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Optimization of a Host Diet for *in vivo* Production of Entomopathogenic Nematodes

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Abstract: To facilitate improved *in vivo* culture of entomopathogenic nematodes, production of both insect hosts and nematodes should be optimized for maximum fitness, quality, and cost efficiency. In previous studies, we developed an improved diet for *Tenebrio molitor*, a host that is used for *in vivo* nematode production, and we demonstrated that single insect diet components (e.g., lipids and proteins) can have a positive or negative impact on entomopathogenic nematode fitness and quality. In this study, we tested components of our improved *T. molitor* diet (lipids, cholesterol, and a salt [$MnSO_4$]) alone and in combination for effects on host susceptibility and reproductive capacity of *Heterorhabditis indica* and *Steinernema carpocapsae*. Our results indicated that moderate levels of lipids (10%) increased host susceptibility to *S. carpocapsae* but did not affect *H. indica*, whereas cholesterol and $MnSO_4$ increased host susceptibility to *H. indica* but not *S. carpocapsae*. The combined *T. molitor* diet (improved for increased insect growth) increased host susceptibility to *S. carpocapsae* and had a neutral effect on *H. indica*; interactions among single diet ingredients were observed. No effects of insect host diet were detected on the reproductive capacity of either nematode species in *T. molitor*. Subsequently, progeny infective juveniles, derived from nematodes grown in *T. molitor* that were fed diets with varying nutritive components were tested for virulence to and reproduction capacity in the target pest *Diaprepes abbreviatus*. The progeny nematodes produced from differing *T. molitor* diet treatments did not differ in virulence except *H. indica* derived from a diet that lacked cholesterol or $MnS0_4$ (but contained lipids) did not cause significant *D. abbreviatus* suppression relative to the water control. We conclude that the improved insect host diet is compatible with production of *H. indica* and *S. carpocapsae*, and increases host susceptibility in *S. carpocapsae*. Furthermore, in a general sense, our results indicate host diets can be optimized for improved *in vivo* entomopathogenic nematode production efficiency. This is the first report of an insect diet that was optimized for both host and entomopathogenic nematode production. Additionally, our study indicates that host diet may impact broader aspects of entomopathogenic nematode ecology and pest control efficacy.

Key words: entomopathogenic nematode, *Heterorhabditis*, host diet, *in vivo*, mass production, *Steinernema*.

Entomopathogenic nematodes (genera *Steinernema* and *Heterorhabditis*) are biological control agents that are used to control a variety of economically important insect pests (Shapiro-Ilan et al., 2002b; Grewal et al., 2005). These nematodes have a mutualistic relationship with a bacterium (*Xenorhabdus* spp. and *Photorhabdus* spp. for steinernematids and heterorhabditids, respectively) (Poinar, 1990). Infective juveniles (IJ), the only free-living stage, enter hosts through natural openings (mouth, anus and spiracles) or, in some cases, through the cuticle. After entering the host's hemocoel, nematodes release their bacterial symbionts, which are primarily responsible for killing the host within 24-48 hours, defending against secondary invaders and providing the nematodes with nutrition (Dowds and Peters, 2002). The nematodes molt and complete up to three generations within the host, after which IJ exit the cadaver to find new hosts (Kaya and Gaugler, 1993).

Entomopathogenic nematodes are cultured *in vivo* or in vitro for large-scale commercial production as well as for laboratory experimentation or field testing (Shapiro-Ilan and Gaugler, 2002; Ehlers and Shapiro-Ilan, 2005). *In vitro* production is accomplished in solid or liquid media, whereas *in vivo* production involves mass inoculation (and harvest from) various insect hosts, primarily the greater wax moth *Galleria mellonella* (L.) or the yellow mealworm, *Tenebrio molitor* L. (Shapiro-Ilan and Gaugler, 2002; Shapiro-Ilan et al., 2002a). One of the components that contributes to successful *in vitro* production is the quality of artificial media (Shapiro-Ilan and Gaugler, 2002; Ehlers and Shapiro-Ilan, 2005), and therefore considerable research has been directed toward nutrient optimization (Han et al., 1992; Yang et al., 1997; Yoo et al., 2000; Abu Hatab and Gaugler, 2001; Gil et al., 2002). Similarly, the efficiency of *in vivo* culture production also relies on the quality of media, i.e., insect hosts. For example, in production operations that produce their own insect hosts for nematode culturing, a host diet that is improved for insect production translates into improved efficiency in the overall process (Morales et al., 2011). Additionally, in a tri-trophic interaction, the nutritional quality of insect host's diet can also impact the quality and fitness of entomopathogenic nematodes that are reared on those insects (Shapiro-Ilan et al., 2008).

Our overall goal is to comprehensively optimize all steps of *in vivo* production from insect culture to nematode packaging; in this study we focus on optimization of insect media and its impact on nematode production. In a separate study we developed an improved diet for *T. molitor* (Morales et al., 2011; unpublished data). The diet consists of a base diet (bran) plus a nutritive supplement that includes a protein source, lipids, $MnS0_4$, and cholesterol (Morales et al., 2011; unpublished data). Additionally, in a previous study, we demonstrated that certain host diet components (e.g., protein and lipids) can positively or negatively impact the virulence and

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reproductive capacity of entomopathogenic nematodes, but we did not determine how the diet ingredients act together. In this study, we build upon our previous findings and investigate further the impact of host diet on *in vivo* production parameters for entomopathogenic nematodes. The objective of this study was to determine if the components of our improved host diet impact entomopathogenic nematode fitness or quality positively, or at least have a neutral impact. Specifically, we investigated whether primary components of the improved *T. molitor* diet (alone and in combination) would affect the virulence and reproductive capacity of *Heterorhabditis indica* (Poinar, Karunakar and David) and *Steinernema carpocapsae* (Weiser). Additionally, an experiment was conducted to demonstrate the superior effects of the improved diet ingredients on *T. molitor* production (as measured by the insect's fecundity).

