Long-Term Survival of the Entomopathogenic Nematode

Heterorhabditis marelatus

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ABSTRACT Entomopathogenic nematodes are important biological control agents for a variety of soil- and litter-dwelling insect pests. A major drawback to their use against pest species is their low level of persistence in many agricultural systems. While a number of studies have examined the persistence of these biological control agents over periods of days and/or weeks, the longer-term survival of these nematodes has received less attention. We report the results of a year-long field experiment testing the long-term survival of infective juveniles (IJ) of the entomopathogenic nematode Heterorhabditis marelatus (Liu and Berry), under a range of initial densities. We buried mesh-covered tubes containing raw field soil with varying densities of H. marelatus IJs in a coastal prairie containing naturally occurring populations of this nematode and then destructively sampled subsets of the tubes for nematode presence five times over 1 yr. H. marelatus IJs lived a surprisingly long time in the absence of prey; some nematodes from the initial cohort were viable after a year in the field. Survival over the year-long course of the experiment was independent of the starting IJ density, suggesting that H. marelatus does not aggregate at high densities to reduce desiccation risk. Our results highlight the fact that IJs vary greatly in their long-term survival; selecting entomopathogenic nematode isolates for persistence as well as virulence could enhance this biological control agent’s long-term effectiveness in agricultural systems.

KEY WORDS Heterorhabditis marelatus, entomopathogenic nematode, biological control, negative binomial

Entomopathogenic nematodes (EPNs) are widely used biological control agents preying on a range of soil-dwelling arthropods (Gaugler et al. 1997). Their economic importance has inspired many studies of their effectiveness in suppressing insect populations (reviewed in Poinar 1979, Kaya and Gaugler 1993, Gaugler 2002). A central factor in determining the suitability of EPNs as biological control agents is their survival under natural conditions. When applied to soil for suppression of pest species, EPN persistence is generally very low (Baur and Kaya 2001, Wilson et al. 2003), and they often require repeated applications for effective control (Fenton et al. 2001). Understanding what factors affect the persistence of entomopathogenic nematode populations in the field is critical to maximizing their effective use.

The extremely high production of dauer infective juveniles (IJ) from a single EPN-infected host cadaver and their mass emergence on exhaustion of the host cadaver’s resources (Lewis 2002) may lead to density-dependent mortality in cases where only a limited number of hosts are available. Density-dependent mortality has been found across a range of taxa and systems and can substantially affect population dynamics (Murdoch et al. 2003). Mortality of individual IJ nematodes can be positively (per capita mortality increases with increased population density) or negatively (per capita mortality decreases with increased population density) density-dependent; it is also possible that local density does not affect IJ survival (e.g., under anaerobic stress; Qui and Bedding 1999).

There are plausible reasons for expecting either positively and negatively density-dependent survival of EPN populations in the field. Desiccation is a major threat to EPN survival (Grant and Villani 2003), and nematode persistence in biological control efforts can be substantially enhanced by watering the soil both before and after nematode application (Baur and Kaya 2001). IJs of the EPN Steinernema carpocapsae (Weiser) clump together, reducing their surface area (i.e., evaporative water loss) and thus decreasing their desiccation-induced mortality (Simons and Poinar 2005).
Similarly, survival of *S. feltiae* (Filipjev) IJs was positively correlated with clump size (Solomon et al. 1999). If this behavior occurs in response to desiccating conditions, survival should generally increase with IJ density; however, evidence for this behavior as an adaptive response to desiccation is equivocal (Womersley et al. 1998).

