AN ASSESSMENT OF THE EFFECTS OF INSECT HOST CONDITION ON ENTOMOPATHOGENIC NEMATODES AND THEIR SYMBIOTIC BACTERIA

by

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ABSTRACT

This study investigated a system comprised of entomopathogenic nematodes (EPN) (Steinernematidae and Heterorhabditidae), their symbiotic bacteria (X*enorhabdus, Photorhabdus*) and an insect host, *M. sexta*. The focus of this investigation was to examine the effects insect host condition on the nematode-bacterium mutualistic partnership as a whole. The EPN used in the study had varying host-searching strategies: an ambusher, *Steinernema carpocapsae*, and a cruiser, *Heterorhabditis sonorensis*. Insect host age and diet were evaluated as factors that affect insect host condition. Wandering 5th instar *M. sexta* were much less susceptible to EPN infection compared to non-wandering 5th instar larvae, regardless of EPN species. Insect host diet had a significant impact on *H. sonorensis* susceptibility to EPN infection, and a non-significant trend was observed for *S. carpocapsae*. EPN and bacterial symbiont fitness were unaffected by insect host condition. This is the first record of the effect of insect host condition on both EPN and their symbiotic bacteria.

INTRODUCTION

Entomopathogenic nematode (EPN) research was originally focused on their potential as biological control agents of insects and other arthropod pests (Tanada and Kaya, 1993). However, recognition that the bacterial symbionts associated with EPN play dual roles as both pathogens of insects and mutualists of nematodes has expanded the research focus to understanding aspects of both pathogenesis and mutualism (Forst and Clarke, 2002; Forst et al., 1997). Mutualisms are mutually beneficial relationships between a pair of species and represent one of the most significant interactions in ecology (Bronstein, 2001; Hoeksema and Bruna, 2000). Services provided by mutualists are crucial for reproduction and survival of both partners, and include pollen transfer, seed dispersal, nutrient provision, and protection from biotic and abiotic factors. The outcomes of mutualisms are quite sensitive to environmental conditions (Bronstein, 1994; Bronstein and Barbosa, 2002; Holland et al., 2005). This study used the EPN-bacteria association to measure how an extrinsic factor, insect host condition, affects the mutualistic partners involved.

For an EPN and its bacteria, the primary environment for growth and development is the insect host (see EPN lifecycle section). The insect host condition is the physiological state of the insect at the time it encounters a pathogen or parasite. Factors such as nutrition, age, and hormone titers are known to influence the physiological status of an insect. For example, it has been shown that insect immune response changes with insect age (Eleftherianos et al., 2008). It has been demonstrated that development and growth

rates of insects are affected by nutrition (Davidowitz et al., 2003). Hormone titers play a role in an insect host's ability to support parasitoids (Strand et al., 1991). These factors may also be involved in determining how well EPN and their symbionts perform within an insect host. By altering the physiological condition of the insect host, the mutualism may exhibit sensitivity and fluctuation, more commonly referred to as conditionality or context-dependency (Bronstein, 1994; Bronstein, 2001). This study focuses on insect host developmental stage as a factor that may affect susceptibility to EPN infection, and nutritional status as a factor that may affect EPN and their symbiotic bacteria.

EPN- Insect-Plant Interactions: Previous Studies

Several studies have presented evidence that the EPN-bacteria mutualistic association plays a key role in important life history events for each partner, including gaining access to insect host resources, dispersal, and gaining protection against various biotic and abiotic factors (Boemare et al., 1993; Flores-Lara et al., 2007; Sicard et al., 2004; Sicard et al., 2006; Snyder et al., 2007). Recognizing other relationships that may influence the nematode-bacterium mutualism will aid in understanding the persistence of this obligate relationship. These relationships include: 1) parasitism (between insect host and nematode); 2) mutualism (between bacteria and nematode); and 3) herbivory or predation (between insects and the host plants on which they feed). Herbivory is a factor involved in insect host condition and there is evidence that it impacts pathogenesis. For instance, the quality and chemistry of host plants are known to impact parasitism and predation. Mira and Bernays (2002) showed that higher quality host plants, assessed by host plant

availability and hatching rates of herbivores, were associated with increased rates of predation and parasitism for the insect host.

As previously mentioned, host plants can modulate interactions between insect herbivores and their pathogens, including EPN (Agrawal, 2005; Bezemer et al., 2005; Mayer, 2004; Rasmann et al., 2005). The 'bodyguard hypothesis' suggests that a plant can defend itself by recruiting the natural enemies of an herbivore via plant volatiles produced post-herbivory. For example, Rasmann et al. (2005) found that EPN were strongly attracted to *(E)*-β-caryophyllene, a compound released after damage by the western corn rootworm, *Diabrotica virgifera virgifera*. Inter- and intra-specific differences in plant chemistry and structure have been known to alter the susceptibility of insects to infection and to pathogen reproduction and persistence (Raymond et al., 2005; Young et al., 1977). Although this study showed that EPN were attracted to *(E)*-β-caryophyllene, there was no measure of how well EPN and their symbiotic bacteria developed within the insect host.

The few studies that have examined the impact of an herbivore's diet on EPN virulence and performance did not determine insect host effects on the symbiotic bacteria. Several studies suggest that insect hosts reared on a low-lipid diet are less susceptible to EPN infection (i.e. there was decreased EPN virulence) relative to those reared on high-lipid diet (Barbercheck, 1993; Barbercheck et al., 2003; Barbercheck et al., 1995). Though establishment, development and reproduction of the EPN were measured, the effect of host diet on the EPN's mutualistic partner was not examined.

A few studies have investigated the bacterial symbiont of EPN as models for pathogenesis without accounting for EPN involvement in the process. *Manduca sexta* has served as the insect host in several studies that examined the infection process of *Photorhabdus luminescens,* the bacterial symbiont of *H. bacteriophora* (Blackburn et al., 1998; Eleftherianos et al., 2008; Eleftherianos et al., 2006). Eleftherianos (2008) found that *P. luminescens* proliferate better within older 5th instar *M. sexta,* indicating that insect host age does have an influence on the bacterial symbionts in the EPN-bacteria mutualism. In this study, the bacteria were introduced artificially and there was no measure of how EPN performed in insect hosts of various ages. In addition, this investigation did not examine differences in performance between non-wandering and wandering *M. sexta*, which is a more ecologically relevant question to answer. The probability of wandering *M. sexta* encountering an EPN is much greater than a non-wandering *M. sexta*. Different physiological states (non-wandering vs. wandering) are associated with different habitats (absence vs. presence in EPN habitat) that occur within the 5th instar of *M. sexta*. Other studies have shown that there is an increase in immune defense throughout an insect's larval life (Hung and Boucias, 1996; Jiang et al., 2004).

To further investigate the effect of insect host condition on the EPN-bacteria mutualism and address the gap of knowledge described above, this study focused on 1) the developmental stage of the insect host as a parameter that could impact EPN' ability to infect a host and 2) the effects of the nutritional status of the insect host on EPN fitness and their reassociation with their symbiotic bacteria.

Objectives

Specific objectives in this project were:

Objective 1. Effect of host developmental stage on EPN performance (virulence).

Hypothesis 1: The non-wandering fifth instar of *M. sexta* will be more susceptible to EPN infection than the wandering fifth instar. Non-wandering *M. sexta* live on host plants and in the absence of EPN whereas wandering *M. sexta* are on and below the soil surface, in the primary EPN habitat. As a result, *M. sexta* are less likely to have a defense against EPN when they are in their non-wandering phase.

Objective 2. Effect of insect host diet on EPN and their symbiotic bacteria.

Objective 2.1—**To assess effects of host diet on components of EPN fitness (virulence, progeny production and emergence time)**.

Hypothesis 1: A nutritionally-challenged insect host (fed on a diet deficient in lipids, proteins and carbohydrates) will be more susceptible to EPN infection. Insects reared on a low-nutrient diet will have allocated more resources to nutrient storage than to development of an immune response, resulting in greater susceptibility to pathogens.

Hypothesis 2: EPN that successfully infect nutritionally challenged insects will have reduced reproductive fitness (in terms of progeny production and emergence time). Though EPN will infect more of the low-nutrient reared insects, there will be fewer nutrients available for growth and development of nematode progeny. Nutrients available for EPN will be depleted in a shorter amount of time when developing in an

insect host reared on a low-nutrient diet; hence, emergence time will be shorter for EPN progeny.

Objective 2.2—**To assess the effects of insect host diet on bacterial symbiont proliferation and reassociation with IJ.**

Hypothesis: Nutritionally-challenged insect hosts provide a less suitable environment for bacterial symbiont proliferation and subsequent colonization of nematode progeny. Fewer nutrients will be available for growth and development of bacteria in insect hosts reared on a low-nutrient diet resulting in lower levels of symbionts within IJ emerging from those insect hosts.

The Study System

Nematodes

"It's a nematode world." –E.O. Wilson

Nematodes are non-segmented roundworms that belong to the phylum Nematoda. Approximately 25,000 species have been described, and 500,000 to 100,000,000 species are estimated to exist worldwide (Lambshead, 2004). Nematodes are ubiquitous, occupying numerous niches and existing in nearly every available habitat on every continent, utilizing many trophic strategies and lifestyles.

The simply body plan of nematodes including a lack of cilia for motility and the presence of a pseudocoelom (a false cavity lined by mesoderm along the epidermis) were characteristics that initially indicated they were primitive. However, it is currently thought that their simple body plan is a result of a secondary reduction from more than one complex ancestral organism (Wallace et al., 1996). Therefore, nematodes are currently placed in the superphylum Ecdysozoa, along with arthropods and other organisms that build and shed cuticle, in a process called ecdysis (Aguinaldo et al., 1997).

