AN ABSTRACT OF THE DISSERTATION OF

Christine Andrea Armer for the degree of Doctor of Philosophy in Entomology presented on August 28, 2002.

Title: Entomopathogenic Nematodes for Biological Control of the Colorado Potato Beetle, *Leptinotarsa decemlineata* (Say)

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Abstract approved: _____ Ralph E. Berry _____ Sujaya Rao

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say), is the most devastating foliage-feeding pest of potatoes in the United States. Potential biological control agents include the nematodes *Heterorhabditis marelatus* Liu & Berry and *Steinernema riobrave* Cabanillas, Poinar & Raulston, which provided nearly 100% CPB control in previous laboratory trials. In the present study, laboratory assays tested survival and infection by the two species under the soil temperatures CPB are exposed to, from 4-37°C. *H. marelatus* survived from 4-31°C, and *S. riobrave* from 4-37°C. Both species infected and developed in waxworm hosts from 13-31°C, but *H. marelatus* rarely infected hosts above 25°C, and *S. riobrave* rarely infected hosts below 19°C. *H. marelatus* infected an average of 5.8% of hosts from 13-31°C, whereas *S. riobrave* infected 1.4%. Although *H. marelatus* could not survive at temperatures as high as *S. riobrave*, *H. marelatus* infected more hosts so is preferable for use in CPB control.

Heterorhabditis marelatus rarely reproduced in CPB. Preliminary laboratory trials suggested the addition of nitrogen to CPB host plants improved nematode reproduction. Field studies testing nitrogen fertilizer effects on nematode reproduction in CPB indicated that increasing nitrogen from 226 kg/ha to 678 kg/ha produced 25% higher foliar levels of the alkaloids solanine and chaconine. However, the increased alkaloids did not affect nematode infection of, nor reproduction in, CPB prepupae. Nematodes applied to field plot soil at 50 infective juveniles/cm^2 reduced adult CPB by 50%, and increased numbers of dead prepupae in soil samples up to five times more than in non-nematode plots.

Laboratory studies of *H. marelatus* and its symbiotic bacteria in CPB hemolymph indicated that immune responses did not limit nematode reproduction. A 58kD CPB hemolymph protein apparently caused the symbiotic bacteria to switch to the secondary form, which does not produce antibiotics and enzymes necessary for nematode growth and reproduction. Despite heat denaturation of the protein, the nematodes did not reproduce unless lipids were added to the hemolymph. Therefore, while *H. marelatus* may provide high levels of CPB control, nutritional constraints on the nematode and its bacteria inhibit reproduction in CPB and limit long-term multi-generation control.

Entomopathogenic Nematodes for Biological Control of the Colorado Potato Beetle, *Leptinotarsa decemlineata* (Say)

by
Christine Andrea Armer

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented August 28, 2002
Commencement June 2003

Doctor of Philosophy dissertation of Christine Andrea Armer presented on August 28, 2002.

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Christine Andrea Armer, Author

ACKNOWLEDGEMENTS

I would like to thank my parents for putting my education foremost, and always encouraging me and assisting me in choosing my way in the world.

My husband, David Putzolu, has provided endless love and support through the research process. His patience and willingness to help have been invaluable, and his intelligence, logic, and understanding of human behavior have been an inspiration.

No woman is an island, and I could not have completed this work without discussions that sparked new ideas and provided necessary information with students and faculty at OSU, as well as in other entomology departments around the country.

Many thanks to all my friends (ayyyyyy!) at OSU and elsewhere who have provided interesting discussions, ideas, sympathy, understanding, and love.

Innumerable thanks too to my committee members who provided support, knowledge, and friendship: Russell Ingham, Marcos Kogan, Alison Moldenke, Mary Powelson, and Gary Reed, and especially advisors Ralph Berry and Sujaya Rao.

Funding was provided by EPA-STAR Graduate Fellowship #U915727, an Applied Insect Ecologists' Foundation Graduate Fellowship, a National Potato Council Auxiliary Graduate Scholarship, an Agriculture Research Foundation Grant from Oregon State University, and a Yerex Graduate Fellowship. As important as the funding to conduct the research was the support of knowing others believed in the value of my research.

CONTRIBUTION OF AUTHORS

Dr. Gary Reed provided vital guidance for field plot set-up, maintenance, and data collection for the field study presented in Chapter 3. He presented background information on agronomic techniques, grower concerns, and costs involved in agribusiness. He also provided valuable discussions throughout the sampling process and offered ideas on new topics to study.

Sarina Jepsen assisted with data collection and helped develop field monitoring techniques for Chapter 3. Conversations with her, based on her questions and ideas, helped clarify the direction and goals of the field study.

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Entomopathogenic Nematodes for Biological Control of the Colorado Potato Beetle, *Leptinotarsa decemlineata* (Say)

CHAPTER 1: INTRODUCTION

COLORADO POTATO BEETLE (CPB): ECONOMIC IMPORTANCE

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), is one of the most economically damaging insect pests of potatoes in the United States and throughout much of Europe (Hare, 1990). Potatoes (*Solanum tuberosum* L.) are one of the most valuable vegetable crops in the United States, with income of nearly $2.7 billion for growers in 2000 (ERS-USDA). The CPB can cause 100% potato defoliation by August if left uncontrolled, which will reduce tuber yields by 50% or more (Hare, 1980). This damage would amount to at least $1.4 billion in lost income to growers, as well as billions of dollars of lost income to potato processors.

CPB BASIC BIOLOGY AND BIOGEOGRAPHY

The beetle feeds on foliage of several wild solanaceous plants, and on tomatoes (*Lycopersicon esculentum* Mill.) and eggplant (*Solanum melongena* L.) (Weber et al., 1995), although it prefers feeding on potato (Hare, 1990). The four larval instars as well as the adults feed on foliage, and a single insect eats at least 100 cm^2 of potato foliage in its lifetime (Tamaki and Butt, 1978; Ferro et al., 1985). The larvae develop from neonate to late fourth stage in about two to three weeks

(Ferro et al., 1985), and when they complete feeding they drop and burrow into the soil prior to pupation. The prepupal stage lasts three to five days, and the pupal stage is about five days. The adults live one month or more during the summer in the field, and the beetles overwinter underground as adults. Each mated female lays 30-60 eggs per day (Peferoen et al., 1981) on the underside of leaves, and a total of 2000 eggs or more in her lifetime (Wegorek, 1959). There are usually three generations per year in the potato growing regions of north central and eastern Oregon and Washington (Berry, 1998). Potatoes are usually planted in March and April and harvested from September through early November. The beetles emerge from overwintering as early as April and as late as June, and begin hibernation in late September.

The CPB is native to Mexico, where it feeds on native solanaceous plants. The CPB was first found in the United States in 1811 feeding on buffalobur (*Solanum rostratum* Dunal) near the Nebraska-Iowa border (Casagrande, 1987). The potato, *S. tuberosum*, was introduced into the United States in 1621 in Virginia (Casagrande, 1987), and by the mid-1800's its use as a crop had spread widely, and the beetle began to include this plant in its host range. The CPB was not noted as feeding on potatoes until 1859 when potato-feeding was discovered to the east of Omaha City, Nebraska (Wegorek, 1959). By 1865, the beetle had spread as far east as Pennsylvania and New York, west to the Rocky Mountains, and south into Kentucky (Wegorek, 1959). CPB densities in the east in 1876 were so high that at times train traffic had to be halted to avoid slipping on the swarms of beetles

covering the area, including the train tracks (Casagrande, 1987). The beetle was present throughout the Atlantic region from Quebec into Florida by 1880, and then traveled to the north along the eastern side of the Rocky Mountains and into British Columbia by 1918. Wegorek (1959) noted that migration eastward proceeded much more rapidly than either to the north or the west, and that there was a "distinct tendency to migrate eastwards" through walking and flight. Additionally, migration coincided with wind patterns and human transportation via train, horse, wagon, and ship traffic. Strict quarantines slowed the beetles' movement to Europe, although individual beetles were found in Germany and Holland as early as 1877. CPB were found in England several times, but rapid response and unsuitable climate seem to have eliminated them from the British Isles. Potato beetle populations finally established in Europe during World War I, when beetles thought to have been transported on troop ships from the United States were introduced into France. By 1950, the beetle was found throughout the European potato-cultivating region from Spain to Denmark, and Italy to Poland. The CPB is now a serious pest of potatoes through much of the United States and Canada, as well as all of Europe except Britain.

CPB HOST PLANTS

The CPB feeds exclusively on plants in the family Solanaceae, which are characterized by high levels of foliar alkaloids (Carman et al., 1986). Potato glycoalkaloids cause cell membrane lysis (Roddick et al., 1988) and are

acetlycholinesterase inhibitors (Roddick, 1989), which produce continual nerve firing and hence nervous system disruption. The high mammalian and insect toxicity of solanaceous plants has led to extensive study of the chemical compounds found in the leaves. Alkaloids are a broad group of organic compounds containing nitrogen in a ring (Robinson, 1974). Several specific categories of alkaloids are found in solanaceous plants: glycoalkaloids, which are nitrogen-containing steroids with glycosidic linkages, include solanine, demissine, tomatine, chaconine, and leptine (Shapiro et al., 1983; Gregory, 1984); atropine, a tropane alkaloid (Harrison and Mitchell, 1988); and nicotine, a pyrrolidine alkaloid. Alkaloids are toxic to many microbes (Costa and Gaugler, 1989), insects (Robinson, 1974; Hare, 1987), and mammals (Gregory, 1984; Kuc, 1984). Here, the alkaloids will be discussed only with respect to CPB.

Many alkaloids affect both insect physiology and behavior. Tomatine and soladulcine slow the development of CPB larvae, and nicotine is highly toxic, causing direct larval mortality (Buhr et al., 1958). Tomatine's main effect is as a feeding repellent. Atropine and some of its derivatives, as well as scopolamine, increase larval mortality at high concentrations of 0.20 to 1.5% of fresh leaf weight (Buhr et al., 1958). Such high concentrations are not normally found in leaf tissues. However, atropine at lower levels causes a significant increase in mobility and decreased acceptance of leaves treated with atropine (Harrison and Mitchell, 1988).

The leptine found in *Solanum chacoense* (wild potato) appears to be the primary glycosidic alkaloid conferring resistance of this plant to CPB (Sturckow

and Low, 1961). This species also contains 0.35% of solanine and chaconine, but these substances do not deter feeding by CPB (Buhr et al., 1958). Leptines may be a good target for breeding resistance in crops because leptines are produced only in the foliage, and so tuber edibility for humans is not affected (Tingey and Yencho, 1994).

Another category of chemical defenses found in some solanaceous plants is the wound-induced increase in polyphenol oxidase (PPO). Tomato plants (as well as tobacco and several non-related plants) display a systemic increase in PPO as a result of wounding and of methyl jasmonate (an intermediate in the wound-response pathway) application (Constabel and Ryan, 1998). Polyphenol oxidase serves as an antifeedant for many herbivores as it oxidizes phenolic compounds to quinones, which are reactive molecules which can alkylate essential amino acids in the diet, making them nutritionally unavailable to the feeding insect. Low levels of PPO are inducible in potatoes, and almost no induction occurs by either wounding or methyl jasmonate application in peppers (Constabel and Ryan, 1998). Although PPO can provide resistance to some herbivores, the effects on CPB have not yet been studied.