Negatively density-dependent survival can occur if nematode natural enemies numerically respond to increases in IJ abundance and increase the per capita rate of predation. Laboratory studies of the nematode-trapping fungus *Arthrobotrys oligospora* (Fresenius) showed that the fungi aggregates outside of *Galleria mellonella* (Linnaeus) cadavers infected by the EPN *H. marelatus*, increasing per capita nematode mortality as IJ density rose (Strong 2002); the fungus responds similarly to *S. feltiae* emerging from infected mole crickets (Fowler and Garcia 1989). Density-dependent parasitism of the plant-parasitic nematode *Heterodera schachtii* (Schmidt) by the fungus *Hirsutella rhossiliensis* (Minter and Brady) occurred under field conditions, but the slow rate of fungal growth largely precluded effective control of nematode populations (Jaffee 1992, 1996). Given the complex, reticulate nature of many underground foodwebs (De Ruiter et al. 1995), however, it is also possible that nematode natural enemies may themselves be suppressed by predation, preventing them from numerically responding to increases in IJ abundance.

We describe the results of a long-term field experiment in which we tested the hypothesis that survival of the entomopathogenic nematode *H. marelatus* is density-dependent. By varying *H. marelatus* density in field conditions while preventing population recycling through the infection of new insect hosts, we isolated the effect of initial density on the long-term survival of this economically important biological control agent.

### Materials and Methods

Our experiment was conducted in the coastal grasslands of the Bodega Marine Reserve (BMR; Bodega Bay, CA, described in detail in Barbour et al. 1973). The entomopathogenic nematode *H. marelatus* (Liu and Berry) is native to this area, and its role in the soil foodweb at BMR has been extensively studied (Jaffee et al. 1996, Strong 1997, Preisser and Strong 2004). The experiment consisted of four nematode density treatments: low density (25 *H. marelatus* IJs/tube; low); high density (2,500 *H. marelatus* IJs/tube; high); a waxworm (*G. mellonella*; cadaver infected with *H. marelatus* (~100,000 *H. marelatus* IJs/tube; cadaver); and a non-nematode control (control). The *H. marelatus* IJs were cultured from waxworms exposed to BMR soil in which nematodes were present. We included the cadaver treatment because a host cadaver may act as a refuge from both desiccation and natural enemies (Koppenhofer et al. 1997). If so, IJ survival within the relatively sheltered microenvironment of a host cadaver should be greater than that of IJs in the surrounding soil.

We assessed nematode survival using 50-ml Falcon plastic centrifuge tubes with 5 mm of the tubes’ tapered bottom removed and covered with plastic mesh (1-mm² mesh size) that was held in place using a rubber O-ring. The mesh ensured gas and moisture exchange between the tube and surrounding soil without allowing potential nematode prey to enter the tube. After the mesh was fastened on each tube, we added 30 g of moist (0.2 ml H₂O/g soil) soil. The soil added to the tubes was gathered from areas on the reserve where *H. marelatus* has never been detected despite extensive sampling (D.R.S. and E.L.P., unpublished data) and was coarsely sieved (4-mm² mesh size) immediately before its addition to the tubes to remove any large debris or potential nematode prey. We did not autoclave or pasteurize the soil because such processes change the soil considerably from its natural condition. We prepared two standardized solutions of distilled water containing nematodes in suspension and applied nematodes to the low- and high-density treatments through the addition of 0.5 ml of the appropriate solution to the experimental tubes. We also added 0.5 ml of distilled water to each tube in the control and cadaver treatments to standardize the amount of water being added. After adding the appropriate treatment to each tube, we sealed the tubes using screw top lids.

We began the experiment on 27 March 2001. After dividing a 4 by 4-m area in the coastal grassland into 16 1-m² blocks, we buried 40 sampling tubes (10 tubes/treatment × 4 treatments) in each of the 16 blocks (640 sampling tubes in total). We used shovels to lift the top 5–10 cm of the grassland soil of each block, placed the tubes haphazardly in the exposed root zone, and replaced the soil immediately to minimize disturbance. On 1 May 2001, we used a random number generator to randomly select 2 of the 16 blocks for removal. After removing all of the tubes from beneath the sod in each of the two blocks, we immediately transported them inside and sampled each for surviving nematodes. We chose this procedure over repeated removal of subsets of tubes from each of the 16 blocks because this disturbance may affect the remaining tubes. We repeated this procedure seven more times, removing the tubes from two more randomly selected blocks on 1 July, 1 August, 1 September, 27 September, 5 November, and 5 December 2001 and 9 April 2002.