Materials and Methods

Insects and nematodes: Tenebrio molitor were cultured at 27°C on a wheat bran (coarse grade, Siemer Milling Co., Teutopolis, IL) diet with or without dietary supplements. Insects were introduced to the experimental diets as early instars (3^{rd}-5^{th}). Early instars were obtained from the laboratory colony by separating small larvae using sieves. Larvae that passed through a standard number 25 sieve (600 μm openings) and failed to pass through a standard number 35 sieve (500 μm openings) were selected. *T. molitor* were cultured in uncovered plastic boxes (16 x 16 x 4.5cm) containing 125 insects and 50 g of diet. Each of the boxes received approximately 6.25 ml water (added by spray bottle) 5 d per week. Insects were then cultured in the diets for approximately 2 months until their weights ranged from 0.08 to 0.12 g.

Prior to experimentation, nematodes *H. indica* (HOM1 strain) or *S. carpocapsae* (All strain) were reared on commercially obtained last instar *Galleria mellonella* (L.) at 25°C according to procedures described in Kaya and Stock (1997). Following harvest, nematodes were stored at 13°C for less than 2 wk before experimentation. All culture conditions and experiments were conducted at approximately 25°C.

Nutrition regimes and experimental approach: The basic approach for assessing host diet effects on entomopathogenic nematode quality and fitness were adapted from Shapiro-Ilan et al. (2008). Three *T. molitor* diet experiments were implemented: 1) a comparison of lipid treatments, 2) testing of MnS04 and cholesterol treatments, and 3) a comparison that included a combined nutritional diet. The components of all diet treatments for the three experiments are listed in Table 1. All experiments included a control diet of wheat bran only (Siemer Milling Co., Teutopolis, IL). All other treatments contained 90% bran and 10% nutritional supplement. The base supplement treatment, which was also included in all experiments, consisted of 1.0% protein, i.e., 0.5% egg white powder (P. No. 40586, Bulkfoods.com) and 0.5% soy protein (unflavored/unsweetened, GNC, Pittsburgh, PA) and 9.0% potato (P. No. 12854, Bulkfoods.com). The base supplement ingredients were found previously to increase *T. molitor* production and were previously shown to have a neutral effect on nematode fitness (Shapiro-Ilan et al., 2008; Morales et al., 2011; unpublished data). All other diet treatments also contained 1.0% protein (0.5% egg white and 0.5% soy powder) as well as other additives and varying amounts of potato to make up remainder of the supplement. In the first experiment, a comparison of lipid amendments, the experimental diets included 0.5% peanut oil (Ventura Foods, LLC, Opelousas, LA) or 0.5% salmon oil (Lenier Health Products, LLC, Carson, CA). In the 2^{nd} experiment, 0.015% $MnS0_4$ (P. No. M114, Fisher Sci. Atlanta, GA) and or 0.016% cholesterol (P. No. C3045, Sigma-Aldrich, Saint Louis, MO) were included alone or in combination. The third experiment compared addition of lipids (0.5% peanut oil), or $MnS0_4$ and cholesterol, and a combined diet that included all three components (lipids, $MnS0_4$, and cholesterol). Experiments addressing diet effects on *H. indica* and *S. carpocapsae* were

Table 1. Percentages of ingredients in *Tenebrio molitor* diets.

Experiment	Treatment[a]	Peanut oil	Salmon oil	MnS04	Cholesterol	Potato
1) Lipid	Peanut	0.5	–	–	–	8.5
	Salmon	–	0.5	–	–	8.5
2) Mn-Cholesterol	Ch	–	–	–	0.015	8.985
	Mn	–	–	0.016	–	8.984
	Mn-Ch	–	–	0.016	0.015	8.969
3) Combined nutrition	Lipid	0.5	–	–	–	–
	Mn-Ch	–	–	0.016	0.015	8.969
	Lipid-Mn-Ch	0.5	–	0.016	0.015	8.469

[a] All treatments also contained 90% bran, 0.5% egg white powder and 0.5% soy powder. A control was also included in each experiment that consisted of 100% bran. Additionally, in each experiment, a basic supplement treatment was included, which consisted of 90% bran, 0.5% egg white powder, 0.5% soy powder, and 9% potato.

conducted separately. In all experiments we tested the impact of diet treatments on host susceptibility and nematode reproductive capacity.

Host diet effects on host susceptibility: Tenebrio molitor cultured on the different diets described above were tested for susceptibility to *H. indica* and *S. carpocapsae*. Ten insects from each diet were placed in each of eight 100 mm petri dishes lined with filter paper. Four of the dishes received 1 ml of water each only as a control. The other four dishes received nematodes in 1 ml of water, i.e., 4,000 IJ of *H. indica* or 200 IJ of *S. carpocapsae*, except 500 IJ of *S. carpocapsae* were used in the first experiment (lipid comparison); the different rates were based on preliminary data indicating higher virulence of *S. carpocapsae* to *T. molitor*. Dishes were stored at 25°C and host susceptibility (insect mortality) was recorded 2, 3 and 4 d post-inoculation. The experiments (for *H. indica* and *S. carpocapsae*) were each repeated once in time (in two trials).

Host diet effects on nematode reproductive capacity: Host diet effects on nematode reproduction were determined by measuring progeny production in nematode-infected insects from the host susceptibility experiments. Five dead *T. molitor* that showed signs of nematode infection from each replicate in the host susceptibility experiments were placed together on modified White traps (Shapiro-Ilan and Gaugler, 2002). The date of first IJ emergence was noted, and nematodes were collected up to 7 d post-emergence. Harvests from each White trap were pooled (four replicates per treatment) and IJ yield per insect was calculated. The experiments (for *H. indica* and *S. carpocapsae*) were each repeated once in time (in two trials).

