays although we cannot completely exclude the possibility that EPNs may be more successful at infecting *P. virginalis* caterpillars during other times or conditions.

Our result, that EPNs killed native *P. virginalis* caterpillars (instars 2–4) in the laboratory but not in the field, has several consequences. First, for EPNs, laboratory assays

should be coupled with appropriate field studies to produce reliable conclusions (Spence et al., 2008). Second, *P. virginalis* may avoid infection behaviorally by reducing contact with moist soil or through morphological (hairs) or immunological features that reduce susceptibility. We found support for at least the first of these mechanisms as *P. virginalis* caterpillars remained in the litter, above the soil surface (Figure 1); this behavior of *P. virginalis* probably put them out of reach of soil-inhabiting nematodes such as *S. feltiae* when it was permitted in the field. Ideally, we would conduct an experiment that separated effects of food from escape from soil-dwelling nematodes. However, this experiment is difficult because lupine simultaneously provides both of these benefits.

We still have a very limited knowledge of the role of EPNs in controlling the populations of native insect species that are not agricultural pests, but which inhabit soil, litter, or cryptic habitats where EPNs are endemic. This study is unusual in examining the relationship between a native insect and the EPNs with which it shares the soil environment (Peters, 1996).

This study was motivated by variation in the annual estimates of population abundance of *P. virginalis* caterpillars that could not be explained by other ecological factors. Populations of *P. virginalis* caterpillars vary by several orders of magnitude in annual surveys at the Bodega Marine Reserve (Karban & de Valpine, 2010). Based on the results of the current study, we conclude that EPNs are unlikely to explain the population dynamics of *P. virginalis* caterpillars in nature, despite the insect's susceptibility to infection in the laboratory and the presence of EPNs at the appropriate time and place in the field.

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