Plant resistance mechanisms involve not only chemical, but also physical, defenses. Potatoes have trichomes which exhibit both antixenosis and antibiosis for feeding insects (Sanford et al., 1984). Trichome-mediated resistance in wild potatoes protects against the CPB. Two types of trichomes are found in the wild Bolivian potato species *Solanum berthaultii*. Type-A trichomes have a membrane-

bound four-lobed gland at the tip which, when broken open, combines oxidases with phenolic substances to create a compound which hardens onto the mouthparts and tarsi of an herbivore. Up to 60% of the total proteins in type-A trichome exudate are polyphenol oxidases (PPOs). Trichomes found in *S. tuberosum* are similar to *S. berthaultii* type-A trichomes, but those of commercial potato cultivars produce little PPO and cannot polymerize to form a hardening exudate to trap insects (Kowalski et al., 1993). Type-B trichomes are taller and produce droplets of sucrose esters of short-chain fatty acids (Kowalski et al., 1993) in *S. berthaultii*. However, *S. tuberosum* type-B trichomes are sparse and produce no exudate. The expression of secretion in potato trichomes may have had a negative effect on yield, and so trichome exudation was lost during domestication. The two trichome types work alone and synergistically to reduce herbivore feeding, increase locomotory activity, induce digestive disorders, reduce fecundity, and to entangle the herbivore (Kowalski et al., 1993).

CPB RESISTANCE TO TOXINS

Just as solanaceous plants have developed chemical products to deter herbivores, insects which feed on these plants have developed various mechanisms for detoxifying or avoiding the plants' chemical defenses. The CPB is not affected by many of the alkaloids found in low levels in solanaceous crops. Similarly, this beetle has developed resistance to most pesticides used for control (Metcalf, 1989). The beetle may use the same physiological pathways to detoxify both pesticides and

alkaloids in the solanaceous plants on which it feeds exclusively. CPB resistant to DDT were also found to be resistant to leptine, the glycoside responsible for wild potato's resistance to CPB attack (Sturckow and Low, 1961). Leptine causes CPB larvae to stop feeding, but when this compound is converted to leptinine through the loss of an acetyl group, the substance also loses its feeding-suppressant properties. The enzymes responsible for DDT and leptine resistance may cause leptine to degrade to the non-toxic leptinine. CPB was found to produce varying concentrations and ratios of gut proteolytic enzymes based on diet (Overney et al., 1997), which may explain its ability to detoxify both alkaloids and synthetic pesticides.

PLANT BREEDING FOR RESISTANCE TO CPB

Glycoalkaloids concentrate in leaves and flowers of potatoes, as well as in the outer layers of the tuber (Gregory, 1984). The glycoalkaloids can diffuse throughout the tuber, especially when the plant is stressed. High glycoalkaloid content in the foliage is generally closely associated with alkaloid content in the tuber (Sanford et al., 1984). The fact that tuber alkaloid levels parallel foliar alkaloid levels has restricted attempts to breed for insect resistance based on alkaloids, because distribution of alkaloids in the part of the plant intended for human or domesticated animal consumption clearly must be considered before intentionally increasing alkaloid content as an anti-herbivory mechanism.

Plant breeding through transgenic insertion of non-native genes into solanaceous plants has had limited success. The *Bacillus thuringiensis* var. *tenebrionis* (Btt) delta-endotoxin genes (Perlak et al., 1993) provided good control against the CPB feeding on transgenic foliage. However, consumer concern about the safety of transgenic plants has effectively removed transgenic potatoes from the market. Additionally, the CPB can develop resistance not only to the truncated Btt entomocidal toxin in transgenic potato plants, but also to the unmodified toxin in the living bacteria (Whalon et al., 1993; Whalon et al., 1994). Insertion of an antinutritive proteinase inhibitor gene (Vaeck et al., 1987) into plants protects against herbivores, but this technology has not been marketed for widespread use.

S. tuberosum has been crossed with *S. berthaultii* to attempt to create insect resistance in cultivated potatoes. Type-A trichomes have been bred into crop potatoes (*S. tuberosum*), and provide successful reduction of CPB oviposition and feeding. Experiments with extracts of type-A trichomes proved that the deterrence was not merely physical, but that chemical properties of the trichome exudates also deterred CPB feeding (Yencho et al., 1994). Pelletier and Smilowitz (1990) found that type-B trichome extracts reduced feeding by CPB. However, type-B trichomes are difficult to breed into *S. tuberosum* populations. The genes for type-B trichomes are recessive, and are on the same chromosome as the genes which require short days for tuber growth in *S. berthaultii*, and linkage makes separating the desired trichome genes from short-day genes difficult.

Breeding for trichome-based insect resistance still holds promise. Bonierbale et al. (1994) reported that herbivore resistance due to trichomes is a stable means of crop protection, because after ten generations of feeding on *S. berthaultii*, the CPB displayed no signs of developing reduced susceptibility to trichome-induced feeding deterrence. Insects have a much lower chance of developing resistance to physical control methods than to chemical control, and physical traits of foliage will not affect mammalian consumers of the fruits and tubers of solanaceous plants. Physical herbivore resistance could, however, affect other insects living on plants.

Tritrophic effects of plant traits intended to affect herbivores must be considered. The transgenic integration of a proteinase inhibitor in potatoes reduced the fecundity and egg hatch rates of the predator *Perillus bioculatus* F. (Heteroptera: Pentatomidae) when fed on CPB larvae fed on transgenic plants (Ashouri et al., 1998). However, the proteinase inhibitor also increased feeding rate of the predator, and although its populations may not survive well in transgenic crop fields, it can provide increased control when supplied to fields augmentatively. Other tritrophic affects may be found when predators and parasites interact with physical herbivore resistance. Glandular trichomes derived from *Solanum berthaultii* in *S. tuberosum* hybrids reduced mobility of coccinellid and chrysopid larvae, and decreased survival of these predators and a CPB egg parasite (Obrycki, 1986). Finally, high solanine and tomatine levels inhibited colony growth and

formation in *Beauveria bassiana*, an important fungal biological control agent of CPB (Tingey and Yencho, 1994).

CPB CHEMICAL CONTROL

Potato growers commonly rely on pesticides to control CPB. The CPB was the first agricultural insect pest to be widely controlled with pesticides, beginning with Paris green in the 1870's (Casagrande, 1987). The beetle was also one of the first pests to develop insecticide resistance, beginning with the possible resistance to Paris green in 1912. The beetle, which developed resistance to DDT within seven years (Casagrande, 1987), has developed resistance to each new pesticide increasingly rapidly (Hare, 1990), so that some populations of CPB are now resistant to almost all pesticides (Forgash, 1985). Without careful crop and pesticide rotations, many insecticides become ineffective. Although the CPB has not shown pesticide resistance in the north central Oregon potato-producing region, the beetle clearly has the potential to render insecticides ineffective as a control measure. Additionally, consumer concerns about health and non-target ecological effects make the reduction of pesticide use desirable. Plant resistance in combination with biological and cultural control techniques may reduce the dependence on pesticides for long-term CPB control.

CPB CONTROL BY PREDATORS, PARASITES, AND PATHOGENS

The CPB is controlled by predators and parasites in Mexico (Cappaert et al., 1991), but these natural enemies either do not live in the United States, or do not have synchronous development with the beetle as they do in Mexico. Therefore, CPB populations are not controlled by naturally-occurring enemies in northern North America. Biological control researchers have examined both natural and supplemented populations of heteropteran predators (Tamaki and Butt, 1978; Biever, 1992a; Biever, 1992b; Biever and Chauvin, 1992a; Biever and Chauvin, 1992b), tachinid larval parasites (Kelleher, 1966; Tamaki et al., 1983; Horton and Capinera, 1987; Lopez et al., 1993), an eulophid egg parasite (Lashomb et al., 1987; Schroder and Athanas, 1989), and several beetle predators (Heimpel and Hough-Goldstein, 1992), for CPB population reduction, but have not found consistent success with these insects. Other workers have studied beetle control with pathogens such as the bacterium *Bacillus thuringiensis* var. *tenebrionis* (Ghidiu et al., 1994; Lacey et al., 1999; Nault et al., 2000), the fungus *Beauveria bassiana* (Poprawski et al., 1997; Lacey et al., 1999), and a spiroplasma (Hackett et al., 1996). Some of these pathogens provide high levels of control, but have not been widely adopted by potato producers, often due to high costs and low field persistence of the pathogens (Moldenke and Berry, 1999).

CPB CONTROL BY ENTOMOPATHOGENIC NEMATODES

Several species of both steinernematids and heterorhabditids have been used successfully under experimental conditions to control the CPB (Toba et al., 1983; Wright et al., 1987; Cantelo and Nickle, 1992; Nickle et al., 1994; Berry et al., 1997; Stewart et al., 1998). Entomopathogenic nematodes survive longest and find hosts most readily in damp sandy loam soil (Kaya, 1990). The eastern Oregon and Washington potato-growing regions have sandy loam soils, and are irrigated daily during the hottest, driest periods. These soil conditions, as well as the potential efficacy of nematodes for CPB control, make nematodes strong candidates for CPB biocontrol.

ENTOMOPATHOGENIC NEMATODE LIFE CYCLE

Parasitic nematodes in the families Heterorhabditidae and Steinernematidae are obligate parasites of insects. The nematodes live in moist soil as infective juveniles (IJs) or dauers, the only form which survives outside the host. The IJs are third-stage nematodes enclosed in the second-stage cuticle. The extra cuticle layer protects the IJs against predators and pathogens while in the soil (Kaya, 1990). Additionally, the outer cuticle layer allows the maintenance of a relatively sterile third-stage cuticle. As the IJ enters a host, it sheds its second-stage cuticle, and so does not carry pathogens into its host on its cuticle. The surface sterility of the nematode also helps it avoid recognition and encapsulation by its host's immune responses (Peters et al., 1997).

Heterorhabditids enter the host through the spiracles, anus, mouth, or directly through the intersegmental cuticle by using an apical tooth to cut through the integument. The nematodes move into the host's hemocoel (Akhurst, 1980), where the IJ halts the host's immune system, and then releases the symbiotic bacteria, *Photorhabdus luminescens* (a second species of *Photorhabdus* has been proposed (Fischer-Le Saux et al., 1999; Liu et al., 2001)) (Enterobacteriaceae) (Boemare et al., 1997) that is carried in the nematode's foregut (Boemare and Akhurst, 1988; Boemare, 1997). The primary form of the bacteria reproduces, utilizing nutrients from the host tissues, and kills the host within two days through bacterial septicemia (Akhurst, 1980). The bacteria also produces an antibiotic to inhibit the growth of competing pathogens (Akhurst, 1982) that could enter from the nematode's entry wound or from the gut once the host's tissues begin to degrade.

The heterorhabditid nematode feeds on the bacterial/host tissue slurry (Akhurst, 1994), developing into a hermaphroditic adult within the host. Eggs in the hermaphrodites are fertilized by sperm from the same individual (Johnigk and Ehlers, 1999), so only one IJ is necessary to initiate reproduction within a host. Hermaphrodites usually die by endotokia matricida (Johnigk and Ehlers, 1999), in which eggs hatch within the mother's uterus, and the juveniles feed on the mother's internal organs, causing her death. Hermaphrodites provided with more nutrients grow larger and develop more mature eggs before the offspring hatch and begin feeding on the mother (Johnigk and Ehlers, 1999). The offspring exit the mother's

body once they have consumed most of the nutrients, and they develop in the bacterial/host tissue slurry, and mature as amphimictic, or male or female, adults. These adults mate and lay eggs within the original host. Nematodes normally develop through two to three generations within a single host before the host's tissues and the symbiotic bacteria are fully utilized for nutrients.