Host diet effects on efficacy of nematode progeny: Following the three experiments described above, an additional experiment was conducted to determine the effects of insect host diets (used during *in vivo* nematode production) on subsequent pest control efficacy. To address this issue, we compared the virulence and reproductive capacity of nematodes produced in *T. molitor* fed modified and unmodified diets. Virulence trials for nematodes produced on modified diets were focused on laboratory suppression of the diaprepes root weevil, *Diaprepes abbreviatus* (L.). Laboratory trials in protected environments are well suited to detecting differences in virulence (Shapiro and McCoy, 2000; Shapiro-Ilan et al., 2002b). The virulence evaluation was conducted according to Shapiro and McCoy (2000). Briefly, experimental units consisted of 30 ml plastic cups containing 27 g of sand and one larva (40- to 60-d-old). Approximately 500 *H. indica* IJ or 1,000 *S. carpocapsae* IJ were added to each cup in 0.5 ml of water; the final soil moisture was 10%. The cups were incubated at 25°C, and insect mortality was recorded 14 d post-inoculation. Treatments included nematodes produced in the combined nutrition experiment (experiment # 3) as well as a water-only control. Each experiment consisted of four replicates of 10 cups/treatment, and the experiments were each repeated once in time. Additionally, reproductive capacity in *D. abbreviatus* cadavers was determined in White traps using the procedures described above (four replicates of five cadavers per White trap).

Diet effect on host fecundity: In addition to measuring the tri-trophic impact of *T. molitor* diets on nematode fitness, we conducted an experiment to demonstrate the potential of the improved diet ingredients to enhance *T. molitor* production efficiency; specifically, we measured the effects of diet on host fecundity. Adult *T. molitor* were obtained from groups of larvae developing in plastic square boxes as described above. Groups of approximately 150 4th to 6th instars were fed with one of four diets as described in experiment 3 and one group (control) was fed with bran only. Pupae resulting from each of the boxes were collected sexed and grouped according their food treatment. Seven groups of 3 emerging adult females and 3 emerging adult males per treatment were transferred to plastic boxes (140 x 102 x 37 mm). The boxes were modified by replacing the bottom with a nylon screen standard No. 20 with 850 μm openings. Hatching *T. molitor* first instars fell through the screen openings to a second unmodified box of the same dimensions as described by Morales-Ramos et al. (2011). Each group of adults was provided with 3g of wheat bran and 1g of the corresponding dry diet treatment. Control groups were provided with 4g of wheat bran only. Each group was also provided with 500 μl of water twice a week.

Adult groups were kept in an environmental chamber at the same conditions described above. First instars recovered from the bottom box of each group were

TABLE 2. Analysis of variance results from experiments investigating *Tenebrio molitor* diet effects on host susceptibility to *Heterorhabditis indica* and *Steinernema carpocapsae*.

Experiment[a]	Nematode	DPI[b]	*F*	df	*P*
1	*H. indica*	2	113.10	7, 55	0.0001
1	*H. indica*	3	197.58	7, 55	0.0001
1	*H. indica*	4	323.12	7, 55	0.0001
1	*S. carpocapsae*	2	156.53	7, 55	0.0001
1	*S. carpocapsae*	3	312.70	7, 55	0.0001
1	*S. carpocapsae*	4	1117.78	7, 55	0.0001
2	*H. indica*	2	40.75	7, 55	0.0001
2	*H. indica*	3	67.45	7, 54	0.0001
2	*H. indica*	4	175.66	7, 53	0.0001
2	*S. carpocapsae*	2	176.35	7, 55	0.0001
2	*S. carpocapsae*	3	66.24	7, 55	0.0001
2	*S. carpocapsae*	4	71.57	7, 55	0.0001
3	*H. indica*	2	121.60	9, 69	0.0001
3	*H. indica*	3	358.0	9, 69	0.0001
3	*H. indica*	4	72.75	9, 69	0.0001
3	*S. carpocapsae*	2	91.91	9, 69	0.0001
3	*S. carpocapsae*	3	93.26	9, 69	0.0001
3	*S. carpocapsae*	4	114.91	9, 69	0.0001

[a] See text for details describing experiments.
[b] DPI = d post-inoculation.

counted daily for a 60 d period. The total number of progeny produced per group was obtained by adding the total number of first instars recovered and the progeny produced per female was calculated by dividing total progeny by the number of females (3 per group).

Data analysis: Treatment effects in all experiments were detected through analysis of variance (ANOVA); if the F value was significant ($P \leq 0.05$), then treatment differences were further elucidated through the Student-Newman-Keuls' test. Data from experiments repeated in time were combined, and variation among trials was accounted for as a block effect (PROC GLM, SAS Institute, Cary, NC). Percentage data (mortality) were arcsine transformed, and numerical data (nematode yield) were square-root transformed prior to analysis (Southwood, 1978; Steel and Torrie, 1980). All means and standard errors presented represent untransformed data.

Results

Host diet effects on host susceptibility and nematode reproductive capacity: In all three experiments measuring host diet effects on host susceptibility, *T. molitor* mortality in dishes treated with nematodes was higher than *T. molitor* in control dishes that did not receive nematodes (Table 2; Figs. 1–6). In experiment 1, lipids had