We moistened the soil in the collected tubes to ~20% (0.2 ml H₂O/g soil) and added two waxworms (larvae of the wax moth *G. mellonella*) to each tube in each treatment. We recapped each tube after adding the waxworms to keep the soil from desiccating. After 1 wk, we unsealed the tubes and visually assessed each waxworm as mentioned above. Both waxworms in each tube were removed and replaced with two new waxworms; we repeated this procedure twice for a total of six waxworms per tube over a 3-wk period.

Waxworms infected by *H. marelatus* were set aside and chilled to 2–4°C to stop nematode development.
They were placed in a dilute pepsin solution and heated at 40°C for 2 h to facilitate finding and counting nematodes (Kaya and Stock 1997). After the cadaver had been partially digested, we counted the nematodes within each cadaver.

We analyzed nematode survival using as our response variable the total number of nematodes recovered from 10 sampling tubes in each of the blocks per treatment per sampling date. Because the distribution of nematode abundances in the experimental data was skewed, we used a negative binomial model for our analyses. This distribution is appropriate for biological count data because of its ability to accommodate both overdispersed and skewed data (White and Bennetts 1996). The negative binomial distribution is described by:

\[ P(Y = y) = \frac{\left( \frac{r + y - 1}{y} \right) \left( \frac{r}{m + r} \right)^y \left( \frac{m}{m + r} \right)^m}{r(y + 1)} \]

where \( m \) is the mean of the distribution and \( r \) is the dispersion parameter. The variance is given by \( m(1 + \frac{m}{r}) \). We used the SAS statistical software v.8.2 GENMOD procedure to fit a negative binomial distribution to the data. The mean of the distribution was allowed to decay with time because of nematode death, \( m(t) = C^{-kt} \), where \( C \) represents the initial number of nematodes and \( k \) is the daily mortality rate. We assessed the goodness-of-fit using Pearson’s \( \chi^2 \) statistic, and used residual plots to check for outliers and trends in the residuals.

Previous work on entomopathogenic nematodes has suggested that only a fraction of nematodes have the potential to infect hosts (Campbell et al. 1999). In addition, nematode production in host cadavers is highly variable. To address these concerns, we also used the data gathered on nematode survivorship to back-calculate the initial number of nematodes in the tubes. This provides an alternate fit to the model—one derived without any assumptions regarding the initial nematode density.

The fit to the data with a fixed initial number of nematodes was good (Pearson’s \( \chi^2 \) value/df = 0.99), but there was a clear trend to the residuals over time. Fitting the data using a back-calculation of the initial number of nematodes/replicate (fixed intercept) produced a similarly good fit (Pearson’s \( \chi^2 \) value/df = 1.07) but did not display a trend in the value of the residuals. We present results from both the fitted-intercept and fixed-intercept model.

**Results**

Infected juvenile nematodes were recovered from all three of the density treatments (Fig. 1). There was an effect of time and initial density for the fitted-intercept model (time: log-ratio \( \chi^2: 56.5, \text{df} = 1, P < 0.001 \); density: log-ratio \( \chi^2: 56.5, \text{df} = 2, P = 0.002 \)). However, the effect of density did not differ over time; thus, the exponential decay rate of all three treatments over the course of the year was similar, and initial density did not affect population mortality rates (time \times density: log-ratio \( \chi^2: 3.23, \text{df} = 2, P = 0.20 \)).

The daily mortality rate for nematodes in all treatments was \( k = 0.016 \pm 0.0015/\text{d} \) (SE), corresponding to a cohort half-life of \( \sim 43 \text{ d} \). In the fitted-intercept model, the dispersion parameter \( (r) = 1.13 \pm 0.25 \) and the fitted initial number of nematodes \( (C) = 1369 \pm 2.0 \) for the cadaver treatment. 727 \pm 2.0 for the high-density treatment, and 33.9 \pm 0.96 for the low-density treatment.