Though the majority of nematodes are aquatic (Lambshead, 2004), there are many terrestrial species, some of which are of agricultural, veterinary and medical importance. Some terrestrial nematodes are free-living, while others parasitize vertebrates and/or invertebrates as well as plants. Plant-parasitic nematodes are of great economic

importance, as they annually cause billions of dollars in crop losses worldwide. These plant parasites are adapted to live inside or outside the plants, feeding on roots, leaves, or any other plant tissue. For example, plant parasitic nematodes in the genus *Meloidogyne*, commonly known as root-knot nematodes, invade the roots of plants, establish within the xylem and result in gall formations (Bird, 1961). Formation of galls decreases and/or stops plant nutrient uptake, debilitating a plant or causing its death. Significant losses in crop yield due to these nematodes have been reported worldwide. It has been estimated, based on an extensive international survey, that overall yield loss averages 12.3% annually and more than 20% for crops such as banana. Monetarily, these losses are estimated to exceed 100 billion dollars annually (Bird and Bird, 2001).

Among vertebrate parasites, filarid nematodes in the family Onchocercidae are of medical importance. For example, *Onchocerca volvulus* causes "river blindness," or Onchocerciasis, in humans, which results in chronic pain and vision loss. These nematodes are transmitted to humans through (*Simulium* spp.) bites. Though the relationship between *O. volvulus* and its human host is antagonistic, *O. volvulus* has a mutualistic partnership with a bacterial endosymbiont, *Wolbachia pipientis*. Recently, it has been shown that it is not the nematode but it's the secondary metabolites of the bacterial endosymbiont that causes the acute inflammatory responses that leave many affected patients blind (Taylor et al., 2000). Filarid nematodes and their bacterial symbionts are model systems for investigating mutualistic/parasitic partnerships.

Among invertebrate parasites, there are 30 nematode families that are associated with insects and other invertebrates (Stock and Hunt, 2005). Seven of these have the potential for being considered as biological control agents. Of these, the most widely studied group are the so-called "entomopathogenic nematodes", also known as EPN. These nematodes are ubiquitously distributed, both geographically and temporally, existing in many habitats, and with a wide range of tolerance to abiotic parameters such as temperature and soil moisture (Grewal et al., 1994; Hominick, 2002). At present, two nematode families within the order Rhabditida are placed in the EPN group: 1) Heterorhabditidae Poinar, 1975 and 2) Steinernematidae Travassos, 1927.

EPN Life cycle

Steinernematidae and Heterorhabditidae nematodes are entirely dependent on the internal environment of an insect host to complete their life cycle. The exception is the third-stage infective juvenile (IJ), the only free-living stage in the life cycle of these nematodes. The IJ is also the stage that actively seeks an insect host in the soil. Once the IJ finds a host, it can enter through any natural opening, including the mouth, anus or spiracles. There is also evidence that IJ of *Heterorhabditis* nematodes can enter a host through intersegmental membranes, using a cuticular 'tooth' to aid in host entry (Bedding and Molyneux, 1982). Once inside the host, *Heterorhabditis* IJ expel their symbiotic bacteria via regurgitation (Ciche and Ensign, 2003), while the bacteria associated with *Steinernema* are defecated (Snyder et al., 2007). Insect death resulting from the pathogenic, symbiotic bacteria generally occurs quickly, in approximately 24 – 48 hours

(Poinar, 1975). *Heterorhabditis* IJ develop into hermaphrodites, while most *Steinernema* spp. are amphimictic (i.e., males and females are present in the first adult generation) (see Figure 1). Though the first generation of *Heterorhabditis* is a hermaphrodite (i.e., a female that has a sperm-filled spermatheca), the second generation is amphimictic, with both males and females present that reproduce sexually.

It has been estimated that up to two adult generations of nematodes may exist within one insect host. It usually takes 15 – 21 days for heterorhabditid progeny to emerge from their host cadaver. In contrast, it takes 8 – 12 days for steinernematids to complete their life cycle within their host. Once nutrients are depleted, IJ re-associate with the bacteria and depart the cadaver in search of a new host (Akhurst, 1980). They remain in the soil (without feeding), moving through a water film (Ishibashi and Kondo, 1990) searching or waiting for a suitable host to parasitize.

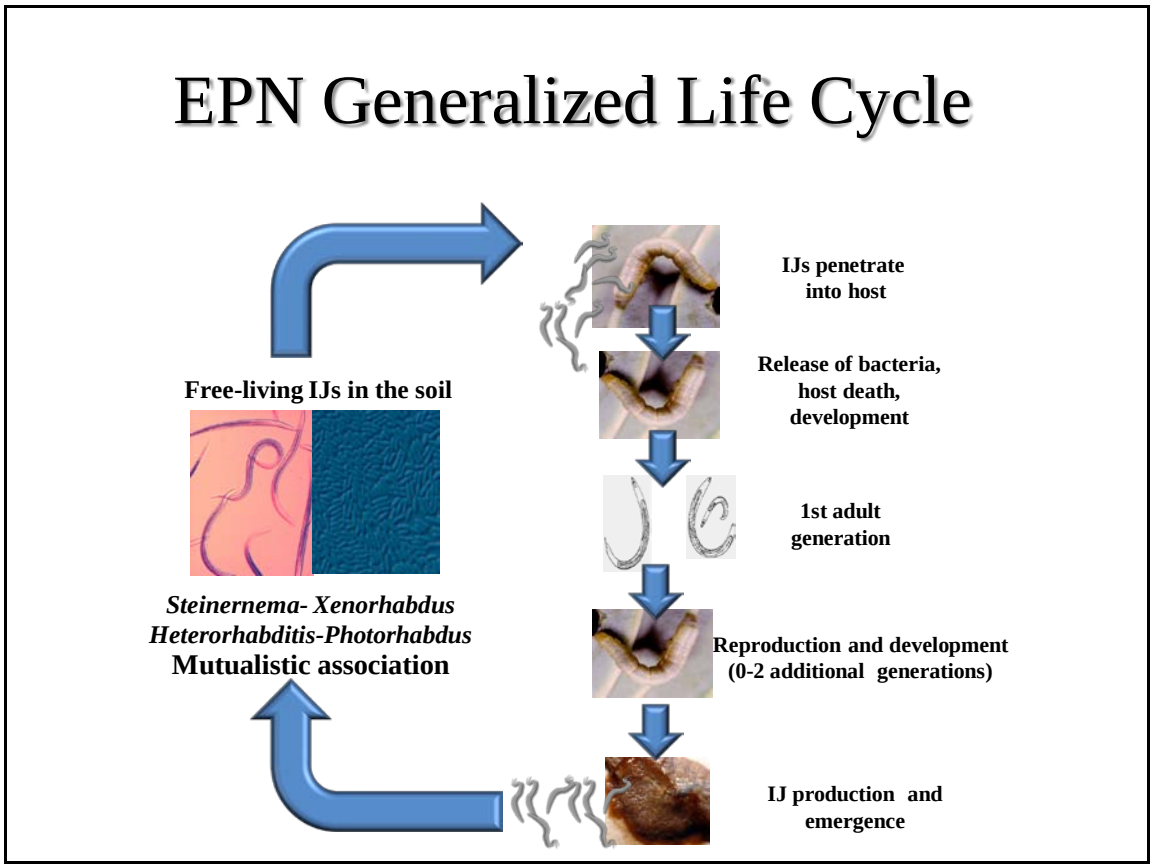

Figure 1.Generalized life cycle of an entomopathogenic nematode (S.P. Stock).

Based on host-searching strategies, EPN are classified into one of three behavioral categories: a) ambusher, b) cruiser, or c) intermediate. Ambusher nematodes live close to the soil surface, swinging their bodies (nictation) to reach active, moving insect hosts on or in the top 10 cm of the soil. Infective juveniles with an ambusher search strategy lift their bodies, wave in the air and jump to reach their insect hosts (Ishibashi and Kondo, 1990). Cruiser IJ, in contrast, tend to be found deeper (up to 28 cm for *H. bacteriophora*) in the soil (Georgis and Poinar, 1983) and actively seek their hosts primarily via CO_2 cues. IJ with intermediate behavior have features of both cruisers and ambushers.

Steinernema spp. have a wide range of host searching strategies, while *Heterorhabditis* spp. are predominantly cruisers (Campbell and Gaugler, 1993).

In addition to exhibiting different host searching strategies, the groups of bacteria associated with these EPN groups vary. EPN are associated with mutualistic, symbiotic Gram-negative Gamma-Proteobacteria in the class Enterobacteriaceae. Members of the Steinernematidae are associated with bacteria in the genus *Xenorhabdus* (Thomas and Poinar, 1979), while the Heterorhabditidae are associated with bacteria in the genus *Photorhabdus* (Boemare et al., 1993). The IJ is the only stage that carries the bacteria and is involved in this symbiosis. At this stage in the life cycle, the IJ is providing shelter and nutrition to the bacteria. It is hypothesized that the IJ houses and nourishes the bacterial symbionts to allow them to grow and multiply. The bacteria do not provide anything to the IJ at this point. *Xenorhabdus* bacteria are found within a specialized intestinal structure known as the 'bacterial receptacle' located at the anterior portion of the intestine. *Photorhabdus* are not restricted to a specific receptacle, and instead are primarily located in the anterior portion of the intestinal lumen. Once the IJ enters its insect host, its symbionts are expelled into the hemocoel. The IJ has invested in carrying and nourishing the bacteria so that it would have a killing agent upon entering the host. The bacteria then proliferate, becoming pathogenic to the insect host and killing it via septicemia (Poinar and Thomas, 1966). The bacteria contribute to the degrading of insect tissues through the production of various enzymes and secondary metabolites which prevent other bacteria from reproducing by antibiotic production (Akhurst, 1982). Once the bacteria have become established as the dominant microbe and processed tissues

within the insect host, they become the nematodes' food source, permitting nematode growth, development and reproduction. Thus, the EPN-bacteria relationship is both 1) antagonistic when IJ are consuming bacteria, and 2) mutualistic when IJ provide shelter and nourishment for the bacteria.