When the available nutrients decline and the bacterial/nematode waste products increase, the bacteria is triggered to develop to a secondary form which differs significantly from the primary form which is vectored by the nematode. In contrast to the primary form, the secondary form of *P. luminescens* does not provide as many nutrients for the nematodes, and does not produce the antibiotics (Akhurst, 1980). The switch to the secondary form of the bacteria triggers the nematodes to develop to the IJ stage, so they can exit the host and survive in the soil until a new host is found. Neither the nematode nor the bacteria can complete their life cycle without the other; the bacteria needs the nematode as a vector to find an appropriate host, and the nematode requires an intact host killed by bacterial septicemia in order to feed on the by-products of bacterial digestion of host tissues.

Steinernematids are not closely related to heterorhabditids (Poinar, 1990), but due to convergent evolution, the two genera have similar life cycles. However, the steinernematids generally have sit-and-wait habits for attacking passing hosts, whereas heterorhabditids actively search for hosts (Kaya and Gaugler, 1993). The steinernematids have no apical tooth with which to cut through the host's integument, so host entry must be through the mouth, anus, or spiracles only.

Additionally, there are no hermaphrodites, so at least one male and one female must enter the same host to reproduce. Steinernematids carry bacteria in the genus *Xenorhabdus*, also in the family Enterobacteriaceae, and these bacteria do not luminesce in the manner of *P. luminescens*.

INSECT HOST RESISTANCE TO ENTOMOPATHOGENIC NEMATODES

Nematodes can be stopped from infecting a host through a variety of mechanisms. Nematodes normally enter the host through spiracles, the mouth, or the anus. The nematodes then search for thin spots in the cuticle to penetrate through pressure, or in the case of heterorhabditids, by using an apical tooth to pick a hole in the host's cuticle. Spiracular plates with openings too small for nematodes to enter can inhibit infection (Forschler and Gardner, 1991). The thickness of the peritrophic membrane in the host's midgut can limit infection (Forschler and Gardner, 1991). Many nematodes that enter the gut are simply carried out by movement of food through the gut. Insects with more rapid feeding and digestion rates may be able to expel more nematodes through this removal process.

In addition to physical deterrents to nematode entry into the host, nematode host-finding abilities can be affected by the host insects' behavior and physiological state. Nematodes are attracted to CO_2 produced by respiring insects (Gaugler et al., 1980). Pupating insects are often entirely enclosed with no open spiracles, and hence do not produce CO_2 outside the pupal cuticle. Nematodes may be attracted to the small amounts of metabolic heat produced by insects (Byers and Poinar, 1982).

Inactive insects will produce less heat than active ones, and so may be less attractive to nematodes. Nematodes can detect volatiles and contact chemicals produced by potential hosts (Schmidt and All, 1978; Lewis et al., 1992; Grewal et al., 1993), and sometimes by insects already infected by other nematodes (Grewal et al., 1997).

Once the entomopathogenic nematode has entered a host, several lines of defense still exist, based on the host's immune responses. Single-cell invaders can be phagocytized (Gotz and Boman, 1985). Insects have two phases of the immune response to respond to invaders not phagocytized: an encapsulation of invading organisms, and an inducible bacteriase enzyme system. A capsule is formed by hemocytes ('blood' cells) sticking to the invader and bursting to spread the cellular contents (Poinar et al., 1968). More hemocytes are attracted by the lysed cells, and hemocytes continue to bind to the capsule until it is 20-60 cell layers thick (Gotz and Boman, 1985). The cells gradually melanize, developing a tough protein-phenol lattice as well as a darkened pigmentation. The invader, such as a nematode, is contained within the capsule and eventually dies from lack of nutrients. In addition to multicellular organisms being encapsulated, bacteria can also be encapsulated after being enclosed in cell contents from autolyzing hemocytes (Gotz and Boman, 1985). Enzymes in the host's hemolymph can alter an invading bacterium's coat to make it more susceptible to encapsulation (Dunphy and Webster, 1991). The inducible enzyme system is a series of membrane-disrupting

lysozymes and peroxidases that acts as rapidly as 4 hours post-invasion (Gotz and Boman, 1985).

Nematodes and their symbiotic bacteria survive host immune responses in several ways. Nematodes are not immediately recognized as non-self by some hosts (Dunphy and Webster, 1988), and so the immune response simply is not triggered until the symbiotic bacteria have reproduced too extensively to be halted. The bacteria can inhibit phagocytosis by the host's immune cells (van Sambeek and Wiesner, 1999). The nematobacterial complex can produce toxins to destroy host hemocytes, the cells which cause an immune response (Ribeiro et al., 1999). The nematodes and bacteria can also limit the hemocytes' encapsulation response by proteolytically inhibiting host cells from sticking to the nematodes, so the capsule cannot be formed (Ribeiro et al., 1999). Nematodes may produce surface coat proteins which inhibit the host's encapsulation response (Wang and Gaugler, 1999). The nematode also produces an immune inhibitor to destroy immune proteins, thus protecting the symbiotic bacteria from the host's immune response (Gotz et al., 1981). The bacteria can, at times, degrade the lysozyme-based inducible defenses of the host (Jarosz, 1998), as well as inhibiting enzyme activation of the encapsulation response (da Silva et al., 2000).

SEQUESTRATION OF HOST-PLANT CHEMICALS

Many specialist herbivorous insects sequester toxins from the plants on which they feed (Duffey, 1980). Chrysomelids other than the CPB sequester plant

toxins, such as salicins from poplar and willow (Pasteels et al., 1986), juglone from walnut (Pasteels et al., 1988), and cucurbitacins from cucumber and squash (Ferguson and Metcalf, 1985).

Research suggests that alkaloids might be sequestered in the CPB to protect against attack by natural enemies such as pathogenic fungi (Hare and Andreadis, 1983; Costa and Gaugler, 1989). Sequestering or otherwise manipulating plant secondary compounds can be energetically expensive for an insect, and could potentially reduce the insect's resistance to natural enemies. Although Rothschild (1972) stated that CPB does not sequester glycoalkaloids, the research to support that assertion was not published. Possibly the beetles sequester the alkaloids in a form not found in potato foliage. The alkaloids could be easily converted to the aglycone form for storage, and these aglycones may be more toxic to some organisms than are the original α-solanine and α-chaconine.

RESEARCH OBJECTIVES

Heterorhabditis marelatus Liu & Berry (Liu and Berry, 1996) provides better and more consistent CPB control than do other nematode species (Berry et al., 1997). *H. marelatus* kills nearly 100% of host prepupae, but does not reproduce in the CPB (pers. obs.). Preliminary studies indicate that the nematode species *Steinernema riobrave* Cabanillas, Poinar & Raulston also does not reproduce in CPB (unpublished data). It is likely that other nematode species respond in a manner similar to that of *H. marelatus* when infecting CPB. The research presented

in this dissertation provides information on why *H. marelatus* (and possibly other nematode species) does not reproduce within the CPB. A natural enemy population that can sustain control must be able to reproduce in the host, survive to attack the next generation of hosts, and be able to adapt to changes in the host population physiology. These studies were completed with the goals of understanding constraints on nematode development and reproduction in hosts, and how to improve nematode reproduction in otherwise inhospitable hosts. It may be possible to alter the nematode's habitat so the nematode populations can reproduce and adjust to changes in the highly adaptable CPB.

The CPB is commonly thought to sequester glycoalkaloids from the solanaceous plants on which it feeds to protect against predators and parasites. Although alkaloid content of the beetles has not been directly examined, alkaloids inhibit the fungal pathogen *Beauveria bassiana* which has limited reproduction in CPB, suggesting that alkaloids are sequestered by CPB (Costa and Gaugler, 1989). Alkaloids reduce survival in leafhoppers feeding on potato plants (Raman et al., 1979), which may be due to alkaloids killing the leafhoppers' symbiotic gut bacteria needed for digestion. Alkaloids found in potato plants affect cell membrane structure and permeability in fungi (Costa and Gaugler, 1989) and plants and animals (Roddick et al., 1988), and are acetylcholinesterase inhibitors in animals (Gosselin et al., 1988; Roddick, 1989).

Parasitic nematodes such as *H. marelatus* require symbiotic bacteria (*Photorhabdus* sp.) to provide nutrients for nematode development. Preliminary

studies (Armer, unpublished) indicated that the symbiotic bacteria could not reproduce in the CPB, even when directly injected into the beetle's body. Another secondary plant compound, cucurbitacin, inhibits the growth of parasitic nematodes (Barbercheck, 1993; Barbercheck et al., 1995) and their symbiotic bacteria (Barbercheck and Wang, 1996). Parasitic nematodes are susceptible to toxins that their hosts ingest, and alkaloids inhibit the growth of many organisms. This evidence supports the hypothesis that alkaloids ingested by the CPB may inhibit the reproduction of *H. marelatus* and its symbiotic bacteria. Alternately, the CPB may expend enough energy for handling the alkaloids, either through sequestration, detoxification, or elimination, that its immune system can be made weaker by ingesting large amounts of alkaloids. This might render the CPB more susceptible to nematode attack. If the CPB does not sequester the alkaloids, but instead detoxifies or excretes them, the alkaloids would not affect the nematodes directly, as the CPB voids its gut as a prepupa (pers. obs.). Finally, a combination of factors may affect nematodes and their symbiotic bacteria attacking a CPB prepupa. The CPB may synthesize toxic compounds unrelated to plant defensive chemicals (Hsiao and Fraenkel, 1969; Daloze et al., 1986), and if these toxins are available to internal parasites, the toxins could reduce the ability of the bacteria and nematode to obtain nutrients. If the CPB is expending energy to produce novel defensive compounds, as well as to manipulate alkaloids from its host plant, it may have a reduced immune capacity at the same time the chemical defenses are stronger. A nematode's ability to develop and reproduce successfully in its host therefore may

depend not only on the nematode and its symbiotic bacteria, but also on the balance of stresses and defenses of the host CPB.

H. marelatus controls the pupating CPB in the soil, but does not attack or directly compete with above-ground predators of the larval and adult CPB. This nematode can cause local extinctions and then survive long enough in the field (Berry et al., 1997) to attack recolonizing beetles, which it can locate through at least 20 cm of soil. Its strong searching ability makes this an ideal biological control agent (Murdoch et al., 1985; Gaugler et al., 1997) that may, either alone or in concert with other natural enemies, provide adequate sustainable control of CPB. Like other parasitic nematodes, *H. marelatus* survives best in moist sandy soils (Kaya and Gaugler, 1993). Any potato-growing region in the US that has suitable soil texture and temperature range could benefit from the successful establishment of *H. marelatus* breeding populations.

Little is known about *H. marelatus* and *P. luminescens*, and their application to CPB control, so this dissertation research was undertaken to understand CPB control with this nematode. A series of growth-chamber studies examined how *H. marelatus* and another nematode that controls CPB, *Steinernema carpocapsae*, respond to soil temperature extremes (Chapter 2). Field experiments tested CPB control efficacy by *H. marelatus* under typical potato-growing conditions in Hermiston, in the north-central Oregon potato-growing region (chapter 3). In-vitro laboratory studies tested the effects of CPB immune responses and hemolymph chemistry on *H. marelatus* and *P. luminescens* developing in CPB hemolymph

(Chapter 4). These chapters provide information that helps elucidate the potential of this nematode and its symbiotic bacterium for control of CPB, as described in the thesis conclusions (Chapter 5).