Because in the fixed-intercept model we do not estimate the initial density, we only consider the effect of time and the effect of the density treatment on the mortality rate (i.e., the time \times density interaction) in the low- and high-density treatments. Although there was a significant effect of time, the density treatment did not affect the mortality rates (time: log-ratio \( \chi^2: 48.73, \text{df} = 1, P < 0.001 \); time \times density: log-ratio \( \chi^2: 1.32, \text{df} = 2, P = 0.074 \)). The daily mortality rates for the low- and high-density treatments were \( k = 0.032 \pm 0.0041/\text{d} \) and \( k = 0.040 \pm 0.0060/\text{d} \), respectively, and the dispersion parameter \( r = 2.54 \pm 0.64 \).

Our three consecutive assays for nematode presence likely caught the vast majority (>99%) of viable IJs present in the sampling tubes. Over the 11 mo of the experiment, we counted a total of 6296 nematodes from 676 infected waxworm cadavers. The number of nematodes found per assay decreased dramatically with each of the three consecutive assays; 3925 of the 6296 nematodes (62% of the total) were caught during the first assay, 1950 (31%) during the second, and 421 (7%) during the third. There was a negative linear relationship between the number of nematodes caught per assay and the number of consecutive assays (no. nematodes/assay = 5603 - 1752 \times \text{assay number}; \( r^2 = 0.995 \)). This formula predicts that adding a fourth consecutive assay would not have affected the total nematode yield.

The tubes used for assessing nematode survival were largely effective in excluding nematode immigration. A single nematode was found in one of the zero-nematode control tubes during the first sampling date; the other 159 control tubes sampled during the course of the experiment tested negative for nematode presence. While we cannot exclude the possibility that some nematode immigration into the tubes occurred during the experiment, the fact that >99% of the control tubes showed no evidence of H. n REUTERSUS presence indicates that immigration is unlikely to have affected our results.

It is probable that the within-tube microenvironment was very similar to that found in the surrounding soil. The tubes were buried under at least 5 cm of grassland soil, and the soil within each tube was in contact with its surroundings through the mesh-covered end. Although we chose not to sample within-tube soil moisture in this experiment because of the risk of accidentally removing viable IJ nematodes, we sampled both within- and outside-tube soil moistures in a subsequent experiment nearly identical to this one (the same coastal grassland, same sampling tubes and experimental design, over the same seasonal range, in
Within- and outside-tube soil moisture differed significantly across dates (repeated-measures analysis of variance \( F_{1,135} = 49.52, P < 0.0001 \)), but there was no tube effect \( F_{1,135} = 1.19, P = 0.292 \) and no significant date × tube interaction \( F_{1,135} = 0.49, P = 0.481 \). (August 2002: within tubes \([n = 40], 0.84 ± 0.15\); outside tubes \([n = 12], 1.26 ± 0.19\); October 2002: within tubes, 1.64 ± 0.11; outside tubes, 1.19 ± 0.12; February 2003: within tubes, 7.38 ± 0.41; outside tubes, 7.64 ± 0.44). It is thus unlikely that the within-tube soil microenvironment in this experiment differed substantially from its surroundings.

**Discussion**

Local density did not affect the mortality rate of IJ *H. marelatus* nematodes over the year-long course of the experiment (Fig. 1). This may seem surprising in light of the desiccation-related decrease in per capita mortality associated with increased IJ clump size (Simons and Poinar 1973, Solomon et al. 1999) and the adaptive advantage that such a behavioral clumping response might provide. Several species of plant-parasitic and mycophagous nematodes aggregate in response to environmental stress (Womersley et al. 1998). This behavior creates a protective eggshell when the nematodes in the outer layers of the clump die and create a barrier around the aggregation, decreasing the per capita mortality rates of the remaining nematodes (Ellenby 1969). Such behavioral responses are rare, however, even among nematodes feeding on relatively abundant resources such as plants or fungi (Womersley et al. 1998). In addition, such aggregations should require a high threshold nematode density for their protective effects. Because IJs must disperse from host cadavers in search of new prey, such clumping behavior would likely reduce the rate of new EPN infections and decrease the probability of long-term population persistence (Dugaw, Preisser, Hastings, and Strong, unpublished data).

