# Susceptibility of *Aethina tumida* (Coleoptera: Nitidulidae) Larvae and Pupae to Entomopathogenic Nematodes

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**ABSTRACT** In this study, we evaluated the potential use of entomopathogenic nematodes as a control for the beetle *Aethina tumida* Murray (Coleoptera: Nitidulidae). In particular, we conducted 1) four screening bioassays to determine nematode (seven species, 10 total strains tested) and application level effects on *A. tumida* larvae and pupae, 2) a generational persistence bioassay to determine whether single inoculations with nematodes would control multiple generations of *A. tumida* larvae in treated soil, and 3) a field bioassay to determine whether the nematodes would remain efficacious in the field. In the screening bioassays, nematode efficacy varied significantly by tested nematode and the infective juvenile (IJ) level at which they were applied. Although nematode virulence was moderate in screening bioassays 1–3 (0–68% *A. tumida* mortality), *A. tumida* mortality approached higher levels in screening bioassay 4 (nearly 100% after 39 d) that suggest suitable applicability of some of the test nematodes as field controls for *A. tumida.* In the generational persistence bioassay, *Steinernema riobrave* Cabanillas, Poinar & Raulston 7-12 strain and *Heterorhabditis indica* Poinar, Karunaka & David provided adequate *A. tumida* control for 19 wk after a single soil inoculation (76–94% mortality in *A. tumida* pupae). In the field bioassay, the same two nematode species also showed high virulence toward pupating *A. tumida* (88–100%) mortality. Our data suggest that nematode use may be an integral component of an integrated pest management scheme aimed at reducing *A. tumida* populations in bee colonies to tolerable levels.

**KEY WORDS** *Apis mellifera*, *Aethina tumida*, entomopathogenic nematodes, *Steinernema* spp., *Heterorhabditis* spp.

In its native range of sub-Saharan Africa, the beetle *Aethina tumida* Murray (Coleoptera: Nitidulidae) is an occasional pest in honey bee, *Apis mellifera* L. (Hymenoptera: Apidae), colonies (Lundie 1940, Neumann and Elzen 2004, Ellis and Hepburn 2006). Since 1996, *A. tumida* has been found and become established in North America and Australia where it can cause significant colony losses to beekeepers (Neumann and Ellis 2008). *A. tumida* females oviposit inside host colonies, after which the emerging larvae feed on bee brood, food stores and dead bees in the bee nest (Lundie 1940). When the larvae reach the postfeeding wandering phase (Lundie 1940), they leave the hive to find suitable soil in which to pupate (Lundie 1940, Pettis and Shimanuki 2000).

Beekeepers traditionally have used insecticides containing permethrin to control *A. tumida* in the soil (Hood 2004). This treatment regime bears the risks of pest resistance (Hemingway and Ranson 2000) and undesirable side effects on honey bees, other insects (Hassan et al. 1983), and humans (WHO 1990). Therefore, an alternative, sustainable control such as the use of entomopathogenic nematodes is desirable. Precedent exists for the control of other coleopteran pests using entomopathogenic nematodes (Georgis and Manweiler 1994, Martin 1997). Moreover, the infectivity of entomopathogenic nematodes has been tested against nitidulids of the genus *Carpophilus* (Vega et al. 1994, Glazer et al. 1999) and against *A. tumida* (Cabanillas and Elzen 2006). Regarding *A. tumida*, the infectivity of three species of nematodes toward wandering *A. tumida* larvae was shown to be moderate.

Advancing from the findings of Cabanillas and Elzen (2006), we evaluated the potential use of entomopathogenic nematodes as an alternative control for *A. tumida* both in the laboratory and in field studies. In particular, we conducted four screening bioassays to determine which nematode species and application

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level most affected larval and pupal *A. tumida*s. We hypothesized that higher concentrations of nematode species from the genus *Heterorhabditis* would be the most efficacious because of their documented efficacy against Coleoptera in general (Glazer et al. 1999).

We also conducted a generational persistence assay to determine whether single soil inoculations of nematodes would control subsequent migrations of *A. tumida* larvae into treated soil. Expecting some efficacy of nematodes in light of previous data on nitidulids in general (Vega et al. 1994, Glazer et al. 1999) and on *A. tumida* in particular (Cabanillas and Elzen 2006), we hypothesized that *A. tumida* larvae and pupae would be controlled by the nematodes with *A. tumida* mortality decreasing over subsequent generations.

Finally, we conducted a field bioassay to determine whether the tested nematodes remain efficacious in the field. Within this bioassay, we varied soil moisture and test site (forested or unshaded field) expecting these environmental parameters to affect nematode virulence (Grant and Villani 2003, Koppenhöffer and Fuzy 2007, Shapiro-Ilan et al. 2007). Using these routine bioassays, we chose to test nematode strains that are commercially available already and known to infect other Coleoptera in an effort to provide beekeepers with a nonpesticidal management tool against *A. tumida* population increases in apiaries.

## Materials and Methods

We used wandering *A. tumida* larvae in all investigations. For screening bioassays 1–3, we reared the *A. tumida* larvae according to Mürrle and Neumann (2004). For screening bioassay 4, the generational persistence bioassay, and the field bioassay, we reared *A. tumida* on an artificial diet composed of honey/pollen/Brood Builder (Brood Builder, Dadant and Sons, Inc., Hamilton, IL) (1:1:2). We initiated all rearing programs using adult *A. tumida* collected from honey bee field colonies at the locations where the bioassays were conducted.