The similarities observed in the life history and morphological traits of these two nematode families have arisen by convergent evolution, since molecular phylogenetic studies have demonstrated that these nematode families are not closely related (Blaxter et al., 1998).

The Insect Host: Manduca sexta

Manduca sexta (Sphingidae: Lepidoptera), the tobacco hornworm, is found abundantly throughout the Americas (Mechaber and Hildebrand, 2000). Sphingid moths are economically important as agricultural pests (Lange and Bronson, 1981). *Manduca sexta* primarily feeds on plants in the Solanaceae, including, in southern Arizona, sacred Datura (*Datura wrightii*) and desert thorn-apple (*Datura discolor*). It has also been documented to feed on devil's claw (*Proboscidea parviflora*), in the family Martyneaceae (Mechaber and Hildebrand, 2000).

Manduca sexta is a model organism in insect physiology. It has been especially important in studies of hormonal regulation, neurobiology, oviposition choice, plant-insect interactions and resource allocation (Abrell et al., 2005; Baldwin et al., 2001; Bollenbacher et al., 1975; Slansky and Angelo, 1984). This insect has been used as a

model system in neurobiology because its brain is relatively large and easy to manipulate. The development and physiology of *M. sexta* is well known (D'Amico et al., 2001; Davidowitz et al., 2004). In addition to these attributes, the artificial diet that has been developed to rear this insect in the laboratory is easily manipulated to alter the nutritional composition of the diet, thereby inducing physiological differences measured by a shift in critical weight and growth rate (Davidowitz et al., 2003). Critical weight is the minimum weight in which additional feeding and growth is not required for normal development time for completion of metamorphosis (Nijhout and Williams, 1974).

The *M. sexta* life cycle includes five larval instars. From egg stage to pupation, *M. sexta*'s life cycle at 25°C with a 16L:8D photoperiod lasts approximately twenty-five days; this is then followed by a three-week pupation period, and ends with an average adult life span of about one week (see Figure 2). Larvae feed both day and night, while the adult moth is nocturnal (Bernays and Woods, 2000).

The last larval instar is very interesting from a behavioral and physiological perspective. Many changes take place subsequent to a rapid increase in prothoracicotropic hormone (Truman, 1972). At this time, the larva purges its gut, wets its body, climbs down from its host plant, and begins to wander in search of a pupation site (Truman and Riddiford, 1974).

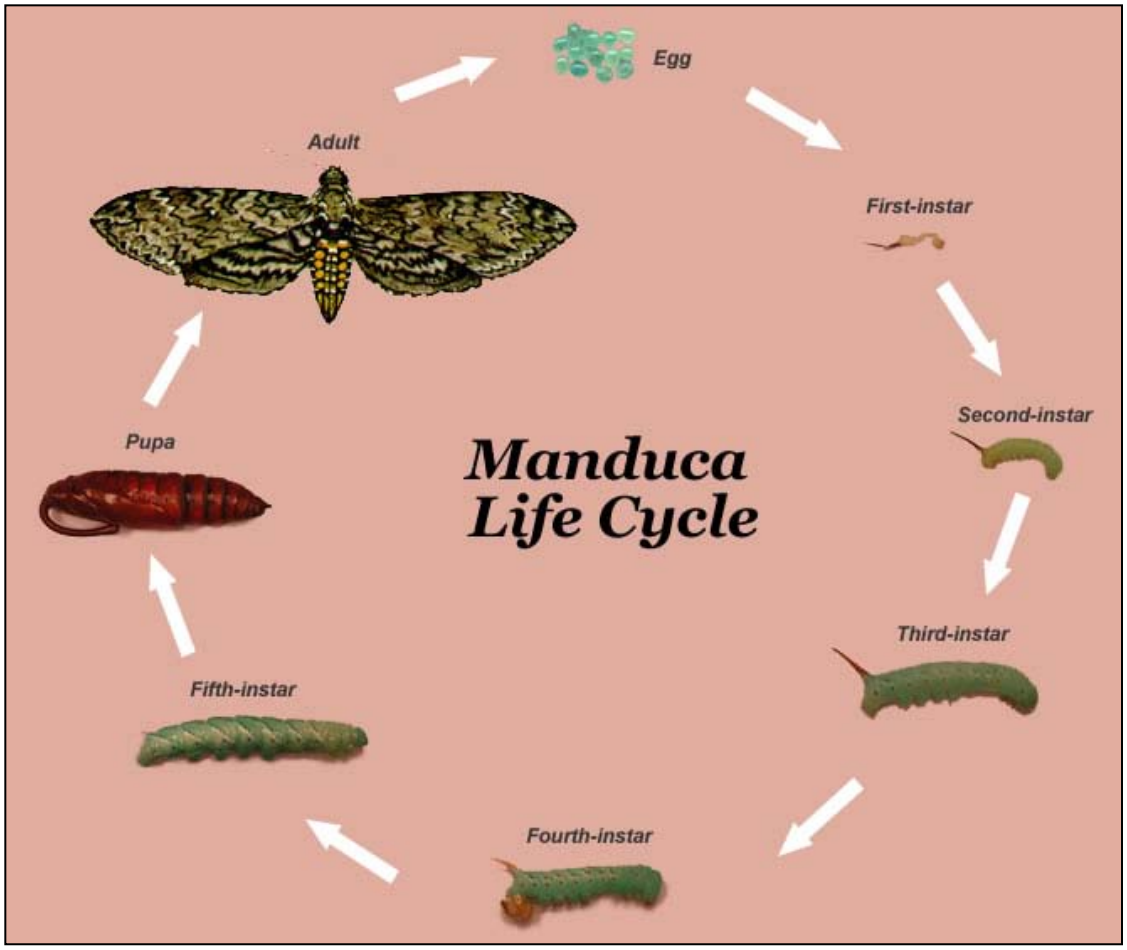

Figure 2. Life cycle of *Manduca sexta* (from www manduca.entomology.wisc.edu/images/lifecycle.jpg).

When the 5th instar larva transitions to the wandering stage, it is lighter in color and weight, the dorsal blood vessel becomes visible, and there is often a pink pigment on the periphery of the dorsal blood vessel (see Figure 3). The induction of a "wandering" phase occurs around midnight, presumably to avoid predators and extreme temperatures (G. Davidowitz, personal communication).

Figure 3. Fifth-instar larvae of *M. sexta* in non-wandering (left) and wandering (right) stages.

MATERIALS AND METHODS

Nematode Cultures and Rearing Conditions

Two EPN species were considered in this study: *Steinernema carpocapsae* (Weiser, 1954) (Steinernematidae) and *Heterorhabditis sonorensis* Stock, Rivera-Orduño and Flores, 2008 (Heterorhabditidae). Both EPN species were obtained from Dr. P. Stock's nematode collection housed in the Department of Entomology, University of Arizona. *S. carpocapsae* is a ubiquitous EPN that has been found in diverse geographic regions. Moreover, this species has been used in numerous studies so that we know a lot about its ecology and efficiency as a biological control agent. Based on its host-finding behavior, this nematode is considered an ambusher. *H. sonorensis* occurs in the state of Sonora in northern Mexico and the Chiricahua Mountains in SE Arizona, USA. This nematode is classified as a cruiser in its host-searching behavior, because it can actively seek its host. It is adapted to high temperatures and is able to tolerate and successfully infect insects at 25 – 30°C (Rivera-Orduño and Stock, in press).

Both nematode species were reared *in vivo* with last instar larvae of the greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae). *Galleria* larvae were obtained from Timberline Fisheries (Marion, IL) and stored at 15°C in a dark incubator until needed. Inoculation of *G. mellonella* larvae was performed in 'infection traps' according to procedures described by Kaya and Stock (1997). Briefly, infection traps consisted of an inverted 10 cm diameter Petri dish layered with two filter paper discs (Whatman® # 1).

One ml of nematode inoculum, containing approximately 1,000 IJ, was applied to the filter paper. Ten *G. mellonella* larvae were then placed on the filter paper soaked with the IJ suspension. The bottom of the Petri dish was used as a cover (Figure 4).

Figure 4. An example of an "infection chamber" used to inoculate *Galleria* with EPN to mass produced IJ for bioassays with *M. sexta*.

Infection traps were maintained in the dark in an incubator at 25°C for approximately 3 days. Dead insects were removed after 2-3 days. If larvae did not show signs of nematode infection, they were kept in the incubator for two additional days. Cadavers parasitized by *S. carpocapsae* had a tan/yellow color and the cadaver had a soft consistency. Cadavers parasitized by *H. sonorensis* had a brick-red coloration and a gummier consistency compared to *S. carpocapsae*. Usually, *H. sonorensis*-infected larvae required a longer incubation time until signs of EPN infection are seen, relative to *S. carpocapsae*. These differences are the result of differences in the duration of the life cycle of this EPN species.

Dead insects with signs of EPN infection were removed and placed into modified White traps (Kaya and Stock, 1997) (Figure 5).

Figure 5. A modified White trap with *S.carpocapsae*-infected *G. mellonella* cadavers.

A modified White trap consists of a 10 cm diameter, standard depth Petri dish in which the lid of a 5 cm diameter Petri dish layered with a single filter paper (Whatman® #1) is placed. The bottom of the 10 cm diameter Petri dish is then filled with 8 ml distilled water.