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CHAPTER 2: LONGEVITY AND INFECTIVITY OF ENTOMOPATHOGENIC NEMATODES AT TEMPERATURE EXTREMES

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For submission to Biological Control: Theory and Applications in Pest Management

ABSTRACT

The entomopathogenic nematodes *Heterorhabditis marelatus* and *Steinernema riobrave* provide effective control for several nursery and agricultural insect pests. The range of optimal soil temperatures for nematode infection and development in a host may depend on the climate of the origin of the nematode. This study examined the temperature range at which *H. marelatus*, from cool coastal Oregon, and *S. riobrave*, from hot southwestern Texas, can infect hosts, and at what temperatures the nematodes can survive. The temperature range limiting reproduction of the symbiotic bacteria that assist nematode development in the host was also examined. Infective juvenile nematodes were applied to moistened sand at 100 nematodes/cm^2, and held at 4°C, and from 13°C up to 37°C in 3°C increments. Portions of inoculated sand were removed periodically and placed in petri dishes with larvae of the highly susceptible host, the waxworm (*Galleria mellonella*), to determine temperature limits to host infection and to length of nematode viability. This experiment indicated that, although the optimal development temperature for

H. marelatus ranged from 16°C to 22°C, the nematode could survive at temperatures from 4°C to 31°C. *S. riobrave* survived from 4°C to 37°C. Both nematode species infected and developed in hosts from 13-31°C, but *H. marelatus* rarely infected hosts at 28°C and above, and *S. riobrave* rarely infected hosts at temperatures of 16°C and below. *H. marelatus* infected more hosts and remained infective for longer periods of time over a range of temperatures than *S. riobrave.* The symbiotic bacteria for both nematode species did not appear to limit the nematodes' ability to develop and reproduce at low temperatures, in that the nematodes did not infect hosts at temperatures below those at which the bacteria could develop. However, the bacteria might restrict *H. marelatus* development at high temperatures. A tightly linked longevity-infective ability trade-off was found for both species within their normal temperature ranges, whereby the nematodes remained viable longer at temperatures at which they infected fewer hosts. In conclusion, although nematodes may be exposed to extreme soil temperatures at which they cannot infect hosts, they can survive to attack hosts at more moderate temperatures when the soil heats or cools with daily and seasonal temperature cycles.

KEY WORDS: *Heterorhabditis marelatus*; *Steinernema riobrave*; Heterorhabditidae; Steinernematidae; Rhabditida; Nematoda; *Photorhabdus* sp.; *Xenorhabdus* sp.; biological control; soil arthropods

INTRODUCTION

Entomopathogenic nematodes in the families Steinernematidae and Heterorhabditidae provide high levels of biological control of some agricultural and ornamental insect pests in the soil (Kaya and Gaugler, 1993). These nematodes can be applied like a chemical pesticide, either through irrigation water (Reed et al., 1986) or via a backpack or tank sprayer. Depending on the species, the nematodes either sit and wait for a host to pass nearby, or actively search for a host to attack. The infective juvenile (IJ) stage of the nematodes enters the host through the mouth, anus, or spiracles. Once inside the host, the steinernematid or heterorhabditid nematode releases symbiotic bacteria of the genera *Xenorhabdus* and *Photorhabdus*, respectively, into the host's hemocoel (Akhurst, 1994). The bacteria reproduce, killing the host by bacterial septicemia. The nematodes then feed on the bacterial/host tissue slurry, developing through one or more generations, before leaving the host as IJs to find new hosts.

Host infection by heterorhabditids and steinernematids is dependent on a strict range of environmental conditions (Kaya, 1990). Nematodes find hosts most readily and survive longest in sandy loam soils (Kaya, 1990), and require moisture to survive. Entomopathogenic nematodes are temperature-sensitive, and because they are poikilotherms they survive longer at cool temperatures but attack more hosts at warmer temperatures (Kaya, 1990). Nematodes that originate from warm areas tend to infect hosts through a range of higher temperatures than nematodes

native to cooler regions (Molyneux, 1985). Hence, selection of an appropriate nematode species for control of a specific pest should be based, in part, on the soil temperature at the time of pest susceptibility. Additionally, the nematodes' symbiotic bacteria may limit nematode control of hosts. The bacteria must be able to reproduce at the soil temperatures at which the nematode attacks a host, as the bacteria are the organisms which actually kill the host and which break down host tissues for nematode consumption. Soil temperature extremes could affect both an entomopathogenic nematode and its bacteria, and hence could affect several integral steps in the host-infection process.

Two nematode species, *Heterorhabditis marelatus* Liu & Berry (Liu and Berry, 1996) and *Steinernema riobrave* Cabanillas, Poinar & Raulston (Cabanillas et al., 1994) (name modified from *S. riobravis* as discussed in Liu et al., 1998) provide exceptionally high levels of Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) control, killing nearly 100% of the beetle population under laboratory conditions (Berry et al., 1997). The CPB serves as a good model for estimating the temperature extremes a nematode must endure in order to control a pest insect. However, although the CPB is killed readily by *H. marelatus* and *S. riobrave*, the beetle does not support nematode reproduction (pers. obs.). Hence, the highly-susceptible host *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) is used here for testing the nematodes for survival and infective ability. The CPB is a serious pest on solanaceous crops that can be controlled by entomopathogenic nematodes as a prepupa in the soil (Toba et al., 1983; Wright et

al., 1987; Cantelo and Nickle, 1992; Nickle et al., 1994; Berry et al., 1997). Between one and three generations are found in the various potato-growing regions of the United States, from north-central Oregon, Washington, and Idaho, to Minnesota, Wisconsin, and Michigan, and through much of the northern East Coast. Soil temperatures 7.5 cm deep in unirrigated soil where CPBs pupate can range from 13-37°C during the CPB's susceptible stage in the potato-growing regions of north-central Oregon (http://www.orst.edu/Dept/hermiston/archive.htm). These two nematode species may vary in the temperature range at which they can infect hosts. *H. marelatus*, native to the coast of Oregon (Liu and Berry, 1996), is expected to be most active in cool climates, whereas *S. riobrave*, from the Rio Grand Valley of Texas (Cabanillas et al., 1994), is adapted to hot conditions. This study examines the ability of these two nematode species to infect waxworms over the range of soil temperatures found during the north central Oregon potato-growing season. Additionally, correlations between period of infectivity and range of infection temperatures are analyzed to provide an understanding of the trade-off between infecting a host under extreme soil temperatures and waiting until soil conditions are more moderate.

MATERIALS AND METHODS

Field temperatures: CPB pupates at 7.5 cm or deeper (pers. obs.). The high and low daily average temperatures at this soil depth are shown in Figure 2.1

for Hermiston, OR (HAREC website: http://www.orst.edu/Dept/hermiston/archive.htm), which is representative of the potato-growing region in the Columbia Basin of Oregon and Washington. Data were collected in unirrigated areas, and indicate temperatures more extreme than those found in irrigated areas. Data were compiled from daily averages from four years. Temperatures can vary by 10°C or more during one day at a soil depth of 12 cm (pers. obs.), and Figure 2.1 gives an average range of temperatures rather than the full range of temperatures that nematodes may experience during a given 24 hours.

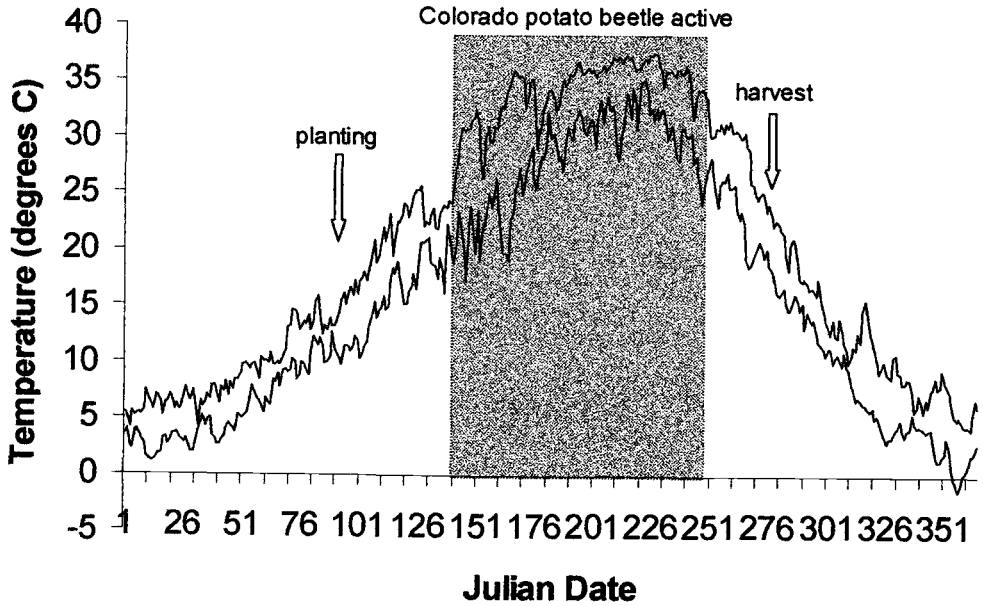

Figure 2.1. Four-year (1998-2001, inclusive) average maximum and minimum soil temperatures at a depth of 7.5 cm in unirrigated soil. Data taken from Hermiston Agricultural Research Extension Center website (http://www.orst.edu/Dept/hermiston/archive.htm). Approximate dates for potato planting and harvest are indicated. The range of soil temperatures when the Colorado potato beetles are active in eastern Oregon are shown by the gray box.

Organisms tested: *S. riobrave* and *H. marelatus* nematodes were obtained from lab colonies (strains TX and OH10, respectively) maintained at Oregon State University. Waxworms (*G. mellonella*) were used as hosts to produce nematodes tested in the experiment (Woodring and Kaya, 1988). Final-instar waxworms used for rearing nematodes, and for nematode survival and infectivity assays, were purchased from Vanderhorst Wholesale Inc., St. Mary, OH. Waxworms were used to test nematode infection abilities because they are more easily available in large quantities than are CPB, and successful nematode infection and reproduction is monitored more reliably because nematodes reproduce more readily in waxworms than in CPB (pers. obs.). Infective juvenile nematodes freshly collected from waxworms were used to initiate each trial.

Parameters examined: This series of trials tested at what temperatures nematodes could infect hosts (**infective temperature**), how long nematodes could infect hosts at each temperature (**infective period**), and how long the nematodes **survived** at each temperature (**functional longevity**). Nematodes were not examined directly for estimation of longevity, but instead longevity refers to the period of time that the nematodes were able to infect hosts and reproduce. These trials estimated infective temperature, infective period, and longevity for populations of nematodes, not for individuals. Successful infections were verified by dissection of dead waxworms to confirm nematode reproduction. Waxworms which were dead but which did not contain reproducing nematodes were considered

uninfected. Therefore, host 'infection' and nematode 'reproduction' are used interchangeably here.

Infective temperature and infective period: Waxworm hosts were exposed to each of the nematode species from 4-37°C to estimate nematode infection ability through the temperature range. These tests also indicated how long nematodes could infect hosts at temperatures from 4-37°C.

A substrate for nematode movement and survival was created by placing 100 g of autoclaved sand wetted to 10%w/w in 100 mm plastic Petri plates. Nematodes were applied at 50/cm^2 in a total of 1 ml water to each dish. The weight of the plate with sand was recorded for each dish so that a measured amount of sand with a constant number of nematodes could be removed periodically to test for length of nematode infectivity. Additionally, the moisture in the sand was maintained at 10% w/w by adding water as indicated by weight loss due to evaporation between examination dates. The Petri plates were sealed with Parafilm, and were placed in growth chambers at 4, 13, 16, 19, 22, 25, 28, 31, 34, and 37°C. Two plates (one replicate) were set up for each nematode species and storage temperature at a time, and three replicates of all temperatures were set up through time.