**Screening Bioassays.** We conducted four screening bioassays to determine which nematode species warranted further investigation in the generational persistence and field bioassays. The first bioassay was conducted at the Department of Entomology and Nematology, University of Florida (UF), Gainesville, FL, in 2004 by using the following nematode species provided by UF: *Heterorhabditis bacteriophora* Poinar (HP88 strain), *Steinernema riobrave* Cabanillas, Poinar & Raulston (RIO strain), and *H. zealandica* Poinar (ENYZ Florida strain). The experimental nematodes were reared in wax moth, *Galleria mellonella* L., larvae according to routine protocols (Kaya and Stock 1997). The wax moth larvae were in culture at UF.

Sand bioassays were preformed on wandering *A. tumida* larvae 4 d after harvesting the infective juveniles (IJs) as described by Glazer and Lewis (2000). Before the experiments, the viability of the IJs was assessed using a dissecting microscope to determine the number of living nematodes per 1 ml of suspension. We applied concentrations of 0 (control), 5, 10, 20, 40, and 80 IJs/$cm^2$ on 30 g of sterilized sand [28.3 $cm^3$] in petri dishes (60 by 15 mm, Thermo Fisher Scientific, Waltham, MA). These concentrations equaled 0, 28, 56, 112, 224, and 448 IJs/*A. tumida* larva, similar to numbers used by Vega et al. (1994) against *Carpophilus hemipterus* L.. We replicated each species and concentration five times (three nematode species × five concentrations × five replicate petri dishes).

Water was added to the dishes to obtain soil moisture of ≈8% water per weight (wt:wt, Glazer and Lewis 2000). After placing five wandering *A. tumida* larvae onto the sand of each petri dish, we sealed the dishes with Pharmaseal and stored them upside-down in a climate room in total darkness at ≈25°C. Five days later, we counted the number of dead *A. tumida* larvae and dissected them in a 1% NaCl solution to confirm nematode infestations.

The second screening bioassay was conducted at Rhodes University in Grahamstown, South Africa, in 2005. Andermatt Biocontrol AG (Grossdietwil, Switzerland) provided Dickmaulrüssler Nematoden with IJs of *H. megidis* Poinar, Jackson & Klein. This product is applied against the black vine weevil, *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae). We conducted this bioassay similarly to the first bioassay, but here we used a different soil and also tested the product against 7-d-old pupae ($N$ = 5 petri dishes per concentration) reared in autoclaved soil.

The third screening bioassay was performed at the USDA Bee Research Laboratory in Beltsville, MD, in 2005. There, we tested TERRANEM (Koppert Biological Systems, Romulus, MI), which contains IJs of *H. bacteriophora.* This bioassay was conducted similar to that of the first bioassay, but we used a regional soil and all IJ concentrations were tested at 20°C as well as at 25°C to detect possible temperature effects at this narrow range of temperatures.

We conducted the fourth screening bioassay at Southeastern Insectaries, Inc. (Perry, GA) from March to April 2005. This bioassay was composed of three separate parts. In part 1, the following six nematode strains (representing five species) were tested in a study lasting 6 d: *S. feltiae* Wouts, Mracek, Gerdin & Bedding; *S. carpocapsae* Wouts, Mracek, Gerdin & Bedding (Agriotos strain), *S. riobrave* (7-12 strain), *H. indica* Poinar, Karunaka & David, and *H. bacteriophora* (Oswego and Hb). The experimental nematodes were reared in *Tenebrio molitor* L. (Coleoptera: Tenebrionidae; reared by Southeastern Insectaries, Inc.) larvae according to routine procedures described previously (Shapiro-Ilan et al. 2002). We put *A. tumida* larvae into petri dishes having Whitman filter paper in the lid and inoculated with one of three aqueous IJ per larva levels (200, 400, and 600 IJs per larva) for each of the six nematodes ($N$ = 10 larvae per petri dish × 10 dishes × three IJ levels × six nematodes). A level of distilled water necessary to bring the total amount of liquid solution added to each petri dish to 1.5 ml was added. All petri dishes were placed lid- and filter paper-side-down in an incubator at 25°C and no light. Six days later, we examined the petri dishes and

counted the number of dead *A. tumida* larvae in each dish.

We established four control groups. These groups consisted of 10 wandering *A. tumida* larvae × 10 petri dishes for each of the following control groups: petri dish with 1) nothing added, 2) filter paper, 3) 1.5 ml of distilled water, and 4) filter paper + 1.5 ml of distilled water. All control and treatment petri dishes were placed lid- and filter paper-side-down in an incubator at 25°C and no light. Six days later, we examined the petri dishes and quantified the number of dead *A. tumida* larvae in each dish.

Part 2 of this screening bioassay was similar to part 1, with the following exceptions: 1) we added a level of distilled water necessary to bring the total amount of liquid solution added to each petri dish (nematode and control) to 2.0 ml rather than 1.5 ml as in the part 1; 2) the filter paper was placed in the bottom of the dish rather than in the lid; and 3) the duration of this study was 9 d rather than 6 d as in part 1.

Based on our results from parts 1 and 2 of the fourth screening bioassay, we chose *H. indica*, *S. riobrave* (7-12 strain), and *H. bacteriophora* (Oswego strain) for continued investigation in part three of this screening bioassay. Part three was conducted similarly to parts one and two with the following exceptions: 1) a fourth IJ level (800 IJs per *A. tumida* larva) was included; 2) the duration of the study was 39 d (by that time, most treated larvae were dead); and 3) the total amount of liquid solution added to each petri dish was 1.5 ml as in part 1. During this time, we determined larval mortality on 14 different days to calculate total larval mortality over time.