Up to five cadavers were placed in each White trap; hence, one infection trap was split into two White traps. IJ emergence (i.e., the time required for nematode progeny to emerge from insect cadavers) varied between EPN species. On average, *H. sonorensis* required 17 days, while *S. carpocapsae* required 10 – 13 days. When *S. carpocapsae* IJ

emerge from cadavers, they tended to emerge of the mouth and anus in thick streams—forming IJ bridges—that often needed to be moved to the water with the aid of a probe. In contrast, when *H. sonorensis* IJ emerged, IJ were simply present in the water of the White trap and IJ bridges were not observed. Following emergence, IJ were harvested every other day and up to three times.

Nematode Harvesting and Inoculum Preparation

To harvest IJ, the 5 cm Petri dish containing cadavers from individual White traps was temporarily removed, and the water containing IJ poured into a 250 ml beaker.

The volume of the beaker containing the IJ suspension was brought to 150 ml by adding sterile distilled water. Beakers were then tilted to allow nematodes to settle to the bottom for 15 to 20 minutes (Figure 6).

Figure 6. Beaker with decanting IJ (Photograph by B. Eubank).

The IJ suspension was rinsed two additional times by replacing the supernatant with fresh distilled water. Afterwards, the IJ suspension was placed into a 250 ml tissue culture flask. By visual assessment, the IJ concentration was adjusted up to 2,000 IJ/ml in a volume no larger than 60 ml. Tissue culture flasks were closed with a lid that was left slightly open to ensure ventilation. Flasks were stored in a dark incubator at 15 °C until use for the experiments.

To determine IJ concentrations for experiments, a nematode counting chamber was used (see Figure 7).

Figure 7. Nematode counting chamber used to determine IJ concentration. Each cell holds 1 ml of IJ suspension.

The initial concentration of IJ stored in each flask was first determined by creating three 1:10 dilutions. A 1 ml IJ inoculum from the 1:10 dilution was then transferred into the nematode counting chamber and the number of IJ found in that volume was determined using an Olympus BX2 dissecting microscope at 50 X magnification. Counts for each of the three dilutions were averaged and the total number of IJ in the master volume was calculated. The following equation was used to determine the concentration required for each experiment:

$$C_i V_i = C_f V_f,$$

where: C_i is the initial concentration, V_i is the initial volume, C_f is the final concentration desired and V_f is the final volume.

Two concentrations of IJ were used in all experiments: a) high dose = 200 IJ/ml and b) low dose = 25 IJ/ml. Each insect was then treated with 1 ml.

Manduca sexta Rearing Conditions

Manduca sexta was reared on artificial diet as described by (Davidowitz et al., 2003) (see Table 1 for ingredients). The following dry ingredients were added into a 1,893 ml Tupperware® container and thoroughly mixed: wheat germ, casein, torula yeast, Wesson salts, cholesterol, sucrose and Alphacel (depending on recipe). Several containers containing the dry ingredients were made in advance and stored at 4°C until the wet diet was prepared.

Manduca Diet Ingredients for 3L Diet		
Ingredient	**Optimal Standard Diet—100%**	**Low-nutrient diet—60% of optimal macro ingredients**
Wheat germ (g)	**327**	**196.2**
Casein (g)	**147**	**88.2**
Torula yeast (g)	**65.4**	**39.24**
Wesson salts (g)	**49.2**	**29.52**
Cholesterol (g)	**14.4**	**8.64**
Sucrose (g)	**129**	**77.4**
Alphacel (g)	**0**	**146.4**
Methylparaben (g)	4.2	4.2
Sorbic acid (g)	8.1	8.1
Ascorbic acid (g)	20.4	20.4
Streptomycin (g)	0.81	0.81
Penicillin (g)	3	3
Vandersandt vitmain mix (g)	2.01	2.01
Water (l)	1	1
Linseed Oil (ml)	15	15
Formalin (ml)	15	15
Agar (g)	63	63

Table 1. *Manduca sexta* artificial diet ingredients representing optimal standard diet (100%) and low-nutrient diet (60%). The low-nutrient diet contains approximately 60% of the ingredients in bold and filled with a non-nutritive bulk (Alphacel) in place of the lipid, protein and carbohydrate deficiency (from Davidowitz et al. 2003).

Small quantities of methylparaben, sorbic acid, ascorbic acid, Vanderzandt™ vitamin mix, penicillin, and streptomycin were mixed separately from the other dry ingredients in a 118 ml Nalgene® container. As with the previously described dry ingredients, several Nalgene® containers were prepared in advance and stored at 4°C.

Two antibiotics, streptomycin and penicillin, were included in the diet for the experiment that determined susceptibility to EPN infection between wandering vs. non-wandering 5th instar larvae. However, antibiotics were not used for the diet quality experiments since

certain antibiotics are known to affect EPN progeny production and interfere with their bacterial symbionts (van der Hoeven et al., 2008).

To make diet, two liters of boiling water were blended at 25-30 rpm in an industrial blender. Agar was then added (63-67g) and blended for 20 seconds until the agar dissolved. To prevent the diet from being too soft, the agar was adjusted. If the diet is too soft, it is then too moist, and neonate larvae can drown when presented with such a diet. An additional 1L of tap water was added to the blender, while increasing the speed to 45 rpm. This was followed by the addition of 15 ml of formalin and 15 ml of linseed oil. Once the dry diet ingredients were added to the blender, the speed was gradually increased to 70-75 rpm. The vitamin mix was added afterwards, as it would denature if added when the temperature was too high. The mixture was blended for approximately 30 seconds to ensure complete incorporation of all ingredients. Finally, the diet was poured into a 3L rectangular Tupperware container. The diet was then placed into a 4°C refrigerator, with the lid on loosely, to decrease condensation in the container and to allow diet to solidify for approximately 12 hours before use. Diet was stored in the refrigerator for a maximum of 2 weeks after being prepared.

One-day old eggs (see Figure 8) were obtained from the J. Hildebrand laboratory (Department of Neurobiology, University of Arizona). Eggs were split into 3 – 4 containers and placed in a 25°C walk-in temperature controlled chamber set to a 16L: 8D

photoperiod. Artificial diet was placed in the rearing containers 2 days later. Eggs hatched 4 days after being laid.

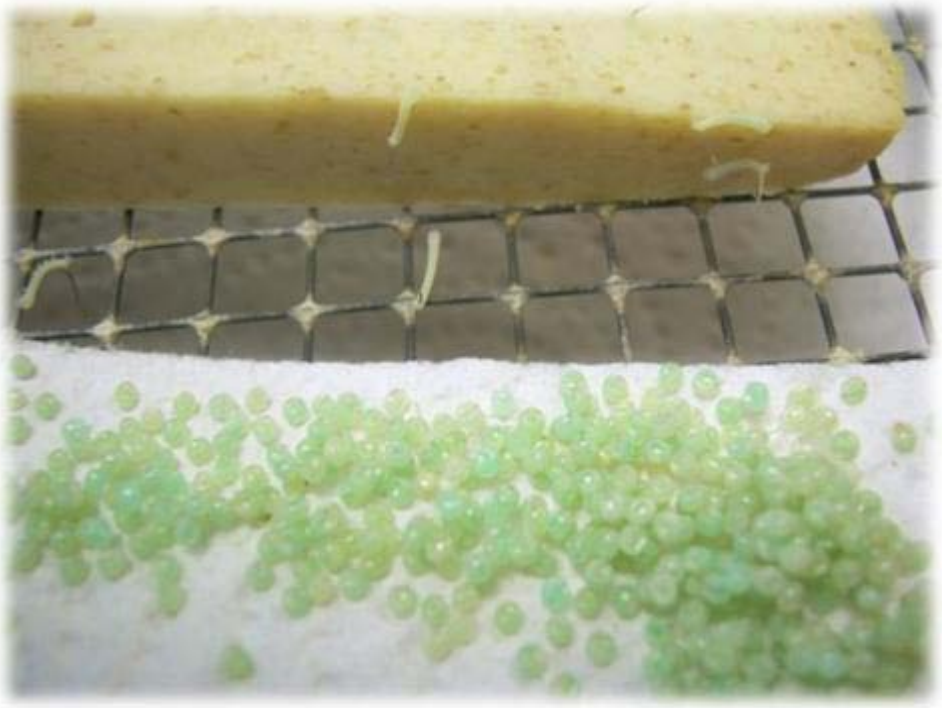

Figure 8. *Manduca sexta* eggs and hatchlings crawling onto artificial diet (photograph by R. Lipson).

The containers used for rearing were 33.02 x 22.86 cm Teflon pans lined with a paper towel. A metallic mesh screen 19 x 27 cm (folded so that it was raised 2.5 cm) was placed on top of the paper towel. Two mesh sizes were considered depending on the size of the *M. sexta* larvae. A smaller mesh (0.5 cm) was used for eggs and 1^{st}- 4^{th} instar larvae, while 5^{th} instars were placed on a larger mesh (1.0 cm). Eggs were placed on top of the mesh. Lids with ventilation holes were used to cover the containers and secured larvae within the container. Paper towels were changed daily and diet was replaced every

1-2 days as needed. Fifth instar *M. sexta* larvae were placed on either of two diet treatments: a) optimal standard diet, which was 100% of all ingredients described above and b) reduced quality diet, which was a 60% reduction in the proportion of macro-ingredients (see Table 1) in the standard diet. Vitamin proportions remained the same for both treatment groups. Insects were maintained in each diet treatment for approximately 67 hours.

The strategy of changing insect host diet at the end of the fourth larval instar was based on preliminary studies conducted by Davidowitz et al. (2003) that showed a significant change in physiological performance when provided diet variations at this point in the lifecycle. Insects could not be reared in low quality diet from hatch because of high mortality (G. Davidowitz, personal communication).