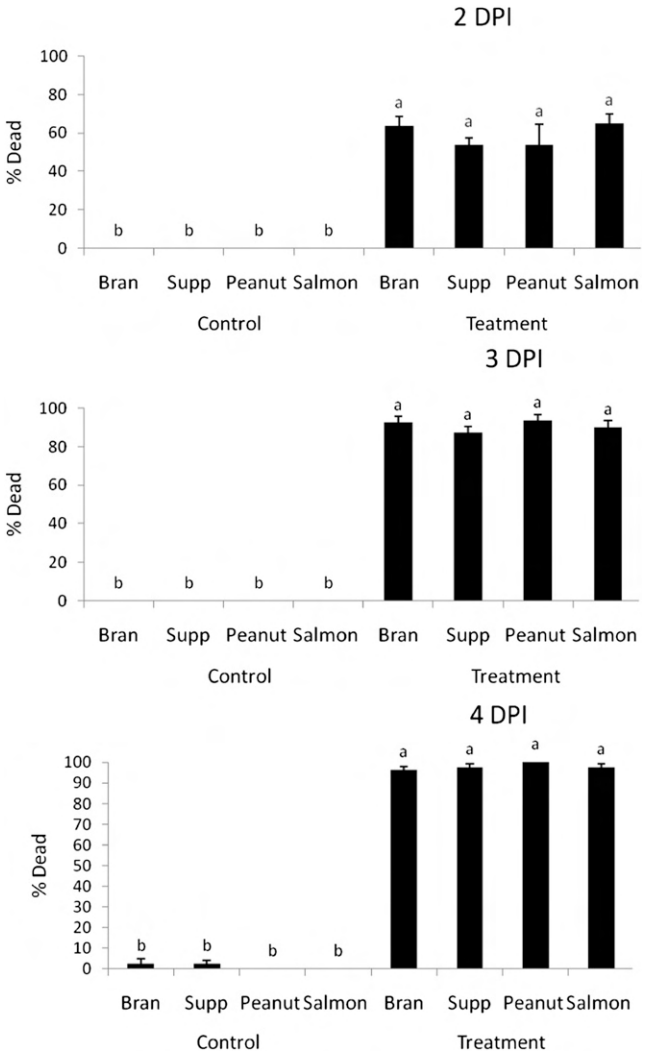

Fig. 1. Susceptibility of *Tenebrio molitor* to *Heterorhabditis indica*, 2 – 4 d post-inoculation (DPI). *T. molitor* were fed diets containing different lipid-based supplements (peanut oil or salmon oil). All lipid treatments also contained bran, egg white powder, soy powder, and dried potato. A base supplement treatment (Supp) did not contain lipids but otherwise contained the same ingredients as the lipid treatments. A bran only diet was also included. Bars above the treatment side of the axis represent percentage (mean ± SE) *T. molitor* mortality following exposure to nematodes; bars above the control side represent *T. molitor* mortality without nematode exposure. Different letters above bars indicate statistically significant differences (SNK test, ~ = 0.05).

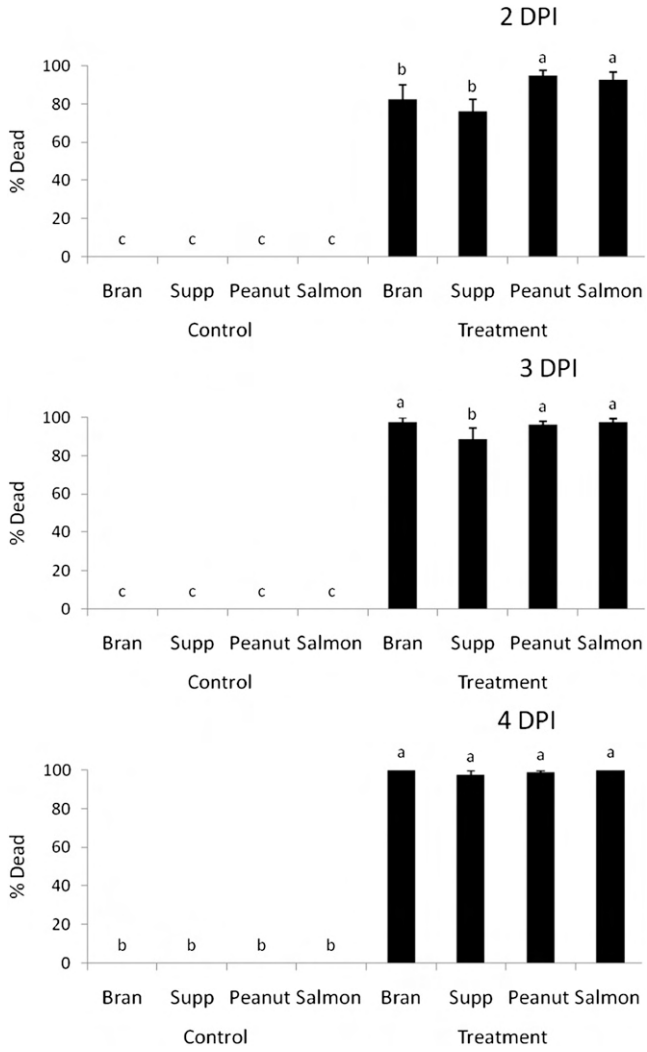

Fig. 2. Susceptibility of *Tenebrio molitor* to *Steinernema carpocapsae*, 2 – 4 d post-inoculation (DPI). *T. molitor* were fed diets containing different lipid-based supplements (peanut oil or salmon oil). All lipid treatments also contained bran, egg white powder, soy powder, and dried potato. A base supplement treatment (Supp) did not contain lipids but otherwise contained the same ingredients as the lipid treatments. A bran only diet was also included. Bars above the treatment side of the axis represent percentage (mean ± SE) *T. molitor* mortality following exposure to nematodes; bars above the control side represent *T. molitor* mortality without nematode exposure. Different letters above bars indicate statistically significant differences (SNK test, ~ = 0.05).