**Generational Persistence Bioassay.** The generational persistence assay was conducted at the University of Georgia's (UGA) Honey Bee Research Laboratory in Watkinsville, GA, from September 2005 to January 2006. We conducted the bioassay in vitro to determine whether single soil inoculations with nematodes would provide continued control of subsequent generations of *A. tumida* larvae and pupae. Based on the results of our screening bioassays, we decided to test only *H. indica* and *S. riobrave* 7-12 in the generational persistence assay. Furthermore, we tested two different methods of soil inoculation (aqueous solution and the use of infected *Tenebrio molitor* cadavers) in 118-ml plastic cups (Thermo Fisher Scientific) of soil to determine the most efficacious method of inoculating the soil with nematodes for *A. tumida* control. The soil was collected from fields surrounding the UGA Honey Bee Research Laboratory and was moistened to 10% (wt:wt) before its use. The cadavers were produced using standard methods described perviously (Kaya and Stock 1997).

In this bioassay, three types of inoculums were created for both species of nematode: an aqueous application of 25,000 IJs, an aqueous application of 50,000 IJs, and a mealworm cadaver containing ≈25,000 IJs ($N = 20$ soil cups per inoculum and nematode species). Water ($N = 40$ cups) and uninfected mealworm cadavers ($N = 20$ cups) were used as controls. In instances where infected cadavers were used, the cadavers were buried ≈0.5 cm below the soil surface.

Five wandering *A. tumida* larvae were put in each of the soil cups 2 d before soil inoculation. They burrowed into the soil and constructed pupation chambers during this time (Lundie 1940, Schmolke 1974). We added 5 ml of water every 3–4 d to each soil cup as needed to maintain adequate soil moisture.

After the introduction of the first round of *A. tumida* larvae, five more larvae were added every 7 d over 15 subsequent weeks. We chose to add the larvae every 7 d because the tested nematode species are known to penetrate the host, reproduce, and their offspring exit the host in ≈14 d (Shapiro-Ilan et al. 2002). Therefore, our design permitted us to determine if the nematodes from the initial inoculation infected and reproduced in larval *A. tumida* subsequently introduced into the soil. We collected and quantified the adult *A. tumida* beginning to emerge the third week after the initial introduction of *A. tumida* larvae. This was continued through the 19th week of the study when all *A. tumida* from the 15th larval introduction had finished pupating.

**Field Bioassay.** The field bioassay was conducted at the UGA Honey Bee Research Laboratory from September to October 2005. We tested *H. indica* and *S. riobrave* 7-12 and three inoculation types to create the same eight treatment combinations used in the generational persistence assay ($N = 80$ soil cups for aqueous control and $N = 40$ soil cups for all other treatments).

Because soil moisture and colony location may affect nematode viability in the field, we wanted to determine whether our nematode applications worked better in a field versus forested setting (location) present at the UGA Bee Lab or periodically wetted versus natural rainfall (soil moisture) situation. To accomplish this, each treatment was divided into four equal groups ($N = 20$ soil cups for aqueous control and $N = 10$ soil cups for all other treatments) with each group going to one of the following combinations: 1) forested × natural rainfall, 2) forested × periodically wetted, 3) field × natural rainfall, and 4) field × periodically wetted. The field location was an ≈1.5-ha field with no trees (partially shaded from 15:00 onward), whereas the forested location was an ≈1.5-ha forest with mixed *Pinus* spp., *Quercas* spp., and *Liquidambar* spp. trees.

We used 118-ml plastic soil cups in this study. All cups were buried in the ground, up to the 118-ml mark on the cup, and grouped according to treatment. The leftover soil from the holes where the cups were buried was put into the cups for use during the study. We cut an ≈4.5-cm-diameter hole in the lids of all cups and glued screen wire (>8 mesh per cm) to the lids to allow natural light and rainfall into the cups while preventing escape of adult *A. tumida.*

Five wandering *A. tumida* larvae were put into each soil cup 2 d before soil inoculation as in the generational persistence bioassay. After this delay, all soil cups were inoculated as in the persistence bioassay. We added 5 ml of water to each "wet" soil cup every

**Table 1. Mortality of small hive beetle larvae and pupae exposed to various nematode species (screening bioassays 1–3)**