Methods

Objective 1. Assessing the effects of insect host developmental stage on susceptibility to EPN

Three-day old *M. sexta* 5^{th} instar larvae (non-wandering) and wandering larvae were used in all experiments. One to two week old (post harvesting age) IJ of each of the EPN species were used in all experiments. Insects were treated individually. The experimental arena consisted of 270 ml clear plastic Solo® cup with a straw-slit lid for gas exchange. Containers were filled with 100g of moistened (10% w/v) autoclaved silica sand. As mentioned previously, two EPN concentrations were used: a) low (25

IJ/ml) and b) high (200 IJ/ml). Insects were weighed and placed individually in each cup prior to inoculation.

A 1ml volume of IJ suspension, either low or high dose, was applied to the sand after placement of insect larvae. Controls received 1 ml of sterilized, distilled water. The order of treatment was completely randomized within diet treatment. Once inoculated, containers were capped with lids that contained a straw hole for ventilation. They were then placed in a 25°C dark incubator. Insect host mortality was assessed over a 5-day period. Insects infected with *S. carpocapsae* infection appeared yellow-green in color while those infected with *H. sonorensis* were pink in the anterior region followed by red after another 24 hours (Figure 9).

Figure 9. Live and EPN-killed *M. sexta* larvae showing signs of EPN infection.

M. sexta mortality was measured with insect stage as the main factor and EPN dose as the subplot factor. For analysis, a split-plot, randomized, complete block design was used. Fifteen insects were used in each insect stage/EPN dose treatment group and the experiments were replicated three times. Time was a random effect present in the model, while diet type and EPN dose were fixed effects; hence, the data were analyzed using a mixed-model ANOVA.

Objective 2.1. Effect of insect host diet on EPN performance and their symbiotic bacteria

Assessing the effect of insect host diet on EPN virulence

Three-day old *M. sexta* 5^{th} instar larvae were used in all experiments. The experimental arena, method of treatment, and signs of infection were the same as previously described. The average weight of insects fed on the reduced--nutrient diet was 0.86 g less than the insects reared on the optimal standard diet (Table 2) (ANOVA, $F_{1,94} = 16.31$, $P < 0.0001$). To ensure that the average weight of the two diet groups was represented, all insects available from each diet group were weighed and the distribution of weights was mapped—the first to third quartile range of larval weights were used in each experiment. The weight used for the low-nutrient treatment group was 3 – 4.5 g while the optimal standard diet group weight range used was 4 – 6 g.

Diet Type	**Weight ± SE (g)**
Low-nutrient	4.036 (± 0.124)
Optimal Standard	4.897 (± 0.177)

Table 2. Weight differences between diet treatment groups. The optimal standard diet insects were fed the 100% recipe while the low-nutrient diet insects were fed the 60% diet recipe.

For analysis, a split-plot, randomized, complete block design was used. The main plot factor was diet type and EPN dose was the subplot factor. Fifteen insects were used in each diet type/EPN dose treatment group and the experiments were replicated six times. Time was a random effect present in the model, while diet type and EPN dose were fixed effects.

Mortality was not corrected using Abbott's formula since control mortality was never greater than 10% in any replicate. Mortality was arcsine-transformed as a result of the proportions ranging from 0% - 100%, and EPN dose and diet type were subjected to a type of mixed-model analysis of variance (partially-nested ANOVA) using JMP Statistical Software, v8 (SAS Cary, NC). When the factor was significant ($P < 0.05$), differences among means were determined by a least square means comparison test—Tukey's HSD. The data was arcsine transformed before analysis, but the untransformed values are reported.

Assessing the effect of insect host diet on EPN time to emergence and progeny production

Once dead insects were observed, they were kept in a 25°C dark incubator for one additional day. Afterwards, insects were rinsed in distilled water and transferred to modified White traps as described above. Insects in White traps were checked on a daily basis to record the first day IJ emerged from cadavers. Emerging IJ migrated into the water. IJ were collected in 50 ml tissue culture flasks every day from the modified White traps for a total of 10 days. For *S. carpocapsae*, there was one flask per White trap, while *H. sonorensis* had such great progeny production that several flasks were used. The total number of IJ emerged per cadaver was recorded. In *S. carpocapsae*, few IJ emerged and they were harvested after the 10-day period. *H. sonorensis* experienced high progeny production; therefore, IJ were harvested every other day for a total of 10 consecutive days. As a result of various mortality percentages for each treatment group, sample size

(*n*) for each nematode species and diet combination was different. The average time to emergence and the number of progeny (IJ) produced was calculated for each diet/EPN inoculum combination for each replicate, hence $n = 6$ for all analyses. Whether time to emergence or progeny production was measured, the average number per host individual per treatment group was subjected to analysis of variance (ANOVA) using JMP Statistical Software, v8 (SAS Cary, NC) similar to the design in estimating differences in mortality, with original values reported.

Objective 2.2—The Effect of insect host diet on EPN symbiont reassociation

Isolation of bacterial symbionts from EPN progeny

Nematode progeny resulting from the experiments performed in Objective 1 were used to assess IJ colonization by bacterial symbionts. Initially, IJ production from each treatment group (by diet type and EPN dose) was going to be measured in these experiments. However, for *S. carpocapsae*- infected cadavers treated with the low inoculum, the mortality and IJ production were too low to generate a sufficient IJ source to account for symbiont proliferation data. For *H. sonorensis* experiments, the cfu/IJ was measured in the High EPN group, as a result of no to low mortality for the Low EPN group. Due to the various levels of mortality and IJ production mentioned above, we only considered high EPN dose with $n = 12$ for each treatment.

To measure bacterial colonization of IJ, 1-ml aliquots of IJ suspension (2 – 3 weeks old) were used. IJ suspension was transferred to a 1.7 ml microcentrifuge tube. Two 1-ml

aliquots were used from each hosts' emerging progeny. Microcentrifuge tubes with IJ suspension were spun down at 13,000 rpm for 6 minutes in a Sorvall® "Pico" model tabletop microcentrifuge. The supernantant (950 µl) was removed and the IJ pellet was rinsed with 1% sodium hypochlorite solution to sterilize the EPN cuticle.

IJ were left in this solution for 2 minutes at room temperature. Then, the IJ/sodium hypochlorite mix was spun down again (same time and centrifugation speed were used throughout this process). The supernatant (approximately 950µl) was carefully removed and IJ were then rinsed in 1X phosphate-buffered saline (PBS) solution twice, following the above mentioned procedure. After the second rinse in PBS, the IJ pellet was examined to determine the volume of PBS to re-suspend the pellet. Depending on the size of the pellet, IJ were resuspended in 100 – 700 µl PBS. Then, IJ concentration was determined by examining 5 µl droplets (6 droplets were averaged for counting; see Table 2 for an example).

Between each sample, the tube was gently vortexed to re-suspend IJ. IJ tended to settle quickly and it was very important to subsequently briefly vortex each sample to maintain homogeneity of the sample. After determining IJ concentration, the volume required to have 200 IJ was transferred to a new 1.7 ml microcentrifuge tube. To this tube, Luria Berthani (LB) broth was added to bring the volume to 250 µl.

8 April 10-- Expt S4_3 Diet Quality Expt. with *X. nematophila*		Count 1	Count 2	Count 3	Count 4	Count 5	Count 6	Average/5 µL	Vol. (µL) to ADD to grind tube	Vol. (µL)LB to Add = 250 - vol. to add to grind	Vol. (µL) resuspended in PBS to count sample
Treatment	Sample #										
Low Diet/ HighEPN	77_1	10	16	22	16	11	25	16.7	60.0	190.0	250
Low Diet/ HighEPN	77_2	21	22	26	12	22	20	20.5	48.8	201.2	250
Low Diet/ HighEPN	85_1	18	14	11	20	24	26	18.8	53.1	196.9	350
Low Diet/ HighEPN	85_2	32	8	24	20	21	16	20.2	49.6	200.4	350
Low Diet/ HighEPN	90_1	12	10	19	19	12	12	14.0	71.4	178.6	200
Low Diet/ HighEPN	90_2	16	10	15	10	11	10	12.0	83.3	166.7	200

Table 3. An example of determining IJ concentration for experiments estimating the number of bacteria per IJ in order to measure symbiont reassociation. Each count is the number of infective juveniles in a 5 µL sample.

Counting bacterial symbiont colony-forming units (cfu)

To allow release of bacterial symbionts, the LB-IJ suspension was throughly ground with a Kontes® motorized grinder and a sterile pestle for 2 minutes. For experiments involving *Xenorhabdus nematophila* (the bacterial symbiont of *S. carpocapsae)*, 50 µl of the homogenate was transferred to 950 µl LB, resulting in a 1:20 dilution. For those experiments that involved *Photorhabdus luminescens* (the symbiotic bacteria of *H. sonorensis*), 25µl of the homogenate was added to 975 µl of LB broth, which resulted in

a 1:40 dilution. This difference was a direct result of the variation in the number of cfu/IJ. A range of 30 – 300 colonies on each plate was desired. Justification for the acceptable cfu range was based on the fact that < 30 cfu increases chances in sampling error, while plate overcrowding is very common with > 300 cfu on a plate, making single colony isolation difficult.

Once the bacterial dilution was made, it was vortexed and 50 µl of the sample was plated onto 3 LB agar plates supplemented with 1% sodium pyruvate and 30 µg/ml ampicillin salts for *P. luminescens* and 50 µg/ml ampicillin salts for *X. nematophila*. Sodium pyruvate was added in LB agar when plating EPN symbiotic bacteria because in its absence, fluorescent light exposure results in the production of toxic photoproducts, including free oxygen radicals, which are toxic to the bacteria and alter bacterial colony numbers (Xu and Hurlbert, 1990). In addition, ampicillin salts were used because they dissolve in LB agar faster than non-salt forms. Plates were kept in a 30°C dark incubator for 24 hours. After this period, the total number of cfu on each plate was counted.