Nematode infectivity at a given temperature was examined by collecting 4 g of sand from each Petri plate, and scattering it on filter paper moistened with 1 ml tap water in a 100 mm Petri plate. Ten waxworm larvae were placed in each dish

with the sand, and the dish placed for 5-7 days in the growth chamber in which the sand had been stored, from 4-37°C. Waxworms that died after 5-7 days were held another 5-20 days, depending on incubation temperature, to allow nematode reproduction prior to dissection. Nematodes develop more rapidly at higher temperatures, and waxworms held at high temperatures could be examined for nematode reproduction sooner than waxworms held at cooler temperatures. The nematodes were checked for infection ability 1, 2, 3, 5, 7, and 10 days post-setup, and weekly thereafter. Nematodes that remained infective for 10 weeks were subsequently checked every two weeks. The number of the original 10 waxworms/plate with nematode reproduction was recorded. Sand from each treatment and replicate was tested for nematode infectivity until no waxworms were killed on two consecutive dates, or no nematodes developed in waxworms on four consecutive testing dates.

Bacterial temperature limits: The temperature ranges for development of the nematodes' symbiotic bacteria (*Photorhabdus* sp. [may be *P. temperata*; see Liu et al. (2001)] in *H. marelatus*, and *Xenorhbadus* sp. in *S. riobrave*) were examined to determine if temperature constraints on the bacteria restricted the nematodes' ability to develop. Each of the two species of bacteria were isolated from four nematode-infected waxworms at different times, and were plated on four plates of MacConkey's agar. The agar plates were placed at each of the temperatures used to

test nematode infection temperature limits. The plates were examined after 2 days and up to 2 months later for growth of the symbiotic bacteria.

Longevity: A second set of assays was performed to determine if nematodes that did not infect waxworms in the experiments described above were killed by the temperatures at which they were stored, or if they had entered an inactive state which they then could exit when temperatures became more moderate. Nematodes were assayed for their ability to infect *G. mellonella* larvae at 22°C, an ideal moderate temperature, after storage at a range of temperatures. The nematodes were not directly examined for longevity, but rather for the period of time over which they remained able to infect and reproduce in hosts.

A second set of Petri plates of sand with nematodes were set up and stored from 4-37°C, as in the infective temperature and infective period trials. However, when 4 g of nematode-treated sand were removed periodically (1, 2, 3, 5, 7, and 10 days post-setup, and weekly thereafter) from the stored plates and waxworms were placed in Petri plates with moistened filter paper, the plates were then placed in a chamber at 22°C, an ideal temperature for nematode development. After 5-7 days, live waxworms were discarded, and dead ones were left 10-15 days for nematode development determination by dissection. Three replicates with two plates each were completed for the longevity tests at each temperature. Separate Petri plates of sand were used for the infective temperature/infective period trials and the

longevity trials because each plate did not have the capacity to hold enough sand to complete both treatments.

Statistical analysis: Infective temperatures of both *H. marelatus* and *S. riobrave* were separately examined using the Kruskal-Wallis rank test to compare percent of waxworms infected by each species per test date at each temperature. The **infective period** for the two species also was examined separately by ranking for the length (days) at each temperature, and comparing with a Kruskal-Wallis test. Differences between individual temperatures were determined by comparing 95% confidence intervals. Comparison of Means tests contrasted the two species for length of time the two species could infect hosts at the range of temperatures, and the number of hosts infected by the two species. Data for infection at 4, 34, and 37°C were omitted from the analyses for both species because no nematodes infected at these temperatures, indicating these temperatures were outside the temperature thresholds for infection.

Longevity of *H. marelatus* nematodes stored at different temperatures and tested at 22°C was compared by one-way ANOVA for temperature treatments after a square-root transformation. Differences in longevity between individual storage temperatures were distinguished by Fisher's LSD. Data for 34 and 37°C were omitted from the ANOVA because no nematodes infected waxworms after storage at those temperatures, and hence the biological threshold for survival of *H. marelatus* had been passed. Longevity of *S. riobrave* was compared with a

Kruskal-Wallis rank test. Percent of waxworms infected by nematodes stored at each temperature, with all infection dates and replicates combined (60 total waxworms/date), was compared by one-way ANOVA for *H. marelatus* infection, and separately for square-root transformed data for *S. riobrave* infection. Differences between individual temperature treatments were determined with Fisher's LSD test. Data from the last replicate at 37°C for *S. riobrave* infections were omitted from the analyses because nematode survival and infection were more than 15 times greater than in the other replicates, indicating the growth chamber probably did not consistently maintain the temperature at 37°C in this replicate. Longevity of the two species and percent of waxworms infected by the nematodes were contrasted with Comparison of Means tests.

The **correlation between longevity and percent of hosts infected** by nematodes at 22°C after storage at each temperature (4-37°C) was compared by calculating the correlation statistic, r, for each species. All statistical analyses were performed with StatGraphics Plus 5.0 (Statistical Graphics Corp.).

RESULTS

Infective temperatures: Trials testing the range of temperatures at which the nematodes could infect waxworms indicated that *H. marelatus* was infective from 13-31°C, but could not infect any waxworms at 4°C, 34° or 37°C (Table 2.1). Although no hosts were infected at 28°C, a small percentage (0.7%) of hosts was

infected at 31°C. Infectivity, or the percent of waxworms infected, varied significantly with temperature (Kruskal-Wallis test statistic=21.77, $p<0.001$, not including data values of zero outside the temperature thresholds for 4, 34, and 37°C), with an average of 15.2% infected at 19°C, 2.8% infected at 13°C, but fewer than 1% of hosts infected at 31°C.

Table 2.1. Ability of two entomopathogenic nematode species to infect waxworms at a range of temperatures. Within each species, numbers followed by different letters were different at the $p<0.05$ level (comparisons of 95% confidence intervals, following Kruskal-Wallace rank tests). Differences between species are not indicated by the letters. Data from nematodes tested at 4, 34, and 37°C were not included in the analyses because these temperatures are outside the biological thresholds at which the nematodes can develop.

Temp.	*Heterorhabditis marelatus*		*Steinernema riobrave*	
°C	Mean infective period (days) ± SE	% waxworms infected	Mean infective period (days)±SE	% waxworms infected
4	0	0	0	0
13	7.5 ± 1.7 ab	2.8 a	1.5 ± 1.5 a	0.3 a
16	39.7 ± 6.5 c	8.8 ab	0 ± 0 a	0 a
19	12.3 ± 3.4 b	15.2 b	2.7 ± 1.4 a	2.0 a
22	16.3 ± 3.1 b	11.7 b	6.3 ± 4.0 a	1.7 a
25	1.7 ± 1.5 a	1.7 a	3.3 ± 2.8 a	1.7 a
28	0 ± 0 a	0 a	4.3 ± 3.9 a	1.7 a
31	3.0 ± 1.9 a	0.7 a	8.7 ± 6.6 a	2.4 a
34	0	0	0	0
37	0	0	0	0

Infectivity did not vary between temperatures in the range of 13-31°C for *S. riobrave* (Kruskal-Wallis rank test statistic=2.6, p=0.76, with values of 4, 34, and 37°C, outside the temperature threshold, omitted) (Table 2.1). No waxworms were infected by *S. riobrave* at 4°, 16°, 34°, or 37° C. Infectivity was uniformly low (0.3%-2.4%) at the other temperatures.

Infective period: *H. marelatus* were infective up to 40 days, on average, at 16°C, for about two weeks at 19 and 22°C, but only for 2-3 days at 25°C and higher (Table 2.1) (Kruskal-Wallis rank test statistic=31.7, p<0.0001). *S. riobrave* was only infective up to an average of 8.7 days, at 31°C (Kruskal-Wallis rank test statistic=5.4, p=0.49). At cooler temperatures, *S. riobrave* was infective for just 1.5-6 days (Table 2.1).

H. marelatus infected a higher average number of waxworms (5.8 ± 2.3 mean ± SE) than did *S. riobrave* (1.4 ± 0.3) over all temperatures (Comparison of Means t= 1.9, p=0.05; data from 4, 34, and 37°C not included in analysis). Additionally, *H. marelatus* infected waxworms over a longer period of time (11.5 ± 5.2 days) than did *S. riobrave* (3.8 ± 1.1 days) at each temperature (Comparison of Means t= 1.44, p=0.05).

Bacterial temperature limits: *Photorhabdus* sp., the symbiotic bacteria isolated from *H. marelatus*, reproduced at temperatures of 4°C-28°C. This *Photorhabdus* species took up to 10 days to reproduce enough to be visible on agar

plates at 4°C, but reproduced in less than 48 hours at 28°C, but not at all above 28°C. *Xenorhabdus* sp., the symbiotic bacteria from *S. riobrave*, reproduced at temperatures from 13°C-34°C. The *Xenorhabdus* species also was slow to reproduce at cool temperatures, requiring 7 days at 13°C, and less than 48 hours at 34°C. After 2 months, no *Xenorhabdus* sp. growth was visible when held at 4°C.

Nematode longevity: Nematodes were stored at a range of temperatures and then used to infect waxworms at 22°C to estimate the length of time the nematodes are likely to remain viable at the range of soil temperatures. Average longevity for *H. marelatus* differed significantly among temperatures (data for 34, 37°C omitted as above biological threshold; $F=5.70$, $df=7, 40$; $p=0.0001$). Nematodes maintained at 13° and 16° were able to infect hosts for an average of 45-48 days, significantly longer than at other temperatures (Figure 2.2). *H. marelatus* held at 31°C were able to infect hosts for just six days on average (Figure 2.2). No *H. marelatus* remained viable after being maintained at soil temperatures of 34°C or 37°C.

S. riobrave remained able to infect hosts after storage at all temperatures for at least 6.5 days (Figure 3), although they survived significantly longer at temperatures below 19°C than at warmer ones (Kruskal-Wallis rank test statistic=38.50, $p<0.0001$). *S. riobrave* maximum longevity was greatest at 13°C and 16°C, with nematodes surviving an average of 104 days and 89 days, respectively (Figure 2.3).

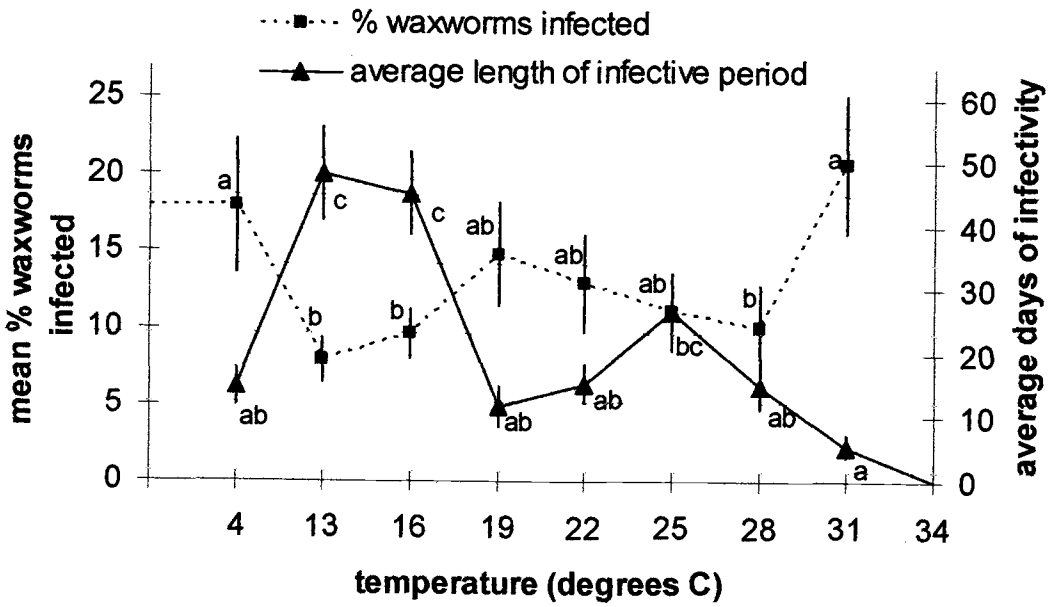

Figure 2.2. Average *Heterorhabditis marelatus* infective period vs. percent of waxworms infected, as mediated by temperature. Nematodes were stored at the listed temperatures, and then used to treat waxworms at 22°C. Longevity (days of infectivity) was measured by the date on which nematodes were removed from storage and used to infect waxworms at 22°C. A nematode considered infective for 25 days was stored at a given temperature for 25 days and then was able to infect a host at 22°C. Different letters above data points indicate significant differences between the temperatures at $p<0.05$ (Fisher's LSD). Note the two y axes describe different data; the percent for waxworms infected at each temperature is provided on the left axis and by the dotted line, whereas the data for average length of infective period is provided on the right axis and by the solid line. The percent waxworms infected and average days of infectivity are strongly negatively correlated ($r = -0.78$).