| Density (IJs/cm$^2$) | Screening bioassay 1 | | | Screening bioassay 2 | | Screening bioassay 3 | |
|---|---|---|---|---|---|---|---|
| | *S. riobrave* (RIO) 25°C Dead larvae | *H. zealandica* (ENYZ FL) 25°C Dead larvae | *H. bacteriophora* (HP88) 25°C Dead larvae | *H. megidis* 25°C Dead larvae | *H. megidis* 25°C Dead pupae | *H. bacteriophora* (Koppert) 20°C Dead larvae | *H. bacteriophora* (Koppert) 25°C Dead larvae |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | 0 | 0.6 ± 0.4 | 0.4 ± 0.2 | 0.6 ± 0.2 | 0.4 ± 0.2 | 0 | 0 |
| 10 | 2.2 ± 0.4 | 0.2 ± 0.2 | 0.4 ± 0.4 | 0.6 ± 0.4 | 0.4 ± 0.2 | 0 | 0 |
| 20 | 1.6 ± 0.4 | 1.4 ± 0.4 | 0.6 ± 0.4 | 1.0 ± 0.6 | 0.4 ± 0.2 | 0 | 0.2 ± 0.2 |
| 40 | 2.2 ± 0.5 | 2.4 ± 0.7 | 1.2 ± 0.4 | 0.4 ± 0.2 | 0.6 ± 0.2 | 0 | 0.2 ± 0.2 |
| 80 | 3.4 ± 0.6 | 2.2 ± 0.2 | 1.0 ± 0.3 | 1.0 ± 0.3 | 1.2 ± 0.4 | 0.2 ± 0.2 | 0.6 ± 0.2 |
| | % mortality of larvae | | | | % mortality of pupae | % mortality of larvae | |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | 0 | 12.0 ± 8.0 | 8.0 ± 4.9 | 12.0 ± 4.9 | 8.0 ± 4.9 | 0 | 0 |
| 10 | 44.0 ± 7.5** | 4.0 ± 4.0 | 8.0 ± 8.0 | 12.0 ± 8.0 | 8.0 ± 4.9 | 0 | 0 |
| 20 | 32.0 ± 8.0** | 28.0 ± 8.0 | 12.0 ± 8.0 | 20.0 ± 11.0 | 8.0 ± 4.9 | 0 | 4.0 ± 4.0 |
| 40 | 44.0 ± 9.8** | 48.0 ± 3.6** | 24.0 ± 7.5 | 8.0 ± 4.9 | 12.0 ± 4.9 | 0 | 4.0 ± 4.0 |
| 80 | 68.0 ± 12.0** | 44.0 ± 4.0** | 20.0 ± 6.3 | 20.0 ± 6.3 | 24.0 ± 7.5 | 4.0 ± 4.0 | 12.0 ± 4.9 |
| $H_4$ | 22.87 | 17.65 | 9.09 | 6.95 | 8.52 | 5.00 | 7.25 |
| *P* | 0.004 | 0.034 | 0.106 | 0.224 | 0.130 | 0.416 | 0.203 |

Data are means ± SE of dead *A. tumida* larvae and pupae and percentage of mortality in the applied concentration of the different nematode strains after 5 d. $N = 5$ petri dishes × five larvae per dish for all means. Nematode densities of 5, 10, 20, 40, and 80 IJs/cm$^2$ equal 28, 56, 112, 224, and 448 IJs/*A. tumida* larvae, respectively. H and *P* values are shown for Kruskal–Wallis tests. Treatment means with asterisks (**) are significantly different from their respective control means at $\alpha \leq 0.01$.

4–5 d to maintain soil moisture. It rained once during the study with all cups being exposed to <2.5-cm rain at that time. All of the soil cups were moved into the lab on day 29 of the study where they remained because night temperatures dropped below 5°C. Consequently, approximately three fourths of the study was conducted in the field, whereas the remaining one fourth was completed in the lab. We quantified the number of emerging *A. tumida* in all soil cups and terminated the study on day 41. The remaining soil was filtered to verify that the beetles not emerging were dead.

**Statistical Analyses.** The statistical tests in screening bioassays 1–3 were performed using STATISTICA (StatSoft 2001), whereas those for screening bioassay 4, the generational persistence assay, and the field bioassay were performed using SAS ( SAS Institute 2004).

In screening bioassays 1–3, proportions of mortality were analyzed for significant differences between the investigated concentrations and the controls for each nematode strain using Kruskal–Wallis test and Mann–Whitney *U* post hoc tests (adjusted $\alpha = 0.01$). Nonparametric tests were applied because the data sets did not meet the assumptions for parametric tests. The same tests also were used to test for differences between the strains of the first screening bioassay for each applied concentration. Analyses of regression were performed between concentrations of IJs and the induced *A. tumida* mortality. The mortality of larvae and pupae in the second screening bioassay as well as the mortality of larvae at the two different temperatures during the third screening bioassay were compared for all concentrations using Mann–Whitney *U* tests.

To test for nematode strain and inoculation level effects in screening bioassay 4, we analyzed data from parts 1–3 by using a factorial analysis of variance (ANOVA). Nematode strain and IJ level were treated as main effects and nematode × IJ level as the interaction term. The main effects were tested against the interaction term. Where significant interactions existed, we analyzed IJ level by nematode strain.

The results of screening bioassays 1–4 were not compared with each other because the tested *A. tumida* larvae and pupae originated from different populations and different soil types (Ellis et al. 2004) were used. These factors may have an influence on nematode performance and survival (Glazer et al. 1999).

In the generational persistence bioassay, we analyzed data using a factorial ANOVA. We considered week (1–15) and treatment (eight nematode treatments) as main effects and week × treatment as the interaction term. We tested the main effects against the interaction term. The whole model showed a significant interaction between week and treatment, so we reanalyzed treatment by week.

The field bioassay was a split plot analysis with location (field or forested) as the whole plot. The treatment (eight nematode treatments) × location interaction tested the location. We analyzed treatment and moisture (routinely wetted or natural rainfall) factorially within location. We tested both by residual error. Where necessary, Tukey tests were used to compare means in screening bioassay 4, the generational bioassay, and the field bioassay.

## Results

**Screening Bioassays.** In the first screening bioassay, all control larvae ($N = 25$) were alive after 5 d. The number and the percentages of dead *A. tumida* larvae in the treatments are shown in Table 1. Compared with the controls, significantly more larvae died in the

treatment with *S. riobrave* at 10, 20, 40, and 80 IJs/cm$^2$ and in the two highest concentrations of *H. zealandica.* The induced mortality at the highest concentrations did not differ between the two strains ($U = 6$, $P = 0.125$). In contrast, none of the treatments with *H. bacteriophora* resulted in an increased mortality over that of the controls. The percentage of mortality was positively correlated with the applied concentrations of IJs for *H. zealandica* ($r_s = 0.73$; $t_{28} = 5.59$, $P < 0.001$) and *S. riobrave* ($r_s = 0.80$; $t_{28} = 7.08$, $P < 0.001$).