The following equation was used to determine the number of cfu per IJ:

$$\text{Number of cfu} = \frac{\text{avg. \# of cfus per plate} * \text{dilution factor} * \left[\frac{\text{totalgrindvol}}{\text{vol plated}}\right]}{\text{\# IJ in total grind vol.}}$$

RESULTS

Objective 1. Effect of insect host developmental stage on susceptibility to EPN infection

Preliminary assays were conducted to assess what insect stage was most suitable for addressing our objectives. Fifth instar larva *M. sexta* would most likely encounter EPN naturally, since they both occupy a soil habitat. This instar has a "wandering" and a "non-wandering" phase, previously described in the insect host section. The "wandering" 5th instar was originally considered to be the most ecologically suitable stage because it is when the larva leaves the host plant and moves on and below the soil surface in search of a pupation site.

Assays showed that 5th instar larvae in the wandering phase inoculated with *S. carpocapsae* are very resistant to EPN infection (ANOVA, $F_{1,\ 2}$ = 34.00, *P*-value = 0.0282) (Figure 10). In addition, there was a non-significant trend that wandering larvae exposed to *H. sonorensis* were more resistant to EPN infection relative to earlier 5th instars (ANOVA, $F_{1,\ 2}$ = 9.4805, *P*-value = 0.0913). As a result, we conducted our experiments with 5th instar larvae in earlier stages of development.

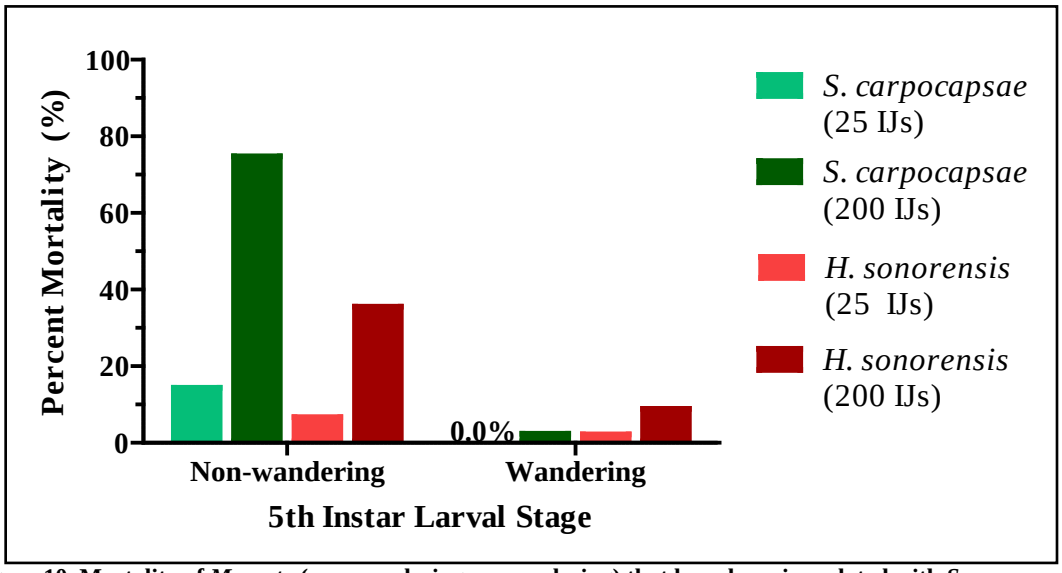

Figure 10. Mortality of *M. sexta* (non-wandering vs. wandering) that have been inoculated with *S. carpocapsae*, an ambusher EPN, or *H. sonorensis*, a cruiser EPN.

Objective 2. Effect of insect host diet on EPN and their bacterial symbionts

Objective 2.1—To assess effects of host diet on components of EPN fitness (virulence, time to emergence and progeny production).

EPN virulence

Steinernema carpocapsae

There was a non-significant trend that nutritionally-challenged insects were more susceptible to *S. carpocapsae* infection than were larvae reared on the optimal standard artificial diet (ANOVA, $F_{1,5}$= 5.03, P = 0.0750) (Figure 11). For all nematode dose/diet type combinations used in this study, mortality ranged from moderate (46.6% for low inoculum) to high (92.8% for high inoculum). Observed insect mortality was

significantly lower for insect hosts given the low EPN inoculum relative to those given the high EPN inoculum (ANOVA, $F_{1,10} = 25.25$, $P = 0.0005$).

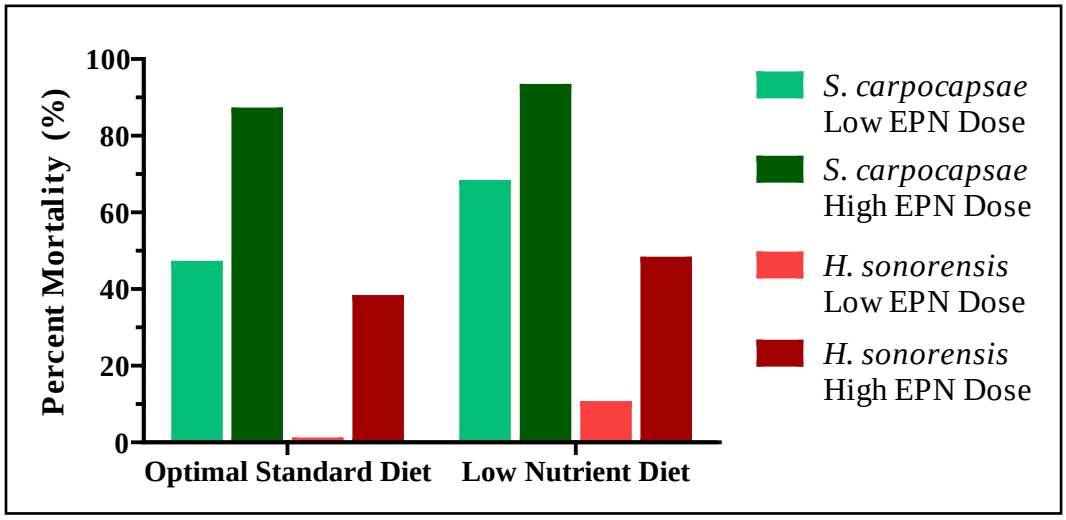

Figure 11. Mortality of *M. sexta* reared on either 1) optimal standard diet or 2) low-nutrient diet that have been inoculated with *S. carpocapsae*, an ambusher EPN, or *H. sonorensis*, a cruiser EPN.

Heterorhabditis sonorensis

Diet had a significant impact on *M. sexta* susceptibility to *H. sonorensis* (ANOVA, $F_{1,5}$= 45.76, P= 0.0011) (Figure 11). For the low EPN inoculum, mortality was higher when insects were fed on the low-nutrient diet (10.0%) compared to those fed with the standard diet (0.5%). The high EPN dose showed that insects reared on low-nutrient diet were associated with an increase in mortality by 10.0% relative to those reared on the optimal standard diet. In addition to diet type having a significant impact on insect susceptibility to *H. sonorensis*, the effect of EPN inoculum was also significant (ANOVA, $F_{1,10}$ = 85.98, P<0.0001), with insects that received the high EPN inoculum experiencing higher mortality than those that received the low inoculum.

Overall, the nutritional status of the insect host (i.e., low vs. standard nutrient diet) had an impact on host susceptibility to EPN. For both EPN species and both inocula tested, mortality was higher for *M. sexta* larvae fed with the low nutrient diet than for those fed the standard diet. This result was significant for *H. sonorensis* ($P = 0.0011$), and a strong trend was observed for *S. carpocapsae* ($P = 0.0750$).

When comparing EPN species, *M. sexta* larvae were more susceptible to *S. carpocapsae* than to *H. sonorensis*. For example, mortality for nutritionally-challenged insects ranged from 10.0% to 47.7% when exposed to *H. sonorensis.* In contrast, for *M. sexta* exposed to *S. carpocapsae,* mortality ranged from 67.7% to 92.8%. Similarly, for insects fed with the standard diet and exposed to *H. sonorensis*, mortality ranged from 0.55% to 37.7% while for those exposed to *S. carpocapsae* it ranged from 46.6% to 86.6%.

Time to Emergence

Time to emergence is the number of days required for progeny to emerge from a cadaver. This time was calculated from the day of IJ inoculation (Day 1) to the first day IJ were first observed emerging from the cadaver. For all experiments, time to emergence data were normally distributed. Therefore, no transformation was necessary.

Steinernema carpocapsae

For *S. carpocapsae*, there were no significant differences observed in IJ emergence time from cadavers with respect to diet quality ($F_{1,5}$= 0.7359, P = 0.4302) (Figure 12). However, emergence time was 2.2 days shorter for insects infected with the higher EPN inoculum ($F_{1,10}$ = 8.1858, P = 0.0169). The average emergence time for insects fed on either low or high quality diet ranged from 17 days (for optimal diet/high nematode dose) to 20 days (for optimal diet/low nematode dose).