The number of waxworms infected by *H. marelatus* varied with sand temperature prior to infection at 22°C (data for 34, 37°C omitted; $F=2.34$, $df=7, 70$; $p=0.03$). Nematodes held at 4°C and 31°C infected more waxworms on average than those maintained at 13° and 16°C (Figure 2.2). The percent of waxworms infected by *S. riobrave* also varied with sand temperature ($F=8.42$, $df=9, 110$;

$p<0.0001$). Fewer waxworms were infected when treated with *S. riobrave* from sand held between 19°-28°C than nematodes maintained at either temperatures below 16°C or at 37°C (Figure 2.3).

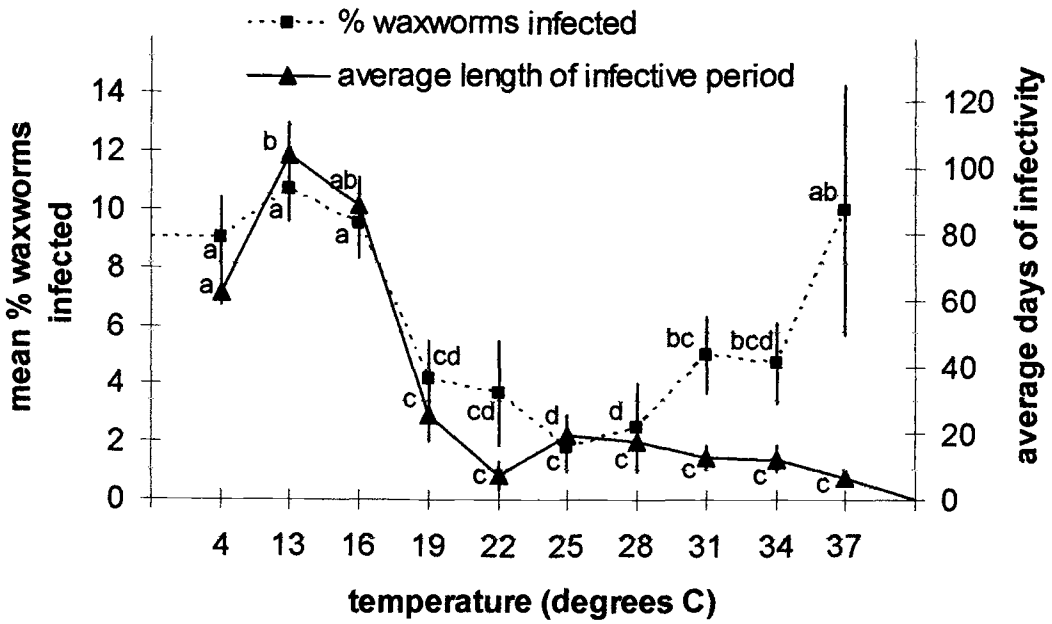

Figure 2.3. Length of *Steinernema riobrave* infective period compared to percent of waxworms infected over a broad range of temperatures. Nematodes were stored at the temperatures listed, and were then used to treat waxworms at 22°C. The average longevity indicates the average number of days (out of 3 replicates, 20 waxworms per replicate per date) that nematodes could be stored and then be able to infect at least one waxworm. A nematode considered infective for 18 days was stored at a given temperature for 18 days and then was able to infect a host at 22°C. Different letters by data points indicate significant differences between temperatures at $p<0.05$ (Fisher's LSD). Note the two y axes describe different data; the percent for waxworms infected at each temperature is provided on the left axis and by the dotted line, whereas the data for average length of infective period are provided on the right axis and by the solid line. The percent waxworms infected and average days of infectivity are negatively correlated ($r = -0.58$) above 16°C.

S. riobrave were able to infect hosts longer on average over all storage temperatures than *H. marelatus* (Comparison of Means $t= -2.14$, $p<0.02$), when the nematodes were held at 4-37°C and then used to infect waxworms at 22°C. *S. riobrave* remained infective an average of 35.6 days (± 5.2 days SE), and *H. marelatus* was infective, on average, only 22.8 days (± 4.1 days SE). However, *H. marelatus* infected more waxworms at 22°C when held throughout the range of temperatures prior to infection than did *S. riobrave* (Comparison of Means $t=3.84$, $p=0.0015$). *H. marelatus* infected an average of 13.1% (± 3.0% SE) of all waxworms, but *S. riobrave* infected only 6.1% (± 1.7% SE) of waxworms over all the storage temperatures.

Correlation between longevity and percent of hosts infected: A strong negative correlation between average longevity and percent of waxworms infected by nematodes stored at each temperature and then inoculated at 22°C was found for both species of nematode (Figures 2.2 and 2.3). Longevity compared with infection rates for *H. marelatus* (after omitting data from 34°C and 37°C at which no waxworms were infected) was inversely correlated ($r= -0.78$). When the temperatures at which *S. riobrave* did not normally infect hosts (Table 2.1) were omitted and only data from 19°C and warmer were compared, the relationship between longevity and infection rates was negative ($r= -0.58$). Nematodes of both species infected more hosts when exposed to temperatures at which they could not

survive for long periods. When nematodes were held at temperatures at which longevity was increased, the nematodes infected fewer hosts.

DISCUSSION

The potato fields of the Columbia Basin of Oregon and Washington have sandy soils and are irrigated daily during host summer weather. Moisture and sandy soils are ideal for nematode survival, movement, and host-finding (Kaya and Gaugler, 1993), suggesting that entomopathogenic nematodes could be used to control Colorado potato beetle. However, high temperatures and large temperature fluctuations in the soil may create an inhospitable environment for nematodes. The nematodes examined in this study were treated as if in an irrigated field in that they were kept moist, and water was added to replace evaporated moisture, and were found to survive for several days to many weeks at the temperature extremes found in this potato-growing region. Figure 2.1 indicates the unirrigated soil temperatures at which the Colorado potato beetle is active, and at which the nematodes would have to survive to provide adequate beetle control. Soil temperatures may be as low as 17°C and as high as 37°C in unirrigated soils during the periods that entomopathogenic nematodes would be applied for Colorado potato beetle control. However, irrigation buffers soil temperatures, and so nematodes would only have to be active from about 20-32°C.

Survival and infectivity over a broad range of temperatures can be directly linked to the behaviors and geographical origins of the two nematode species. *S. riobrave* is native to a locale with a hot climate, and it more readily infects hosts at higher temperatures than does cool-weather-adapted *H. marelatus*. *S. riobrave* infects very few hosts at 13°C, none at 16°C, and constant numbers of hosts from 19-31°C. Although *H. marelatus* also can infect hosts from 13-31°C, infection rates at 25°C and above are very low. Additionally, *S. riobrave* can remain infective after being heated up to 37°C, whereas *H. marelatus* is not able to infect hosts after being in sand above 31°C.

The two species of nematodes respond differently to environmental conditions. *S. riobrave* appears to survive longer by becoming inactive and infecting fewer hosts at a given time. Although *S. riobrave* remains infective about 60% longer than *H. marelatus* over the range of temperatures examined, *S. riobrave* only infects 50% as many waxworms as does *H. marelatus* (Figures 2.2 and 2.3). This is probably because *S. riobrave* displays both cruiser and ambusher host-searching behavior (Cabanillas et al., 1994), often remaining inactive until a host is nearby, whereas *H. marelatus* is an active cruiser species, expending energy to move through the soil to search for hosts. Some nematode species survive extended periods by coiling and entering an inactive state (Molyneux, 1985; Kaya, 1990) but neither of the species in this study are known to enter this quiescent state. The two species are similar in size and probably have similar lipid reserves, so the activity

level of the species most likely is responsible for the differences in longevity. The host-searching behavior may have evolved differently in the two species to allow for differences in host distribution in time and space. If hosts are patchy in time or space, a nematode might infect more hosts if the nematode is able to remain inactive through periods or areas of low host availability, and conserve energy to survive longer until more hosts are available. Higher levels of biological control of pest insects might be provided by selecting a species of nematode which is appropriate either for patchy or for even host distributions.

The temperature range at which a nematode's symbiotic bacteria reproduce might limit the temperatures at which the nematode can reproduce (Grewal et al., 1996). Although Peel et al. (1999) found that 4°C can be lethal to some strains of *P. luminescens*, the *Photorhabdus* sp. symbiotic with *H. marelatus* developed at 4-28°C. No *H. marelatus* reproduced at 4°C, so the bacterial growth rate probably did not limit nematode infection and reproduction at low temperatures. The nematodes may have been inactive at 4°C, not entering the host until placed in warmer conditions. *H. marelatus* reproduced occasionally at temperatures above 28°C, so clearly this *Photorhabdus* sp. could at times also reproduce at high temperatures. The upper temperature limit for *H. marelatus* development may be restricted by temperature limitations on its symbiotic bacteria. *S. riobrave* also may be limited in reproduction at high temperatures by its associated bacteria. The bacteria developed at 13°C-34°C, which was the same temperature range at which

S. riobrave developed (Table 2.1). The nematode rarely developed at the extreme cool temperatures, so probably was not limited by its bacteria, which appeared to develop readily at 13°C.

Several researchers have indicated that not all individual infective juveniles in a population are able to attack a host at any one time (Kaya and Gaugler, 1993). The results presented here support this idea, in that nematodes surviving longer periods infected fewer waxworms (Figures 2.2 and 2.3). Length of infective period for the population of *H. marelatus* (Figure 2.2) dropped at 19 and 22°C, perhaps because this is a temperature range at which they can infect high numbers of hosts and at which they will commonly encounter hosts, and so few individual nematodes are inactive or storing energy for later infection. There was a strong negative correlation between average longevity and infectivity for *H. marelatus* ($r = -0.78$), and for *S. riobrave* ($r = -0.58$) above 16°C. *S. riobrave* is from a hot climate, and so it is not surprising that it does not respond with a clear longevity/infectivity trade-off pattern at low temperatures. At high temperatures, these nematodes remain continually active to infect more individuals in case environmental conditions worsen. These results suggest that increased population longevity is due to inactivation of a portion of the population, which then becomes active at a later time.

Cabanillas et al. (1994) originally found *S. riobrave* infecting corn earworms and fall armyworms in corn fields in southern Texas. The soil

temperatures where *S. riobrave* was isolated were about 35 ± 4°C at the depth of 5 cm. The nematodes used in the present study survived only up to 30 days at 34°C, and a maximum of 20 days at 37°C. Although Cabanillas et al. (1994) did not discuss the longevity of *S. riobrave* in the field, its natural occurrence throughout the year suggests it can survive long periods of extremely high temperatures. Previous laboratory studies indicated *S. riobrave* could infect waxworms and reproduce at 20-35°C (Grewal et al., 1994), whereas the *S. riobrave* in the present study reproduced in waxworms between 13-31°C. The survival and reproduction of *S. riobrave* in both laboratory and field studies at higher temperatures than those found in the present study suggest that the nematodes in this study had developed a lower maximum temperature threshold over time, that nematodes in the field moved deeper in the soil to avoid excessively high temperatures, or that cooler soil temperatures at night in Cabanilla's study allowed the nematodes to survive longer in the field than they would at constant high temperatures. The three factors are not mutually exclusive, and it may be a combination of factors that allow nematodes to survive long periods in the field. Grewal et al. (1996) indicated that nematode temperature range can change rapidly under strong selective conditions. This plasticity in thermal adaptation could be both a bane and a blessing to biocontrol practitioners attempting to utilize nematodes for pest control. Nematodes may not behave as initially expected, but careful selection of nematode populations may allow nematodes to infect and reproduce better than originally proposed under given conditions.