In the second assay, all control larvae ($N = 25$) were alive after 5 d. It seems that *H. megidis* killed both *A. tumida* larvae and pupae (there was some mortality among both), but no treatment caused a significantly increased mortality over that of the controls. Consequently, no significant differences were found between the susceptibility of larvae and pupae to nematodes at all concentrations ($U > 8.5$, $P > 0.418$) (Table 1).

Regarding the third assay, all larvae ($N = 25$) in the controls survived. The mortality of larvae exposed to *H. bacteriophora* did not differ significantly compared with the controls at 20°C or at 25°C. In fact, at 20°C only a single larva was killed at a concentration of 80 IJs per cm$^2$. As a consequence, no differences between the mortalities at the two tested temperatures were detected ($U > 7$, $P > 0.220$) (Table 1).

In the fourth screening bioassay, the six nematode strains did not affect larval mortality differently after 6 d compared with one another ($F_{5,10} = 2$; $P = 0.27$) but did after 9 d ($F_{5,10} = 13$; $P \leq 0.001$). After 9 d, *A. tumida* larvae exposed to *H. bacteriophora* Oswego strain (5.8 ± 0.4 dead larvae) and *H. indica* (5.8 ± 0.5) showed significantly higher mortality than *A. tumida* larvae exposed to any other nematode strain. *A. tumida* mortality was similar in the *S. carpocapsae* Agriotus (3.6 ± 0.4) and *H. bacteriophora* HB strains (2.8 ± 0.4) and between the *H. bacteriophora* HB and *S. riobrave* 7-12 strains (2 ± 0.4). *A. tumida* larvae exposed to *S. feltiae* for 9 d showed the lowest mortality (0.8 ± 0.2). Data are mean ± SE and $N = 30$ petri dishes with 10 *A. tumida* larvae per dish for each nematode strain.

There was a significant interaction between nematode strain and IJ level after 6 d ($F_{10,162} = 11$; $P \leq 0.001$) and 9 d ($F_{10,162} = 3$; $P \leq 0.01$); so, IJ level was tested by nematode type at both lengths of time. At the end of 6 d, IJ effects varied by nematode type (*P* values ranged from ≤0.0001 to 0.3). This was true at the end of 9 d as well (*P* values ranged from ≤0.0001 to 0.8). Even when IJ levels significantly affected the mortality of *A. tumida* larvae treated within a particular nematode strain, no clear trend within IJ levels was observed. No larvae died in any of the control dishes after 6 d, whereas an average of 2.9 ± 0.4 larvae died per control dish after 9 d. The control mortality in the 9-d group was due largely to larvae that were drowning in the pooled water in the water only petri dishes.

In part three of screening bioassay 4, the three nematode strains killed almost 100% of the *A. tumida* larvae after 39 d at all tested IJ levels. *S. riobrave* 7-12 (10 ± 0 dead larvae), *H. indica* (9.9 ± 0.04), and *H. bacteriophora* Oswego (9.7 ± 0.1) affected *A. tumida* larval mortality similarly after 39 d ($F_{2,6} = 1$; $P = 0.4$). Data are mean ± SE and $N = 40$ petri dishes with 10 *A. tumida* larvae per dish for each nematode strain. An average of 0.8 ± 0.2 larvae died per control dish after 39 d. Furthermore, there was a significant interaction between nematode strain and IJ level ($F_{6,108} = 4$; $P = 0.001$) in part three of this screening bioassay. Consequently, IJ effects were investigated within each nematode strain, but no clear trends were observed.

**Generational Persistence Assay.** Toward the conclusion of the generational persistence bioassay, the number of emerging *A. tumida* adults decreased in a few of the control soil cups (fewer than five cups). After a thorough investigation, we noticed that the soil in these suspect cups contained many soil mites (>500 per cup) apparently attacking the pupating *A. tumida.* We sent a sample of the mites to an acarologist at the Florida Department of Agriculture and Consumer Services, Division of Plant Industry. The acarologist identified the mites as *Tyrophagus putrescentiae* Schrank (Acari: Acaridida), which is a stored-product mite (Hubert et al. 2007). We saved some of the infected soil containing the mites into which we continued to introduce five *A. tumida* larvae weekly. No *A. tumida* ever emerged from mite-infested soil ($N = 5$ cups), whereas emergence rates were high (>90%) in nonmite infested soil ($N = 5$ cups where mites were not visibly present). Although we did not look for soil mites in the nematode treated cups, we believe they were present because the soil used in all cups originated from the same location. However, we could not determine which cups contained mites because mortality in all nematode-treated cups was high.

Overall, there was a significant interaction between week and treatment for the whole model ($F_{98,2580} = 2$; $P \leq 0.001$), so we reanalyzed the data by week (Table 2). In general, all nematode treatments significantly lowered the number of emerging *A. tumida* adults below that of the two controls although the level of control provided by each nematode treatment varied by week. This is confirmed further when considering treatment averages across all weeks, which we only compare numerically due to the significant interaction (Table 3). In this instance, *A. tumida* mortality in the control groups (≈8–12%) was lower than in the treatment groups (≈76–94%) (Table 3).