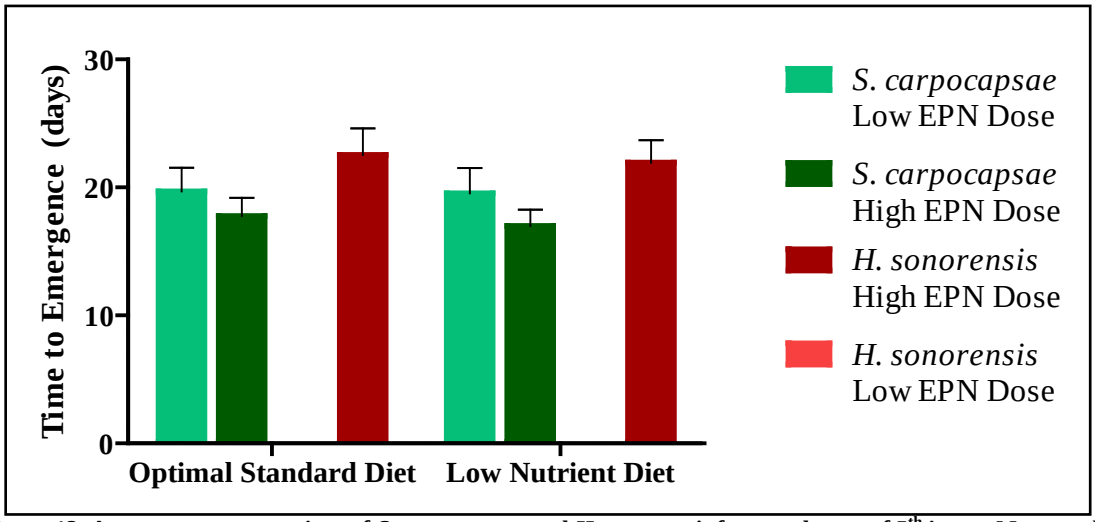

Figure 12. Average emergence time of *S. carpocapsae* and *H. sonorensis* from cadavers of 5th instar *M. sexta* that were reared on either optimal standard diet or low-nutrient diet. Each error bar represents 1 SE above and below the mean. No significant differences were observed between diet types.

Heterorhabditis sonorensis

For this nematode species, very low mortality was observed in the high diet and low EPN dose treatment group (overall mortality with all combined replicates for that treatment group was 0.10%; only 1 insect succumbed to EPN infection in this group). Because of the low sample size in the "high diet, low EPN dose" treatment group, only "high EPN

dose" was compared between diet types. Similar to results obtained with *S. carpocapsae*, diet did not affect the time it took IJ to emerge for either insect diet quality (ANOVA, $F_{1,5} = 0.1205$, $P = 0.7426$) (Figure 12). The average number of days for IJ emergence from insects reared on high quality diets was 22.5 days, while it was 21.9 days for those *M. sexta* larvae reared on low quality diet.

Nematode Progeny production

Progeny production was defined as the total number of IJ that emerged from a cadaver in a given period of time. We calculated progeny production for the first 10 days after initial emergence.

Steinernema carpocapsae

Progeny production by *S. carpocapsae* nematodes did not differ as a function of host nutritional status ($F_{1,5}= 0.0479$, $P = 0.8354$). EPN inoculum had a significant effect on progeny production ($F_{1,10} = 7.0393$, $P = 0.0242$), with an average of 8,262 more IJ produced from a high nematode inoculum relative to a low dose (linear contrast, $t_{10} = 2.6532$, $P = 0.0242$) (Figure 13). The range in progeny production for *S. carpocapsae* from *M. sexta* larvae in either diet type ranged from 5,960 IJ (optimal standard diet/low EPN dose) to 19,003 IJ (optimal standard diet/high EPN).

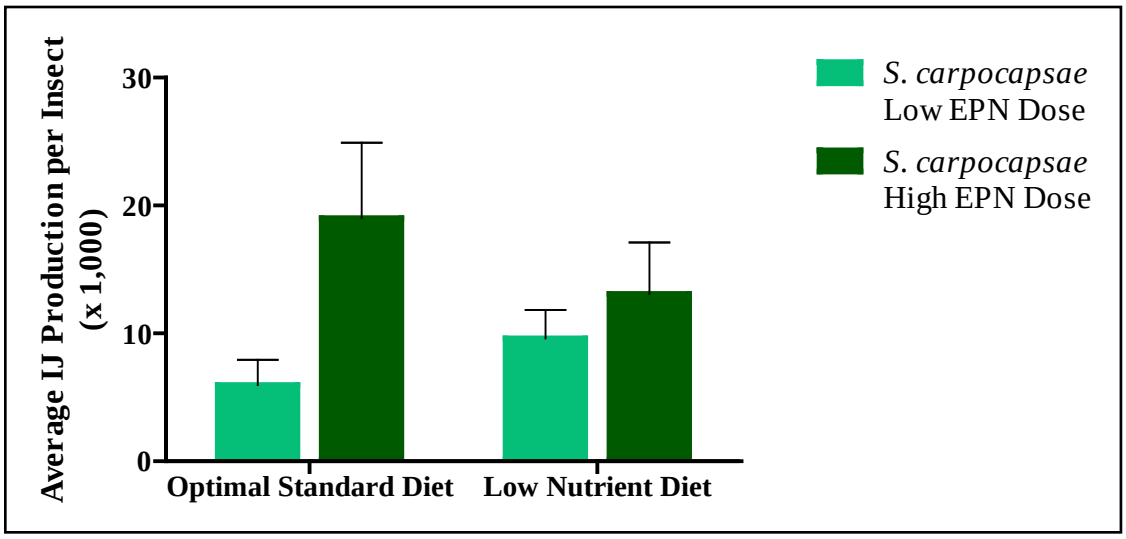

Figure 13. Nematode progeny production from *M. sexta* larvae (Mean ± SE) reared on low vs. high quality diet and different inocula of *S. carpocapsae*.

Heterorhabditis sonorensis

As mentioned previously, only the high EPN dose was used in analysis of emergence and progeny production for *H. sonorensis*. Progeny production in *H. sonorensis* was not significantly related to host diet quality ($F_{1,5} = 0.1205$, $P = 0.7426$). On average, there were 314,951 +/- 125767 IJ, (Mean +/- 1 SE) per *M. sexta* reared on optimal diet and 345,076 +/- 151534 IJ, (Mean +/- 1 SE) per *M. sexta* reared on low-quality diet (Figure 14). The large standard error for each group indicated high variation in IJ production.

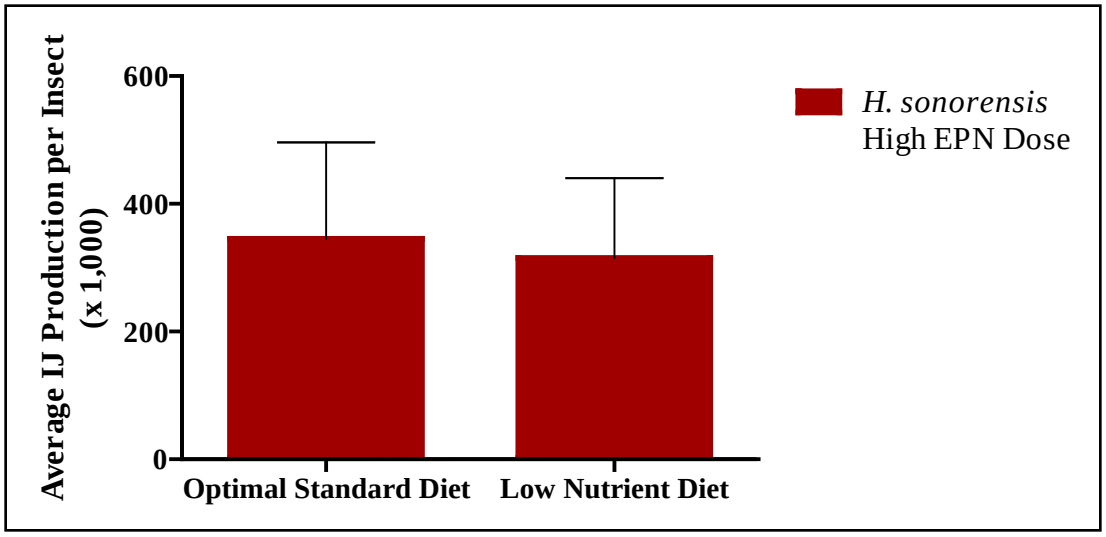

Figure 14. Nematode progeny production per insect (Mean ± SE) of *H. sonorensis* from *M. sexta* insect hosts reared on low-nutrient vs. optimal standard diet. Average IJ production was not significantly different.

There were extreme differences in IJ production between EPN species, regardless of the nutritional status of the insect host. The range in progeny production for *S. carpocapsae* from *M. sexta* larvae in either diet type was 5,960 IJ (optimal standard diet/low EPN dose) to 19,003 IJ (optimal standard diet/high EPN), while the range in progeny production for *H. sonorensis* from *M. sexta* was 314,951 IJ (low-nutrient diet/high EPN dose) to 345,075 IJ (optimal standard diet/high EPN dose).

Objective 2.2—To assess the effects of insect host diet on IJ bacterial symbiont reassociation.

The number of bacterial cells per IJ was measured (cfu/IJ) to determine how the symbiont population was affected by the insect hosts' diet quality. As previously indicated, no

progeny were collected from “low EPN dose” infections for *H. sonorensis*. Therefore, only progeny collected from high nematode dose inoculations was evaluated.

This parameter was normally distributed and therefore not transformed for analysis. Here, the “high EPN dose” treatment group was compared between diet types using linear contrast with diet type as the main factor.