Nematode movement through soil not only allows searching for hosts, but may moderate soil temperature effects on infectivity. Cabanillas et al. (1994) discussed vertical migration of *S. riobrave* as a survival mechanism to avoid extreme conditions. In north central Oregon, soil at a depth of five centimeters versus 20 cm can vary by 6°C or more daily (HAREC website). Cabanillas and co-workers found the majority of infective juveniles within the top 20 cm of soil. *H. marelatus* also can move vertically 20 cm or more in soil, as suggested by Berry et al. (1997), and so both species could potentially search for moderate temperature conditions by ascending or descending the soil column. Movement in soil might allow nematodes to avoid high temperatures during the day, as well as allowing nematodes to infect hosts at night, when soil temperatures near the surface are cooler. Although soil temperatures at the soil depth at which hosts are found can rise above and below the infective temperature range, these temperature extremes generally only last for a few hours to a few days at a time in north central Oregon (Figure 2.1). Soil temperatures at 7.5 cm deep can fluctuate by 10°C or more in one 24-hour period during the summer. The higher temperatures prohibit nematode attack of hosts, but in just 12 hours, the soil temperatures can drop to a level at which the nematodes can successfully infect a host. The results of the current study indicate that nematodes can survive longer at temperature extremes than they can infect hosts. When temperatures become more moderate, as represented by bringing nematodes to 22°C for infection in the longevity trials, nematodes that would not be infective at temperature extremes then become more likely to infect

hosts. Thus, soil temperature fluctuations in time and space probably cause nematodes to infect hosts that they would not attack under temperature extremes.

Both *H. marelatus* and *S. riobrave* can infect hosts over the same range of temperatures, from 13°C to 31°C. *H. marelatus* does not survive at 34 and 37°C, although the nematodes may move downwards during periods of extreme heat to avoid such high soil temperatures. *H. marelatus* survives for several weeks in north central Oregon potato plots during mid-summer when daily irrigated soil temperatures range from approximately 20-32°C (Armer, pers. obs.), supporting the possibility of movement to mediate temperature extremes. *H. marelatus* infects more hosts and is infective for a longer period of time than is *S. riobrave*. Therefore, under the conditions found most commonly in the north central Oregon potato growing region, *H. marelatus* is the stronger candidate for biological control of CPB with entomopathogenic nematodes than *S. riobrave*.

ACKNOWLEDGEMENTS

I thank Sujaya Rao and Ralph Berry for their comments which improved earlier versions of the manuscript. Sarina Jepsen provided lab assistance during final stages of the project. I am grateful for support which was provided by EPA-STAR Graduate Fellowship #U915727.

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CHAPTER 3: FIELD EFFICACY OF THE ENTOMOPATHOGENIC NEMATODE *HETERORHABDITIS MARELATUS* LIU & BERRY FOR COLORADO POTATO BEETLE CONTROL

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For submission to Entomologia Experimentalis et Applicata

ABSTRACT

The Colorado potato beetle (CPB) is the most severe defoliating pest of potatoes in the United States. This study examined the potential for control of the CPB with the entomopathogenic nematode *Heterorhabditis marelatus* in potato fields in north central Oregon. This research also examined possible impacts of varying nitrogen fertilizer levels on nematode control of the beetle and nematode reproduction within the beetle. Nematodes were applied with a backpack sprayer to the soil June 28 and August 17, 2001 at a rate of 50 infective juveniles/cm^2 soil surface as CPB were nearing pupation. In an attempt to manipulate alkaloid levels in potato foliage, nitrogen fertilizer was applied at 226 kg/ha to low-nitrogen plots, whereas high-nitrogen plots received a total of 678 kg/ha of nitrogen. Nitrogen treatments and nematode treatments were applied to plots in a 2x2 factorial randomized block design, with a total of eight plots of each of the four treatment combinations. The higher nitrogen application rate significantly increased foliar levels of the alkaloids solanine (from 2.8 ± 0.3 mg to 3.8 ± 0.4 mg/100 g dried

foliage) and chaconine (from 7.0 ± 0.5 mg to 9.9 ± 1.2 mg/100 g leaf tissue) as measured by HPLC analysis of field-collected foliage. The first nematode application resulted in a 50% reduction of adult CPB populations three weeks later, from more than seven adults per four beat sheets in untreated plots to 3-4 in nematode-treated plots. The second nematode application also killed significant numbers of prepupal CPB. Soil samples collected one week after nematode application had more than 12 ± 10 (mean ± SE) dead prepupae in the nematode-treated soil samples, but only 2 ± 1.4 dead in the untreated samples. As an additional monitoring method, buckets filled with soil and a known number of prepupal CPB were buried prior to nematode application. Only 2.5-12.8% of CPB survived to maturity in the buckets from the nematode-treated plots, whereas 35-45% of the CPB from buckets from untreated plots matured. Non-target effects of nematode applications were positive for above-ground predators, in that heteropteran predators were found in higher densities in the nematode-treated plots than in control plots on August 8, along with the increase in young CPB larvae. The CPB-parasitic tachinid *Myiopharus doryphorae*, which exists naturally in potato systems in the Columbia Basin, did not appear to be affected by nematode applications. Tachinid parasitism rates ranged from 50% of CPB from nematode-treated plots to 45-82% of CPB from untreated plots from soil samples collected a week after the second nematode application. No reproducing nematodes were found in the 303 dead prepupae and pupae collected from soil samples and buckets from nematode-treated plots. Nitrogen fertilizer levels, and the related alkaloid

levels, did not affect nematode infection rates or reproduction in any of the soil samples examined. These results indicate that this species of nematode is effective for controlling CPB in the field, but the low nematode reproductive rate cannot be manipulated through increased foliar alkaloids. Due to limited nematode longevity and nearly complete lack of reproduction in the field, nematode applications would need to be made several times per season for adequate CPB control.

KEY WORDS: *Leptinotarsa decemlineata*, Heterorhabditidae, Nematoda, biological control, integrated pest management, solanine, chaconine

INTRODUCTION

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), is one of the most economically damaging insect pests of potatoes (*Solanum tuberosum* L.) in the United States and throughout much of Europe (Hare, 1990). The four larval instars as well as the adults feed on foliage, and a single insect eats at least 100 cm^2 of potato foliage in its lifetime (Tamaki and Butt, 1978; Ferro et al., 1985). The adults live one month or more during the summer in the field, and there are two to three CPB generations per year in the Columbia Basin potato growing regions of north central Oregon and south central Washington (Berry, 1998). The second and third generations of the CPB can cause 100% potato defoliation several months before the end of the growing season if left uncontrolled, which can reduce tuber yields by 50% or more (Hare, 1980; Ferro et

al., 1983). The CPB is commonly controlled by pesticides, but some populations of the beetle have developed resistance to nearly all chemical insecticides (Forgash, 1985), as well as to *Bacillus thuringiensis* var. *tenebrionis* foliar sprays in the laboratory (Whalon et al., 1993). Biological control, using predators, parasites (Hough-Goldstein et al., 1993), and pathogens (Lacey et al., 1999), is being examined by researchers for reducing CPB populations. Entomopathogenic nematodes are among the pathogens that can provide high levels of CPB control (Berry et al., 1997).

Entomopathogenic nematodes have a life cycle which makes them good candidates for CPB control. The infective juvenile (IJ) nematodes live and attack arthropod hosts in the soil. The CPB feeds on foliage as a larva, and when feeding is completed, it burrows into the soil beneath its host plant. After 3-5 days in the prepupal stage, the CPB pupates underground, and then emerges to continue feeding on foliage as an adult. The prepupal stage of the CPB is highly susceptible to nematode attack, and nearly all prepupae in soil inoculated with appropriate rates of entomopathogenic nematodes are killed. Nematodes enter a host through the mouth, anus, or spiracles, and then push through the peritrophic membrane or tracheoles (Nguyen and Smart, 1991) into the hemocoel. Once inside the host, the nematodes release symbiotic bacteria that kill the host (Kaya, 1990a). The nematodes feed on the bacterial/host tissue slurry, developing through several generations before depleting the food supply and exiting the host to search for a new host. Nematodes survive best and find hosts most readily in sandy, moist soil

(Kaya, 1990b), which are the conditions that CPB prefer for pupation (Wegorek, 1959). Many potato-growing regions, such as those in north central Oregon and central Washington, have sandy loam soils and are irrigated daily during the hottest, driest periods. These conditions provide an ideal habitat for the use of nematodes in biological control.

The nematode *Heterorhabditis marelatus* Liu & Berry (Nematoda: Heterorhabditidae) (Liu and Berry, 1996) kills nearly 100% of CPB when applied at a rate of 50 infective juvenile nematodes/cm^2 of soil surface. Numerous species of nematodes have been examined for CPB control over the years, but *H. marelatus* provides consistently higher levels of control in laboratory and greenhouse studies than do many other species (Berry et al., 1997). However, *H. marelatus* reproduces in less than one percent of infected beetles (Armer, unpublished). The nematodes would need to be applied several times during each season to provide adequate beetle control unless nematode reproduction could be enhanced. In preliminary studies with growth-chamber grown potato plants, higher levels of nematode reproduction were supported in CPB prepupae that had fed on plants grown with high levels of applied nitrogen fertilizer than beetles fed on plants provided with low levels of added nitrogen. The CPB prepupae that had fed on high-nitrogen plants also attained less weight in some replicates. Potato plant alkaloid levels can be elevated by applying high levels of nitrogen fertilizer (Mondy and Munshi, 1990). Although the CPB has evolved to feed on solanaceous plants, it may expend energy for sequestering, detoxifying, or excreting alkaloids ingested from its host

plants. The negative effects of added nitrogen on CPB weight and ability to inhibit nematode reproduction therefore could be due to energy spent handling increased levels of alkaloids in the CPB diet. If foliar alkaloid levels could be increased to encourage nematode reproduction in the beetle, nematode populations could potentially recycle in the field, and maintain themselves for season-long beetle control.

The present study was conducted to evaluate efficacy of *H. marelatus* in reducing CPB populations in the field. In addition, based on the hypothesis that increased nitrogen fertilizer levels reduce the CPB's resistance to successful nematode infection and development, we examined the impact on *H. marelatus* infection and reproduction in CPB prepupae of providing excess nitrogen to the potato plants on which host CPB fed.

MATERIALS AND METHODS

Field site: The experiment was conducted at the Oregon State University Hermiston Agricultural Research and Extension Center in Hermiston, OR. Potato (cv Russet Burbank) seed pieces were planted at an in-row spacing of 23 cm on May 19, 2001 in plots that were 7.8 m x 4.6 m. The plots contained nine rows, with the outer two rows on each edge and the last 0.76 m of each row acting as buffers. The treatments were laid out in a 2x2 factorial randomized block design, with a total of eight replicates per treatment combination. The four treatment

combinations that were applied consisted of high nitrogen fertilizer (N) x nematodes, high N x no nematodes, low N x nematodes, and low N x no nematodes.