We did not notice any trends with regard to larval mortality within the three methods of soil inoculation we tested (25,000 IJs, 50,000 IJs, and mealworm cadavers (Tables 2 and 3). The *S. riobrave* 7-12 cadaver and 25,000 IJ treatments affected pupating *A. tumida* most, with <9% of *A. tumida* exposed to these treatments as pupae emerging as adults (Table 3). In contrast, the *S. riobrave* 7-12, 50,000 IJ treatment affected pupating *A. tumida* least, with ≈24% emerging as adults. All *H. indica* treatments performed similarly (Table 3).

**Field Bioassay.** Location at the UGA Honey Bee Research Laboratory significantly affected the number of adult beetles that emerged from the treatment cups in the field bioassay ($F_{1,9} = 24$; $P \leq 0.0009$) with

**Table 2. Mortality of small hive beetle larvae exposed to nematodes in the generational persistence assay**

| Treatment | Week 1 | Week 2 | Week 3 | Week 4 | Week 5 | Week 6 | Week 7 | Week 8 |
|---|---|---|---|---|---|---|---|---|
| Water (control) | 4.5 ± 0.2a | 4.7 ± 0.2a | 4.8 ± 0.1a | 4.9 ± 0.1a | 4.4 ± 0.1a | 4.6 ± 0.1a | 4.7 ± 0.1a | 4.7 ± 0.1a |
| Uninfected mealworm cadaver (control) | 4.7 ± 0.2a | 4.8 ± 0.2a | 4.8 ± 0.2a | 4.5 ± 0.3a | 4.4 ± 0.2a | 4.5 ± 0.2a | 4.8 ± 0.2a | 4.6 ± 0.3a |
| *H. indica*, 25,000 IJ aqueous | 0b | 0.6 ± 0.3b | 0.2 ± 0.2c | 1.3 ± 0.4b | 1.0 ± 0.3bc | 0.4 ± 0.3bc | 0.3 ± 0.3c | 0.4 ± 0.3c |
| *H. indica*, 50,000 IJ aqueous | 0b | 1.1 ± 0.4b | 0.9 ± 0.4bc | 1.1 ± 0.4b | 1.2 ± 0.3b | 1.0 ± 0.4bc | 0.5 ± 0.3bc | 0.5 ± 0.3bc |
| *H. indica*, 25,000 IJ mealworm cadaver | 0b | 0.2 ± 0.1b | 0.3 ± 0.2c | 1.2 ± 0.3b | 1.3 ± 0.4b | 0.1 ± 0.1bc | 0c | 0.2 ± 0.1c |
| *S. riobrave* 7-12, 25,000 IJ aqueous | 0b | 0.8 ± 0.2b | 0.4 ± 0.1c | 0.3 ± 0.3b | 0.5 ± 0.3bc | 0.5 ± 0.3bc | 0.3 ± 0.2c | 0.4 ± 0.3c |
| *S. riobrave* 7-12, 50,000 IJ aqueous | 0b | 0.5 ± 0.1b | 1.6 ± 0.5b | 1.4 ± 0.5b | 1 ± 0.4bc | 1.3 ± 0.5b | 1.4 ± 0.5b | 1.6 ± 0.5b |
| *S. riobrave* 7-12, 25,000 IJ mealworm cadaver | 0b | 0.5 ± 0.2b | 1.3 ± 0.3bc | 0.8 ± 0.3b | 0c | 0c | 0.1 ± 0.1c | 0.1 ± 0.1c |
| ANOVA *F* values | $F = 348$ | $F = 93$ | $F = 74$ | $F = 40$ | $F = 50$ | $F = 59$ | $F = 96$ | $F = 72$ |

| Treatment | Week 9 | Week 10 | Week 11 | Week 12 | Week 13 | Week 14 | Week 15 |
|---|---|---|---|---|---|---|---|
| Water (control) | 4.5 ± 0.2a | 4.6 ± 0.1a | 4.5 ± 0.2a | 4.2 ± 0.2a | 4.7 ± 0.2a | 4.6 ± 0.2a | 4.3 ± 0.3a |
| Uninfected mealworm cadaver (control) | 4.1 ± 0.3a | 4.4 ± 0.4a | 4.1 ± 0.3a | 4.2 ± 0.4a | 4.6 ± 0.3a | 4.2 ± 0.3a | 4.0 ± 0.4a |
| *H. indica*, 25,000 IJ aqueous | 1.7 ± 0.3b | 1.6 ± 0.5bc | 0.8 ± 0.3b | 0.3 ± 0.2b | 0.9 ± 0.4b | 1.3 ± 0.4bc | 1.2 ± 0.4b |
| *H. indica*, 50,000 IJ aqueous | 1.2 ± 0.4bc | 0.7 ± 0.4bc | 0.7 ± 0.3b | 0.7 ± 0.3b | 1.0 ± 0.4b | 1.5 ± 0.4b | 0.9 ± 0.4b |
| *H. indica*, 25,000 IJ mealworm cadaver | 2.1 ± 0.3b | 2.1 ± 0.4b | 0.8 ± 0.3b | 0.3 ± 0.2b | 0.9 ± 0.3b | 1.6 ± 0.4b | 1.5 ± 0.4b |
| *S. riobrave* 7-12, 25,000 IJ aqueous | 0.9 ± 0.3bc | 0.9 ± 0.3bc | 0.7 ± 0.4b | 0.4 ± 0.2b | 0.3 ± 0.3b | 0.4 ± 0.3bc | 0.2 ± 0.2b |
| *S. riobrave* 7-12, 50,000 IJ aqueous | 1.5 ± 0.5bc | 1.7 ± 0.6bc | 1.4 ± 0.4b | 1.1 ± 0.5b | 1.5 ± 0.5b | 1.2 ± 0.5bc | 1.3 ± 0.5b |
| *S. riobrave* 7-12, 25,000 IJ mealworm cadaver | 0.2 ± 0.1c | 0.4 ± 0.2c | 0.2 ± 0.2b | 0.2 ± 0.2b | 0.2 ± 0.1b | 0.1 ± 0.1c | 0b |
| ANOVA *F* values | $F = 32$ | $F = 27$ | $F = 41$ | $F = 45$ | $F = 43$ | $F = 36$ | $F = 27$ |