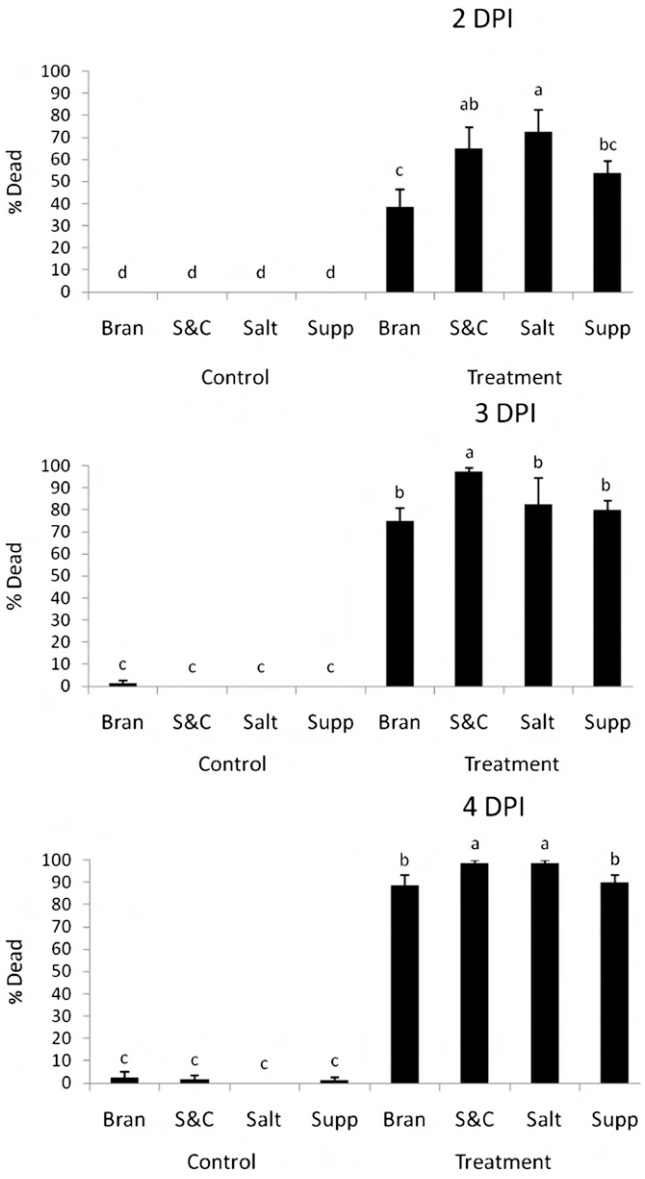

FIG. 3. Susceptibility of *Tenebrio molitor* to *Heterorhabditis indica*, 2 – 4 d post-inoculation (DPI). *T. molitor* were fed diets containing a salt ($MnS0_4$) or the salt combined with cholesterol (S&C). These treatments also contained bran, egg white powder, soy powder, and dried potato. A base supplement treatment (Supp) did not contain salt or cholesterol but otherwise contained the same ingredients. A bran only diet was also included. Bars above the treatment side of the axis represent percentage (mean ± SE) *T. molitor* mortality following exposure to nematodes; bars above the control side represent *T. molitor* mortality without nematode exposure. Different letters above bars indicate statistically significant differences (SNK test, $\sim = 0.05$).

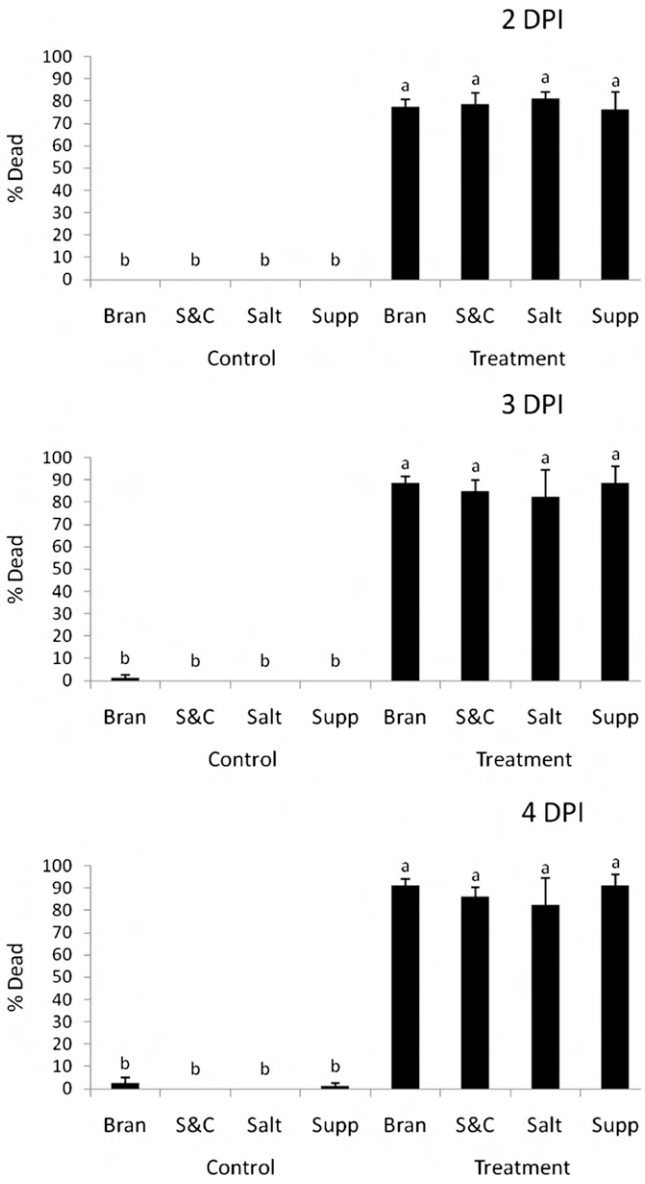

FIG. 4. Susceptibility of *Tenebrio molitor* to *Steinernema carpocapsae*, 2 – 4 d post-inoculation (DPI). *T. molitor* were fed diets containing a salt ($MnS0_4$) or the salt combined with cholesterol (S&C). These treatments also contained bran, egg white powder, soy powder, and dried potato. A base supplement treatment (Supp) did not contain salt or cholesterol but otherwise contained the same ingredients. A bran only diet was also included. Bars above the treatment side of the axis represent percentage (mean ± SE) *T. molitor* mortality following exposure to nematodes; bars above the control side represent *T. molitor* mortality without nematode exposure. Different letters above bars indicate statistically significant differences (SNK test, $\sim = 0.05$).

no effect on the susceptibility of *T. molitor* to *H. indica* (Fig. 1). In contrast, both lipid-based diets (peanut oil and salmon oil) resulted in higher *T. molitor* susceptibility to *S. carpocapsae* at 2 d post-inoculation relative to bran only or the base supplement without lipids added, and at 3 d post-inoculation the lipid treatments caused higher susceptibility relative to the base supplement, whereas no differences were detected at 4 d post-inoculation (Fig. 2).