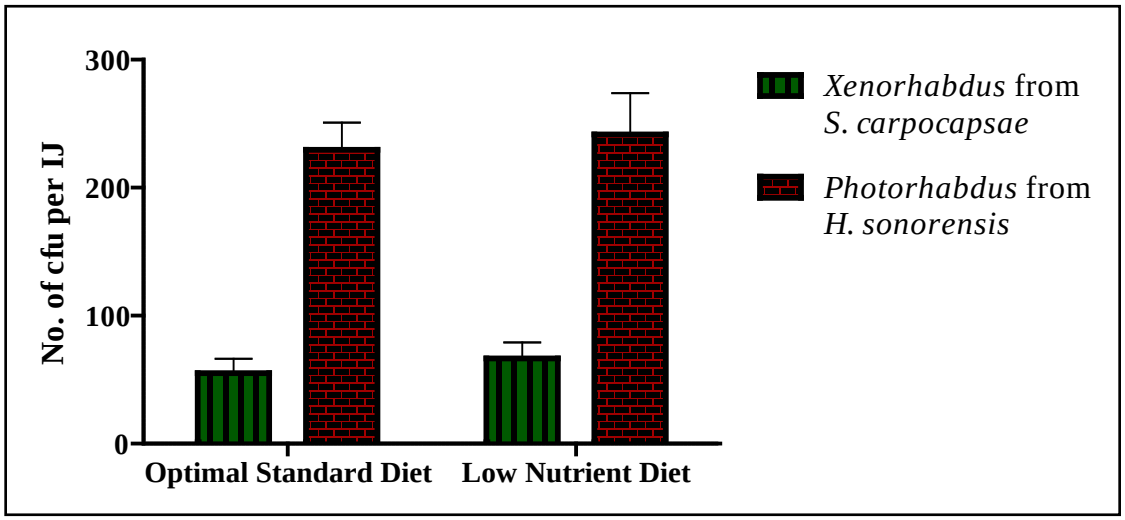

Figure 15. Average number (Mean ± SE) of cfu (*X. nematophila* cfu per *S. carpocapsae* IJ or *P. luminescens* per *H. sonorensis* IJ) emerging from *M. sexta* cadavers reared on either low-nutrient or optimal standard quality diet and exposed to the high nematode inoculum.

S. carpocapsae – X. nematophila

Nutritional status of the host was not a significant factor in determining the number of cfu isolated per IJ (linear contrast, $t_{16} = -0.625$, $P = 0.5408$). The general trend was that the average number of *X. nematophila* cfu per *S. carpocapsae* IJ from *M. sexta* reared on optimal standard diet (55.0 cfu/IJ) was lower than the cfu per IJ emerging from *M. sexta* reared on low-nutrient diet (66.5 cfu/IJ) (Figure 15).

H. sonorensis – P. luminescens

Similar to results obtained from S. *carpocapasae – X. nematophila* assays, nutritional status of the host did not have an effect on number of bacterial symbionts obtained per IJ (linear contrast, t_{20} = -0.331, P = 0.7443). In addition, the trend observed was that the number of *P. luminescens* per *H. sonorensis* IJ was lower if they emerged from *M. sexta* reared on optimal standard diet (229.5 cfu/IJ) than from those reared on the low-nutrient diet (241.6 cfu/IJ) (Figure 15).

In summary, there was a trend for both nematode species to have fewer cfus per IJ when they emerged from *M. sexta* reared on the standard diet relative to those reared on the low-nutrient diet. In addition, there was a trend for fewer IJ to emerge from insects reared on the low-nutrient diet compared to those reared on the optimal diet.

DISCUSSION

Insect host condition, the physiological state of an insect host at the time it encounters a pathogen or parasite, was investigated as a factor that could impact EPN and their symbiotic bacteria. Specifically, the current investigation examined *Manduca sexta* developmental stage and diet as factors that could impact susceptibility to EPN infection. In addition, this study measured the effect of *M. sexta* diet on the fitness of both EPN and their symbiotic bacteria. Both developmental stage and diet were found to be significant factors in determining the susceptibility of *M. sexta* to EPN infection. Yet, the fitness of EPN and their bacteria were not affected by *M. sexta* diet.

The current study showed that non-wandering *M. sexta* are relatively more susceptible to EPN infection when compared to the wandering stage. This is the first record of variation in susceptibility to EPN infection within the 5^{th} instar of an insect host's life cycle. In preparation to pupate, a hormonal spike in 20-hydroxyecdysone at the end of the 5^{th} instar stimulates *M. sexta* to change from a non-wandering stage to a wandering stage. At this time, *M. sexta* purges its gut and ceases feeding. This physiological transformation is associated with a behavioral shift as *M. sexta* transitions from a relatively sedentary larva that resides on its host plant to a wandering larva that occupies a soil habitat, on and below the ground. Once this transition has occurred, *M. sexta* becomes exposed to a completely different set of entomopathogens. Many entomopathogens have arboreal stages for dispersal that provide access to a host. However, EPN are soil-dwelling organisms and they only gain access to their host on or

below the ground surface (Ishibashi and Kondo, 1990). Variation in *M. sexta* immune defense mechanisms associated with a different entomopathogen profile is one of coping with habitat transition. Indeed, *M. sexta* does vary in its immune defense within the 5th instar.

There is evidence of variability in immune defense mechanisms by *M. sexta* throughout its 5th instar. Published studies conducted by (Jiang et al., 2004) showed that wandering 5th instar *M. sexta* larvae have a pattern recognition protein (PRP), β-1,3-Glucan recognition protein-2 (βGRP-2), that is specific to the wandering stage. βGRP-2 recognizes and binds to gram-negative and gram-positive bacteria, as well as to yeasts and fungi. In insects, PRPs identify foreign material and stimulate the prophenoloxidase cascade, which is the precursor for insect immune response. The unique aspect of this specific pattern recognition protein (βGRP-2) is that it is only present in the latter portion of the 5th instar of the *M. sexta* life cycle. The prophenoloxidase cascade and PRPs are not unique to insects, but shared with other invertebrates including crayfish and earthworms (Bilej et al., 2001; Lee et al., 2000). Because of the similarity in pathways involved in immune response for *M. sexta* as well as other invertebrates, *M. sexta* could serve as an immune response model for invertebrates in general.

With respect to insect host diet, this study showed that EPN virulence impacted by the nutritional status of *M. sexta*. *M. sexta* mortality due to EPN infection was higher for those insects reared on the low-nutrient diet. However, the nutritional status of *M. sexta* did not have a negative impact on the fitness of EPN or their bacterial symbiont. We

predicted that insect hosts reared on low-nutrient diet would be more vulnerable to EPN infection, and that EPN progeny production and bacterial reassociation would be negatively affected by such a diet. This would most likely have been a direct result of fewer nutrients available to the EPN and its bacterial symbiont. Research by Davidowitz et al. (2003) has shown that *M. sexta* reared on lower quality diets weigh less and experience delays in physiological development. In the current investigation, the expected weight difference between those reared on low vs. optimal nutrient diet did exist. Our data showed that *M. sexta* larval mortality due to EPN infection was higher for insects reared on low-nutrient diet, regardless of the EPN species. This result was significant for *H. sonorensis*, and a non-significant trend was observed for *S. carpocapsae*. The difference between EPN species could be due to *H. sonorensis* utilizing a cruiser host-searching strategy while *S. carpocapsae* is an ambusher EPN. Host-searching strategies of EPN determine the which hosts they are most likely to infect. Sedentary insects are usually more susceptible to cruiser EPN while active, moving insects are more likely to be infected by ambusher EPN.

Previous studies that investigated EPN performance relative to insect host diet quality found that insects reared on low-lipid diets were generally more susceptible to EPN infection (Barbercheck et al., 1995). However, lipid supplementation of insect host diet leads to increased susceptibility to some EPN species (Shapiro-Ilan et al., 2008). Shapiro-Ilan et al. (2008) found that yellow mealworms, *Tenebrio molitor*, reared on diets supplemented with lipids from canola, corn, peanut, pork, or salmon were more susceptible to *Heterorhabditis indica* relative to those reared on a low-lipid diet.

However, lipid supplementation did not affect *T. molitor* susceptibility to *S. riobrave* (Shapiro-Ilan et al., 2008). In this study, despite differences in EPN susceptibility, there were no differences in EPN fitness, which was measured via nematode progeny production. The current study also found that nematode progeny production was unaffected by diet type. Differences in susceptibility to EPN infection based on insect host diet also depend on the EPN species and isolate.

It can be speculated that *M. sexta* consuming fewer lipids would allocate more resources towards storage rather than growth and development when presented with a low-nutrient diet, since this is true for other organisms (Slansky and Angelo). There is also evidence that *M. sexta* reared on the low-nutrient diet have higher lipid content relative to those reared on the optimal standard diet (Davidowitz, unpublished). In general, the main food reserve for the non-feeding infective stages of many plant- and animal-parasitic nematodes is neutral lipid (Storey, 1983; Storey, 1984). In this respect, Armer et al. (2004) found that the lipid supplementation (in the form of olive oil) to Colorado potato beetle hemolymph allowed for nematode reproduction while no growth and/or reproduction was observed without the addition of lipids. It is clear that one of the most crucial nutritional components for successful growth and development of both EPN and their bacterial symbionts is lipid availability. The possible higher lipid content in *M. sexta* reared on the low-nutrient diet could explain why there were no differences in IJ production or symbiont reassociation in this study.

This is the first study to evaluate the impact of insect host condition on both EPN and their symbiotic bacteria. Even the most recent studies that have evaluated EPN as biological control agents neglect to incorporate the bacterial symbiont in the system, despite the fact that these mutualists are essential for the persistence of EPN in the environment (Fenton et al., 2000). In addition, the EPN-bacteria partnership has been neglected in the most recent reviews regarding entomopathogens (Cory and Hoover, 2006). Other pathogens that solely utilize insects as their hosts include bacteria, viruses, fungi, and some protozoa. Because the EPN-bacteria system is more complex since it consists of a prokaryotic-eukaryotic obligate mutualism, it is often disregarded in models that investigate various biotic and abiotic factors that affect entomopathogen ecology and evolution. However, measurement EPN symbiont fitness is not a difficult task; therefore, it should be considered in future studies that evaluate EPN efficacy as biological control agents, and as organisms that can be evaluated in multitrophic models that investigate factors involved in the ecology and evolution of entomopathogens.

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