Data are mean ± SE of the number of adult *A. tumida* emerging from soil cups (of five possible adults) treated with one of eight nematode treatments. Week, observation week, not the week the nematodes or small hive beetle larvae were introduced into the soil cups. $N = 40$ soil cups for the water treatment and $N = 20$ soil cups for all other treatments. Data within the same week, followed by the same letter are not different at $\alpha \leq 0.05$ by using Tukey tests. For all weeks, the ANOVA df = 7, 172 and $P \leq 0.0001$.

more *A. tumida*s emerging from soil cups located in the forested area (1.3 ± 0.1, 180 [mean ± SE, *N*]) than in the field (0.8 ± 0.1, 180). In contrast, soil moisture did not affect the number of adult beetles that emerged from treatment cups ($F_{1,317} = 1$; $P = 0.36$). Equal numbers of adult *A. tumida* emerged in soil cups that were wetted periodically (1.1 ± 0.1, 180) and in cups wetted once by natural rainfall (1.0 ± 0.1, 180). There were no significant interactions ($P \geq 0.21$) between any of the measured variables (location, treatment, and moisture).

**Table 3. Average mortality of small hive beetle larvae in the generational persistence assay**

| Treatment | No. adult *A. tumida* emerging from soil cups (of five possible adults) | % mortality = ((5 − mean from second column)/5) × 100 |
|---|---|---|
| Water (control) | 4.6 ± 0.04 (600) | 8 |
| Uninfected mealworm cadaver (control) | 4.4 ± 0.1 (300) | 12% |
| *H. indica*, 25,000 IJ aqueous | 0.8 ± 0.1 (300) | 84 |
| *H. indica*, 50,000 IJ aqueous | 0.8 ± 0.1 (300) | 84 |
| *H. indica*, 25,000 IJ mealworm cadaver | 0.8 ± 0.1 (300) | 84 |
| *S. riobrave* 7-12, 25,000 IJ aqueous | 0.4 ± 0.1 (300) | 92 |
| *S. riobrave* 7-12, 50,000 IJ aqueous | 1.2 ± 0.1 (300) | 76 |
| *S. riobrave* 7-12, 25,000 IJ mealworm cadaver | 0.3 ± 0.04 (300) | 94 |

Data in the second column are mean ± SE (*N*).

There was a significant treatment effect on the number of adult *A. tumida* that emerged from the treatment cups ($F_{1,317} = 70$; $P < 0.0001$) (Table 4). *A. tumida* adult emergence in all soils cups receiving nematodes via any delivery method was lower than that in control soil cups (Table 4). Mortality of *A. tumida* in the nematode-treated cups ranged between 88 and 100%. In general, *A. tumida* pupae exposed to *S. riobrave* 7-12 showed higher mortality than those exposed to *H. indica*. Interestingly, *A. tumida* mortality

**Table 4. Average mortality of small hive beetle larvae in the field bioassay**

| Treatment | No. adult *A. tumida* emerging from soil cups (of five possible adults) | % mortality = ((5 − mean from second column)/5) × 100 |
|---|---|---|
| Water (control) | 2.7 ± 0.2 (80)a | 46 |
| Uninfected mealworm cadaver (control) | 2.9 ± 0.3 (40)a | 42 |
| *H. indica*, 25,000 IJ aqueous | 0.5 ± 0.1 (40)bc | 90 |
| *H. indica*, 50,000 IJ aqueous | 0.3 ± 0.1 (40)bcd | 94 |
| *H. indica*, 25,000 IJ mealworm cadaver | 0.6 ± 0.2 (40)b | 88 |
| *S. riobrave* 7-12, 25,000 IJ aqueous | 0 (40)d | 100 |
| *S. riobrave* 7-12, 50,000 IJ aqueous | 0 (40)d | 100 |
| *S. riobrave* 7-12, 25,000 IJ mealworm cadaver | 0.1 ± 0.1 (40)cd | 98 |

Data in the second column are mean ± SE (*N*). Means followed by the same letter are not different at $\alpha \leq 0.05$ by using a Tukey test.

in control soil cups in the field bioassay was comparatively higher (>3 times higher) than that in the generational persistence study.

## Discussion

The data from our screening bioassays are consistent with those of Cabanillas and Elzen (2006) who demonstrated that *A. tumida* larvae and pupae are susceptible to entomopathogenic nematodes. In our study, and consistent with our first hypothesis, nematode efficacy varied significantly by nematode species and the IJ level at which they were applied. Although nematode virulence was moderate in screening bioassays 1–3 (0–68% mortality), *A. tumida* mortality approached levels in screening bioassay 4 (nearly 100% after 39 d) that suggest suitable applicability of some of the nematode species as field controls for *A. tumida.*

In screening bioassay 3, we attempted to induce varied nematode efficacy by altering the temperature at which we conducted parts of the bioassay. Although temperature has been shown to be an important factor influencing the virulence of nematodes (Goude and Shapiro-Ilan 2003), we did not detect any temperature effect on *H. bacteriophora* virulence. This is not surprising in light of the very low mortality experienced by *A. tumida* infected with this nematode and the narrow range of temperatures we tested. As such, we cannot state conclusively that temperature has no effect on *H. bacteriophora* virulence.