In experiment 2, the *T. molitor* diet containing $MnS0_4$, or $MnS0_4$ combined with cholesterol, caused increased host susceptibility in *H. indica* (Fig. 3). Specifically, $MnS0_4$ (without cholesterol) resulted in increased host susceptibility to *H. indica* relative to bran only and the base supplement at 2 and 4 d post-inoculation. $MnS0_4$ combined with cholesterol resulted in increased host susceptibility compared with the bran control on all three evaluation dates, and compared

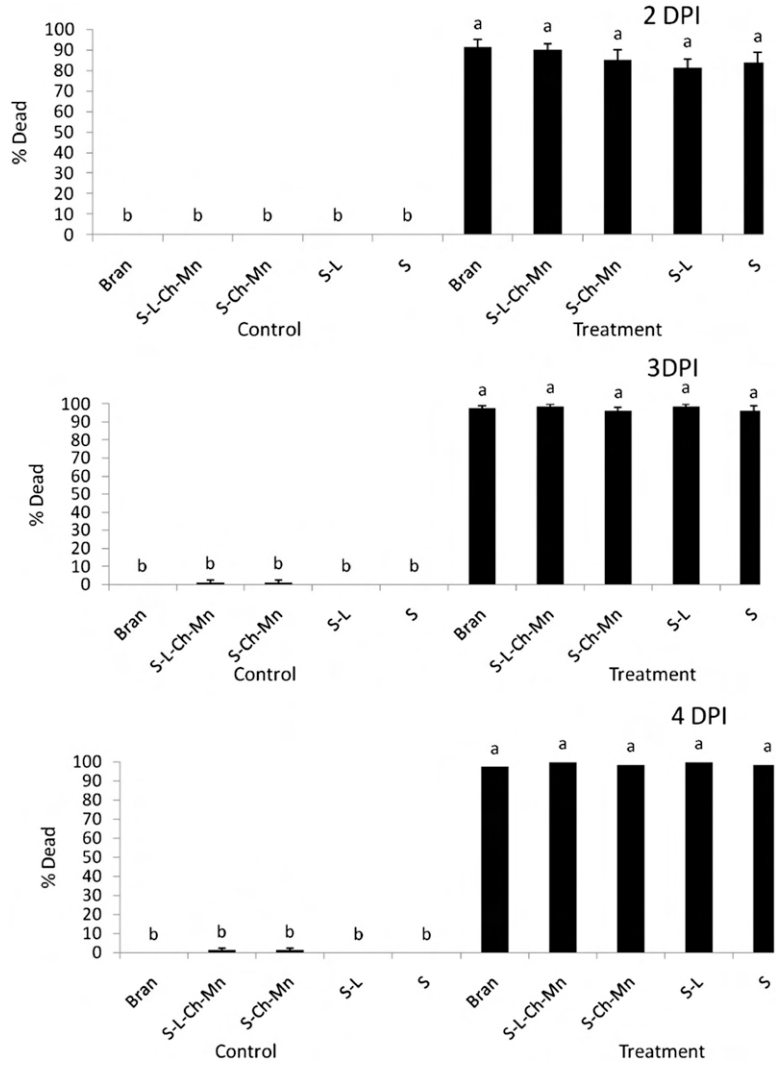

FIG. 5. Susceptibility of *Tenebrio molitor* to *Heterorhabditis indica,* 2 – 4 d post-inoculation (DPI). *T. molitor* were fed varying diets. A basic supplement (S) consisted of bran, egg white powder, soy powder, and potato. Other diet treatments included the basic supplement plus lipids (S-L), cholesterol and $MnS0_4$ (S-Ch-Mn), and a combined diet with all ingredients (S-L-Ch-Mn). A bran only diet was also included. Bars above the treatment side of the axis represent percentage (mean ± SE) *T. molitor* mortality following exposure to nematodes; bars above the control side represent *T. molitor* mortality without nematode exposure. Different letters above bars indicate statistically significant differences (SNK test, ~ = 0.05).

with the base supplement 3 and 4 d post-inoculation (Fig. 3). Conversely, no differences in host susceptibility to *S. carpocapsae* were detected among diet treatments in experiment 2 (Fig. 4).

In experiment 3, comparing the effects of a combined nutritional diet, no effects on susceptibility of *T. molitor* to *H. indica* were detected (Fig. 5). For *S. carpocapsae,* the combined diet including a lipid source, $MnS0_4$ and cholesterol (added to the base supplement) resulted in increased host susceptibility at 2 d post-inoculation; no other differences in host susceptibility were detected among treatments (Fig. 6).

In all three experiments measuring reproductive capacity in *T. molitor,* no effects of host diet were detected for *H. indica* or *S. carpocapsae* ($P > 0.05$). Thus, rather than report the mean IJ yields per insect for all treatments, we report the range (low to high ± SEM) for each experiment as follows: In experiment 1, the IJ yield per insect for *H. indica* ranged from 159,321 ± 46,313 in the bran only control to 213,063 ± 58,821 in the base supplement treatment, and for *S. carpocapsae* the yield ranged from 15,740 ± 2,269 in the base supplement to 26,906 ± 4,388 in the bran only control. In experiment 2, the *H. indica* yield ranged from 90,274 ± 9,325 in the bran control to 109,125 ± 6,891 in the base supplement, and the *S. carpocapsae* yield ranged from 12,375 ± 1,701 in the base supplement to 19,500 ± 1,585 in the $MnSO_4$ treatment. In experiment 3, the *H. indica* yield ranged from 73,000 ± 2,764 in the base supplement to 90,775 ± 8,026 in the $MnS0_4$/cholesterol treatment (without lipids), and the *S. carpocapsae* yield ranged from 15,513 ± 3,004 in the $MnS0_4$/cholesterol/lipid treatment to 25,971 ± 2,125 in the $MnS0_4$/cholesterol treatment (without lipids).