An unexpected result of our analyses was that, as nematode density increased from the low to high to cadaver treatments, the starting density estimates predicted in the fitted model using data from the five sampling dates represent a smaller and smaller fraction of the actual densities. The ratio of estimated to actual starting densities was 1.32 in the low-density treatment (25 IJs/tube), 0.29 in the high-density treatment (1,369 nematodes/replicate), and 0.014 in the cadaver treatment (34 nematodes/replicate).
However, recent research has shown that, although density in the high-density and cadaver treatments increased over the course of the experiment (Wilkinson et al. 2001), the density of predatory mites and collembolans increased over the course of the experiment (Wilkinson et al. 2001). While the nylon mesh precluded the immigration of invertebrate predators, it should have had minimal impact on the naturally occurring suite of nematode-trapping fungi or other microinvertebrate predators. A. oligospora, the most abundant nematode-trapping fungus at BMR (Jaffee et al. 1996), often coats host cadavers in the field; under laboratory conditions, such aggregation on the outside of nematode-infected cadavers leads to high IJ mortality (Strong 2002). In a field experiment testing the persistence of Heterorhabditis bacteriophora (Poinar) IJs, survival was lower in areas where the density of predatory mites and collembolans increased over the course of the experiment (Wilson and Gaugler 2004). Similar processes occurring within our tubes could have caused high initial mortality, lowering our estimates of starting nematode density in the high-density and cadaver treatments. However, recent research has shown, although A. oligospora and other nematode-trapping fungi at BMR increase greatly in abundance in response to high IJ densities, they were largely ineffective at trapping large numbers of nematodes: even the most lethal fungi only reduced nematode densities by 30% (Jaffee and Strong 2005). This relatively weak top-down effect implies that predatory fungi are unlikely to be responsible for our results.

A more likely explanation for this inconsistency involves declining soil moisture levels. Before adding the nematodes to the experimental tubes, we moistened the soil in the tubes to ≈20% by weight (0.2 ml H₂O/g soil). The soil in the tubes was thus wetter than the surrounding field soil, and the moisture levels in the tubes likely decreased to ambient levels over a period of several days. Heterorhabditid nematodes are extremely susceptible to desiccation (Grant and Villani 2003), and they retreat to the core of moist soil aggregates in response to dry conditions (D.R.S. and E.L.P., unpublished data). Even if the number and capacity of such refuges is limited, IJs in the low-density treatment will have little trouble finding suitable areas. Under high-density conditions, however, most IJs will find the refuges already fully occupied and perish. This would explain both the inconsistency between our actual and estimated starting densities and the long-term density independent mortality we observed over the course of experiment: once the within-tube soil moisture had equilibrated with the surrounding soil, the mortality risk was independent of density.

Our results add to a growing body of work on EPN mortality under natural conditions (Strong 2002, Perez et al. 2003, Shapiro-Ilan et al. 2003, Wilson and Gaugler 2004). Our half-life estimate of 34 d is considerably above the mean Heterorhabditid half-life of 34 d calculated by Strong (2002) using data from Baur and Kaya (2001); however, we know of no other long-term studies testing nematode survival under natural conditions in the absence of potential hosts. In a larger sense, the transitory density-dependence we observed challenges the assumption of constant mortality underpinning the idea of a cohort half-life. Even in situations with a low mean IJ survival, we found that a small fraction of nematodes can survive for long periods of time. In our experiment, some IJs were able to infect hosts after nearly a year in the soil; these long-lived individuals may substantially increase the chance of population persistence across seasons and years (Dugaw, Preisser, Hastings, and Strong, unpublished data). Further long-term experiments under field conditions are necessary to determine whether the existence of such long-lived individuals is a general feature of EPN populations. If they are, the success of control efforts using EPNs may be enhanced by including isolates selected for survival as well as virulence.

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