Collectively, the screening bioassays permitted us to determine which nematode species might warrant further field investigations. The cumulative data from all of the screening bioassays suggest that *H. indica* caused the most significant increase in *A. tumida* mortality over time. Consequently, we chose it for further field testing. Also, heterorhabditid and steinernematid nematodes are known to differ in a variety of characteristics (Gaugler and Kaya 1990), including host-seeking behavior. As such, we elected to test *S. riobrave* 7-12 strain in the generational persistence and field bioassays.

In the generational persistence bioassay, we discovered that both *S. riobrave* 7-12 strain and *H. indica* continued to provide adequate *A. tumida* control for 19 wk after a single soil inoculation even though we had hypothesized reduced efficacy over time. In fact, both species at all inoculation types caused ≥76% mortality in pupating *A. tumida.* These findings particularly are encouraging because the soil cups were treated only once. The data indicate, at least under the conditions our tests were conducted, that it may be possible to achieve long-term control of *A. tumida* pupae with only one application of nematodes, even with multiple migrations of wandering larvae into the soil.

In the generational persistence bioassay, we believe the level of control we achieved was possible because of the reproductive habits of the tested nematodes. In general, they are known to penetrate the host and feed/reproduce for ≈14 d (Shapiro-Ilan et al. 2002). After this time, new infective IJs emerge from the host and begin to seek a new host (Shapiro-Ilan et al. 2002). Our data suggest that as long as *A. tumida* pupae are available as a food source, any applied nematodes may persist for significant periods. This situation can be expected in some cases because *A. tumida* adults might reproduce cryptically in honey bee colonies (Spiewok and Neumann 2006). However, any break in host availability in the soil (such as in winter for *A. tumida;* Lundie 1940, Pettis and Shimanuki 2000, De Guzman and Franke 2007) may demand the soil be retreated with nematodes at a later time.

In the field bioassay, both nematode species showed high virulence toward pupating *A. tumida,* even though we had predicted only moderate efficacy. The data further confirms the results from our screening and generational persistence bioassays. It is unclear to us why *A. tumida* mortality was higher in the unshaded field than in the forested area. Our data suggest that it is unrelated to soil moisture, even though soil moisture is known to affect nematode virulence in other pest control systems (Grant and Villani 2003, Koppenhöffer and Fuzy 2007, Shapiro-Ilan et al. 2007). Soil humidity and temperature are known to effect *A. tumida* pupation success (Ellis et al. 2004, De Guzman and Frank 2007). Thus, interactions between marginal suitable soil conditions for *A. tumida* pupation and nematodes may contribute to the higher mortality observed in the unshaded field.

In the generational persistence and field bioassays, all methods of soil inoculation that we tested worked well. Using nematode-infected mealworm cadavers did not seem to give us better control of *A. tumida* than that gained from using aqueous inoculums. However, infected cadavers may be more practical for beekeepers to use in the field because their application is less labor-intensive (they are buried rather than watered into the ground) than applying nematodes aqueously and they may serve as a temporary food source, at least initially, when *A. tumida* are not available. However, ease of application would need to be verified in large scale field trails before a recommendation could be made regarding the advantages of cadaver or aqueous applications.

Glazer et al. (1999) rated the mean susceptibility to nematode infestations poor (mortality <35%), moderate (mortality 35–65%), or high (mortality >65%). Following this approach, *A. tumida* larvae showed a high susceptibility only to the highest concentrations of a few, select nematodes (*S. riobrave* Rio strain, *H. bacteriophora* Oswego strain, and *H. indica*) in screening bioassays 1–3. In general however, the treatments resulted in poor to moderate *A. tumida* mortality. In screening bioassay 4, the generational persistence and field bioassays (all with higher IJ levels than those used in screening bioassays 1–3), the tested nematodes induced high *A. tumida* mortality. Thus, our data are in line with previous studies suggesting that nematodes are generally less effective against sap beetles at low IJ levels (Coleoptera: Nitidulidae, Vega et al. 1994, Glazer et al. 1999). For example, tested *S. riobrave* strains induced a high mortality of larvae of *C.*

*hemipterus* or *C. humeralis* F. only at high concentrations like those used in our generational persistence and field bioassays (≥200 IJs per larva, Vega et al., 1994; 50 IJs per $cm^2$, Glazer et al. 1999).

In conclusion, our experiments demonstrate that entomopathogenic nematodes can infest and kill *A. tumida* wandering larvae and pupae. Because environmental conditions influence the performance of nematodes as control agents, we clearly cannot predict the levels of concentration required for an effective field application. Yet, our data indicate that levels >200 IJs per *A. tumida* larva are sufficient to induce high levels of control within the parameters tested in our study. Despite this, these concentrations may be less feasible for an economic integrated pest management system, although this certainly needs to be tested further. Because *A. tumida* usually pupate in proximity to the infested hives (<180 cm, Pettis and Shimanuki 2000), the treatment area would be relatively small. Thus, the application of higher IJs of nematodes still might be acceptable economically, particularly because nematodes seem relatively innocuous to honey bees on a large scale (Kaya et al. 1982, Baur et al. 1995, Zoltowska et al. 2003).

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