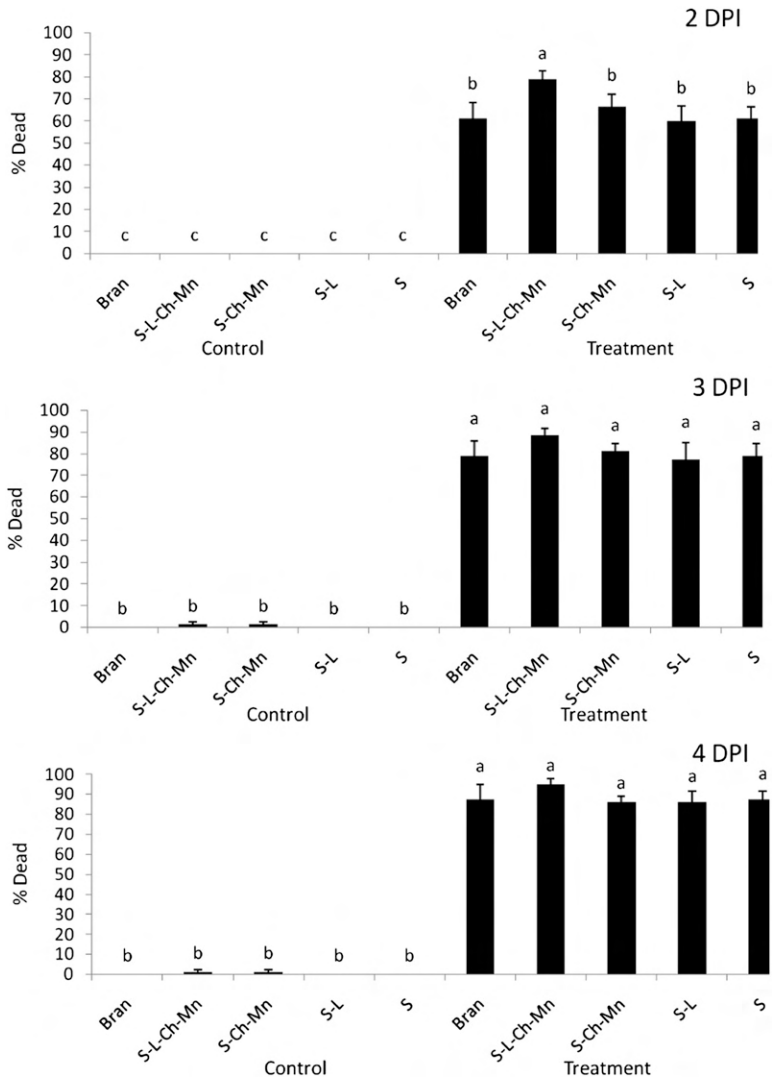

FIG. 6. Susceptibility of *Tenebrio molitor* to *Steinernema carpocapsae,* 2 – 4 d post-inoculation (DPI). *T. molitor* were fed varying diets. A basic supplement (S) consisted of bran, egg white powder, soy powder, and potato. Other diet treatments included the basic supplement plus lipids (S-L), cholesterol and $MnS0_4$ (S-Ch-Mn), and a combined diet with all ingredients (S-L-Ch-Mn). A bran only diet was also included. Bars above the treatment side of the axis represent percentage (mean ± SE) *T. molitor* mortality following exposure to nematodes; bars above the control side represent *T. molitor* mortality without nematode exposure. Different letters above bars indicate statistically significant differences (SNK test, ~ = 0.05).

Host diet effects on efficacy of nematode progeny: In the experiments involving IJ that emerged from *T. molitor* fed different diet treatments, mortality of *D. abbreviatus* at 14 d post-inoculation was reduced in all nematode treatments relative to the no-nematode controls except in the *H. indica* experiment, the lipid treatment (that lacked MnSO4 and cholesterol) did not cause significant suppression (F = 5.65; df = 5, 29; P = 0.0009 for *H. indica*, and F = 14.29; df = 5, 29; $P < 0.0001$ *for S. carpocapsae*) (Fig. 7). There were no differences among the nematode treatments (Fig. 7). Additionally, the reproductive capacities of both nematode species did not differ among treatments (F = 1.53; df = 4, 15; P = 0.2437 for *H. indica* and F = 0.13; df = 5, 15; P = 0.9693 for *S. carpocapsae*). The *H. indica* yield ranged from 39,915 ± 22,305 in the lipid treatment (lacking $MnS0_4$ and cholesterol) to 84,877.5 ± 12,358 in the $MnS0_4$/cholesterol treatment (without lipids), and the *S. carpocapsae* yield ranged from 6,383 ± 1,177 in the $MnS0_4$/cholesterol/lipid treatment to 7,579 ± 1,276 in the lipid treatment (lacking $MnS0_4$ and cholesterol).

Diet effect on host fecundity: Progeny production during a 60 d period was significantly lower in the control group (bran only) than in all the amended diet treatments (F = 7.65; df = 4, 34; P = 0.0002). However, no significant differences were observed in progeny production among the amended diet treatments (Fig. 8). The progeny produced per female was 219.43 ± 27.94 in the control, 349.52 ± 26.51 in the protein only diet, 393.33 ± 33.27 in the lipid diet, 381.76 ± 28.05 in the Mn + cholesterol diet, and 412.57 ± 22.82 in the complete diet (lipid + Mn + Cholesterol).

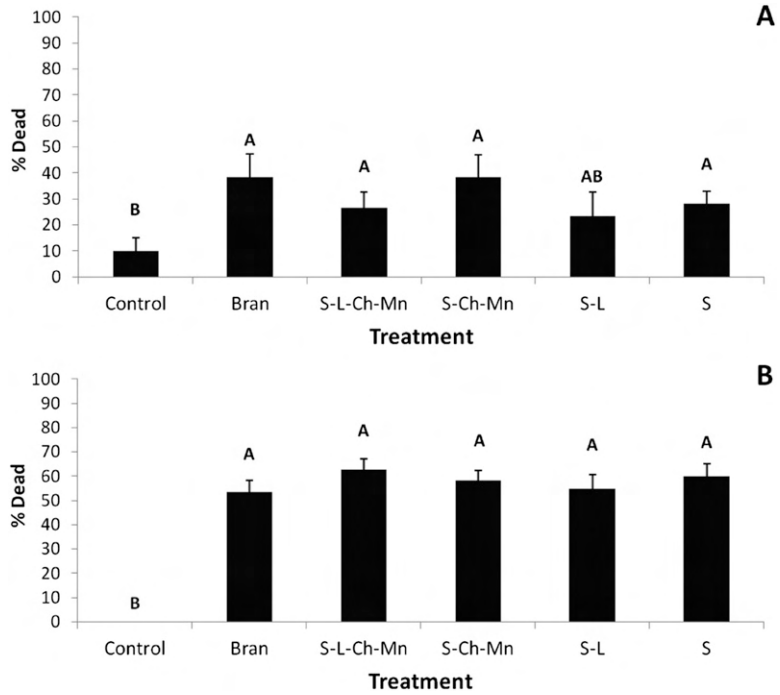

FIG. 7. Efficacy of *Heterorhabditis indica* (A) and *Steinernema carpocapsae* (B) in suppressing *Diaprepes abbreviatus*. Nematodes were applied to *D. abbreviatus* in all treatments except the control (which received water only). Nematodes were derived from *T. molitor* that were fed different diets. A basic supplement diet (S) consisted of bran, egg white powder, soy powder, and potato. Other diet treatments included the basic supplement plus lipids (S-L), cholesterol and $MnS0_4$ (S-Ch-Mn), and a combined diet with all ingredients (S-L-Ch-Mn). A bran only diet was also included. Bars represent percentage (mean ± SE) *D. abbreviatus* mortality 14 days post-inoculation. Different letters above bars indicate statistically significant differences (SNK test, $\sim = 0.05$).

DISCUSSION

Prior studies have reported that tri-trophic effects, e.g., from host plant chemicals, can have a detrimental impact on entomopathogenic nematodes (Barbercheck et al., 1995; Barbercheck and Wang, 1996; Kunkel et al., 2004). In this paper we demonstrated that tri-trophic effects can also have a positive impact on entomopathogenic nematodes, and that these effects can be leveraged to design improved media for *in vivo* production. Specifically, our data indicated that a combined nutritional diet that was developed for improved *T. molitor* production increases host susceptibility to *S. carpocapsae* and has a neutral effect on *H. indica*. Although the diet is especially advantageous for *S. carpocapsae* production (due to increased host susceptibility) it is still beneficial for *H. indica* production because the diet increases *T. molitor* production and therefore improves efficiency (and lowers costs) for the entire process. Thus, improved nutrition is now part of a holistic *in vivo* production process we have developed for entomopathogenic nematodes; the process also includes automated separation of insects from media (Morales et al., 2009), as well as automated inoculation (Shapiro-Ilan et al., 2009) and harvest (unpublished data).

The nature of each diet amendment and its quantity can impact nematode fitness. As expected, certain components tend to have a positive influence, e.g., it was not surprising that Mn based diet components had some positive effects on host susceptibility given that these ions have been shown previously to increase infectivity when exposed directly to the nematodes

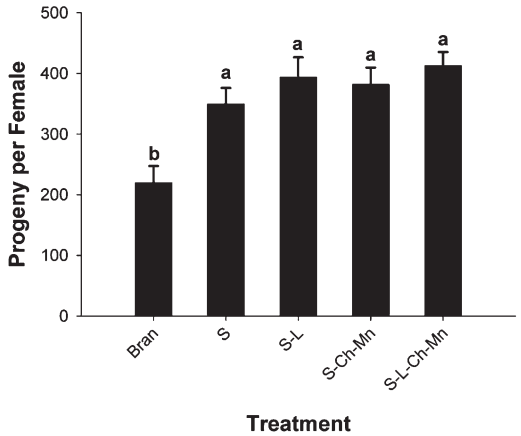

FIG. 8. Progeny produced by *Tenebrio molitor* feeding on different diets. Progeny from newly emerged females was quantified for a period of 60 d. A basic supplement diet (S) consisted of bran, egg white powder, soy powder, and potato. Other diet treatments included the basic supplement plus lipids (S-L), cholesterol and $MnS0_4$ (S-Ch-Mn), and a combined diet with all ingredients (S-L-Ch-Mn). A bran only diet was also included. Bars represent mean progeny produced per female and brackets represent standard error of the mean (mean ± SEM). Different letters above bars indicate statistically significant differences (SNK test, $\sim = 0.05$).

(Jaworska et al., 1997, 1999; Brown et al., 2006). Additionally, based on prior studies (Yang et al., 1997; Yoo et al., 2000; Shapiro-Ilan et al., 2008) we expect lipids to be important for nematode fitness, and this was confirmed for *S. carpocapsae* in the present study. Previously, lipid amendments in a *T. molitor* diet also affected *H. indica* positively, but that outcome was not supported in this study; the discrepancy is likely due to the lower amounts of lipids used in the present study (the amount of lipid was reduced in the newly improved *T. molitor* diet for the sake of optimizing insect growth). Furthermore, along the lines of optimizing quantity of amendments, higher levels of protein in *T. molitor* diets (e.g., 2%) were observed to be detrimental to entomopathogenic nematodes (Shapiro-Ilan et al., 2008), whereas the intermediate levels tested in this study were not harmful.

Our study also indicates that interactions among diet components can be important. For example, the combined nutritional diet was beneficial for *S. carpocapsae* compared with cholesterol-Mn or lipid amendments applied singly. Additionally, progeny virulence in *H. indica* was negatively affected by the lipid component plus base supplement, but this effect was neutralized when cholesterol-Mn was also added. Conceivably, additional combinations of diet components can be utilized to further improve host diets for entomopathogenic nematodes.

In addition to providing benefits for *in vivo* production, our results have broad implications for other aspects of entomopathogenic nematology. For example, host diet impacts on host susceptibility are relevant to studies on entomopathogenic nematode virulence in the laboratory and field because the results may be affected differentially by tri-trophic effects. Moreover, host diet effects may be important for maintaining beneficial traits during repeated culture for laboratory or commercial purposes; although beneficial trait deterioration in entomopathogenic nematodes has been reported to be genetically based (Bai et al., 2005; Adhikari et al., 2009; Chaston et al., 2011), nutritional factors may still contribute to cumulative defects and trait loss (Hopper et al., 1993). Furthermore, host diet effects may impact entomopathogenic nematode ecology, i.e., as fitness is impacted by differential nutrition, the nematode's role in community dynamics will be affected. Additional studies are needed to assess the role of host diet and other nutritional factors on entomopathogenic nematode fitness in nature and artificial settings.

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