Fertilizer treatment: Nitrogen was preplant strip applied to all plots at 68 kg/ha, and sidedressed onto plants at 158 kg/ha on June 10. Additional nitrogen was sidedressed on high-fertilizer plots at 226 kg/ha on June 26, and 226 kg/ha on July 23. The high-nitrogen plots thus received a total of 678 kg/ha of nitrogen, and the low-nitrogen plots received 226 kg/ha. Commercial potato fields in the Hermiston area normally receive about 395-450 kg/ha of nitrogen. The high- and low-nitrogen treatments therefore did not represent commercial practices, but were used to create high and low levels of glycoalkaloids in the foliage (Mondy and Munshi, 1990). We chose not to plant different varieties of potatoes with naturally high and low levels of alkaloids in order to avoid possible confounding factors of different growth rates, maturation dates, foliar nutrient levels and plant architecture found in different potato varieties.

Nematode treatment: *H. marelatus* (isolate OH10) were applied on June 28 and August 17, 2001, when most CPB were fourth instars. Nematodes were produced in waxworms (*Galleria mellonella* L. [Lepidoptera: Pyralidae] purchased from Vanderhorst Wholesale Inc., St. Mary, OH) in the laboratory. Nematodes were collected from infected waxworms one to three days prior to application in the field, and were held in chilled water with air circulation until application. All plots

were irrigated with sprinklers for one hour immediately prior to application, and then for one hour after application. The nematodes were applied to entire plots (including buffer areas) just before dusk to minimize the detrimental effects of UV radiation and desiccation (Smits, 1996). Nematodes were applied at 18,000,000 per plot, equivalent to 50 per cm^2 of soil (Berry et al., 1997). The nematodes were applied at 30 psi with a CO_2 backpack sprayer through a fan nozzle with the screen removed.

The field was treated with fungicides and herbicides typically used in commercial fields. Bravo (chlorothalonil) and Dithane (mancozeb) were applied at label rates to protect against late blight. Matrix (rimsulfuron) was applied at 0.14 kg/ha, Eptam (S-ethyl dipropylthiocarbamate) at 0.57 l/ha, and Sencor (metribuzin) at 0.13 l/ha were applied pre-emergence for weed control. The insecticide Success (spinosad) was applied on July 13, 2001 at 0.05 l/ha (75% of the maximum rate) to slow CPB defoliation by killing early instars. Spinosad targets the insects' nervous system (Salgado, 1998), and degrades in sunlight within several days. Non-target predators are not affected by spinosad (Success Specimen Label).

Foliar alkaloid analysis: Foliar content of chaconine and solanine, the two main alkaloids in potato foliage (Friedman and McDonald, 1997), was measured by HPLC analysis of leaves collected from the field. Every fifth leaf was collected from one random plant within each plot on each of the insect sampling dates except the first. The leaf samples were stored in a cooler up to 12 hours before being

placed in a -20°C freezer in the laboratory. Although leaves were collected from plants in each of the plots in which insects were sampled, alkaloids were only quantified for five to seven plants selected at random from those collected from the high-nitrogen plots, and for five to eight plants in the low-nitrogen plots, for each of the sampling dates. The collected leaflets were stripped from the stems and were dried at 45°C 3-5 days. The foliage was ground with a mortar and pestle, and the alkaloids were extracted based on the protocol from Brown et al. (1999). Approximately 50 mg of the dried foliage powder was weighed to the nearest 0.1 mg, and was stirred with 40 mg 2% acetic acid for two hours. The extract was vacuum filtered through no. 4 Whatman filter paper. Ammonium hydroxide was added to make the filtrate basic (pH 10.05-10.25). The filtrate was partitioned twice with 20 ml water-saturated n-butanol in a 125 ml separatory funnel. The butanol layers were combined, and the butanol was evaporated to dryness in a fume hood. The residue containing the alkaloids was then dissolved in 2 ml methanol, and diluted with 2 ml acetonitrile and 1 ml deionized water. The extracted samples were stored at -20°C until HPLC analysis.

The foliar samples were run through a Guard-Pak Resolve C18 precolumn and a Waters Resolve C18 3.9 x 300 mm column. The eluent was 100 mM monobasic ammonium phosphate in 35% acetonitrile, adjusted to pH 3.5 with phosphoric acid. A Waters model 717plus autosampler, using a Waters 600S controller and a Waters 996 Photodiode Array Detector, injected 20 µl of each sample at 1 ml/min into the column. Each sample was run for 10 minutes with 1

minute between samples, and alkaloid peaks were detected at 200 nm. Millenium[32] software from Waters was used in collecting and graphing the chromatographs. Solanine and chanconine standards purchased from Sigma (St. Louis, MO) were used to calibrate the results.

CPB sampling by beat sheets: CPB populations were estimated with beat sheet samples which were taken on the mornings of June 28, July 11, July 20, August 8, and August 17 prior to nematode application. A beat sheet was placed against the plants and the vines were struck ten times over the sheet for each count. Individual plants were not sampled because the vines were entwined together as they grew. The four beat sheet samples taken in each plot on each date were summed for a single count per plot for each date. All CPB were counted by instar, and were returned unharmed to the plots after counting.

CPB sampling by soil samples: The plants in three of the plots appeared diseased in early August, and by late August, the plants in all plots were defoliated by apparent disease and by CPB feeding. To monitor nematode effects on CPB when no plants remained for beat sheet sampling, soil samples were dug from within 34.5 cm square frames to a depth of 11.5 cm on August 24 and September 3. Each sample contained approximately 13,700 cm^3 of soil. Samples were taken from four plots with each treatment on the first date, and from eight high-N nematode-treated plots, eight low-N nematode-treated plots, three high-N no-

nematode plots, and two low-N no-nematode plots on the second date. Prepupal and pupal CPB were brought back to the laboratory and held for up to two weeks to calculate death rate and cause of death. Prepupae and pupae that matured to adulthood were considered 'live,' whereas those that died prior to maturation were considered 'dead.' The Sun-Shepard correction for control mortality was calculated (% efficacy of treatment = ((% treatment mortality)-(% control mortality)/(100)-(% control mortality))*100) to estimate the percentage of CPB killed by nematodes in treated plots.

CPB sampling by bucket samples: In addition to beat-sheet sampling of above-ground CPB populations in the nematode-treated vs. control plots, we also directly examined prepupal CPB death rate caused by nematode applications. Prior to nematode application, plastic buckets 19 cm in diameter and 19 cm tall with small holes drilled in the bottoms for drainage were filled with field soil and buried in the field plots until the bucket tops were even with the ground. One bucket was placed in each of the 32 plots, in the furrow between rows 4 and 5. The buckets were left uncapped during nematode application. Ten late fourth-instar CPBs that had been field-collected from the corresponding high-N or low-N fertilizer plots prior to nematode application were placed on the soil in each bucket. The buckets were capped with plastic lids with holes drilled for ventilation and to allow irrigation water into the buckets. Two weeks after the nematode treatment all the buckets were returned to the laboratory for sorting. Adult CPB in each bucket were

counted, and other CPB were collected and held at room temperature for emergence. Following the second nematode application, 20 late fourth-instar CPBs were placed in each bucket and treated in the same manner. Half of the buckets (four from each treatment combination) were collected from the field one week after the second nematode application, and the remaining buckets were collected two weeks after treatment. All adult CPBs, dead pupae, and prepupae were counted. Dead pupae and prepupae were dissected to determine the presence of *H. marelatus*. When not all CPB could be found in a bucket, the missing ones were considered dead and disintegrated. Percent CPB survival was calculated.

Beneficial arthropod population sampling in beat sheets: Common arthropod predators were counted in beat sheet samples at the same time as CPB were counted. Predators were sorted by feeding guild into two groups. Heteropteran predators which also obtain some nutrients from plants, *Orius tristicolor* White, *Nabis* spp., *Geocoris* spp., and reduviids, were grouped together, as they could be affected by foliar nitrogen availability in the high-nitrogen plots. The strict carnivores, spiders, coccinellids, and hemerobiids, were grouped together in a second group to determine possible non-target effects of the nematode applications. Predators were returned unharmed to the plots after counting.

Tachinid CPB parasites in soil samples: Many of the CPB collected in the 13,700 cm^3 soil samples were infected by the native prepupal parasitic fly

Myiopharus doryphorae (Riley) (Diptera: Tachinidae) (Tamaki et al., 1983; Horton and Capinera, 1987) by late season. Numbers of emerging tachinids were counted to determine non-target nematode effects. The tachinids and nematodes both obtain nutrients from prepupal CPB and are in direct competition, hence the tachinids are the most likely of the natural enemies to have a direct response to nematode application.

Tachinids in bucket samples: Tachinids that emerged from the CPB in the bucket samples were counted, and percent parasitism was calculated for the CPB from each of the four treatments. Any tachinid that died within the host of unknown causes was included in the counts for percent tachinid parasitism.

Statistical analyses of alkaloids: The potato plant alkaloids solanine and chaconine quantities were compared for each alkaloid separately via one-way ANOVA to determine the effect of nitrogen fertilizer on alkaloid level. The data were first examined with all dates combined, and then for each individual date. Data for all the dates were normalized by a natural-log transformation.

Statistical analyses of beat sheet data: The insect count data were examined separately for each sampling date. Data were omitted for three plots from the August 8 sampling, and for five plots in the August 17 sampling because the plants were dead and could not support normal insect populations. The CPB data

were square-root, ln(x+1) or $\log_{10}(x+1)$ transformed where appropriate and compared in a two-way ANOVA. The remaining data that could not be normalized by transformation were ranked prior to being compared by calculating H=Sum of Squares/total Mean Squares. The variable *H* is calculated as an extension of the Kruskal-Wallis test that compares the results of two treatments, and is examined as a χ^2 variable to test for significance between treatments (Sokal and Rohlf, 1995). CPB counts were examined by individual instars, whereas the other insects were grouped by feeding guild. In addition to examining predator counts to determine the effect of nematodes on the predators, CPB first and second instars were combined and regressed against heteropteran predators and against non-phytophagous predator counts for each plot on each date. Only young CPBs were used in the regressions because most of the predators avoid attacking larger and more active CPB larvae.

Statistical analyses of soil samples: Data collected August 24 by digging 13,700 cm^3 soil samples were compared by a two-way ANOVA for the effects of fertilizer level and nematode application on the total number of live CPB, the number of tachinids that emerged from collected prepupae, and the number of dead prepupae collected. Numbers of live CPB and dead CPB were both log(x+1) transformed, and the number of tachinids was square-root transformed. Numbers for live CPB from September 3 samples were not transformed, whereas the numbers of tachinids and numbers of dead prepupae plus pupae were rank transformed.

Statistical analyses of bucket samples: Nematode efficacy data from bucket samples were compared with a two-way ANOVA for fertilizer treatment and nematode application for effects on percent CPB survival and percent beetles parasitized by tachinids. The two dates were compared separately. The CPB survival data from both nematode application dates were rank transformed and the H variable was calculated as above. Percent of parasitism by tachinids was square-root transformed prior to analysis of data.

Statistical analyses other than calculation of the variable H were done using StatGraphics Plus 5.0 (Statistical Graphics Corp.). The variable H was calculated manually.

RESULTS

Foliar alkaloids: Potato foliage had higher levels of the alkaloids solanine ($F=5.34$, df=1, 48, $P=0.025$) and chaconine ($F=5.48$, df=1, 48, $P=0.023$) season-long in plots with high levels of nitrogen fertilizer than in plots with low levels of nitrogen (Figure 3.1). There was also more chaconine in the highly fertilized plants on July 11 than in the less-fertilized plants ($F=5.42$, df=1, 11, $P=0.04$). No other individual dates had different levels of alkaloids in the plots with high and low nitrogen fertilizer.

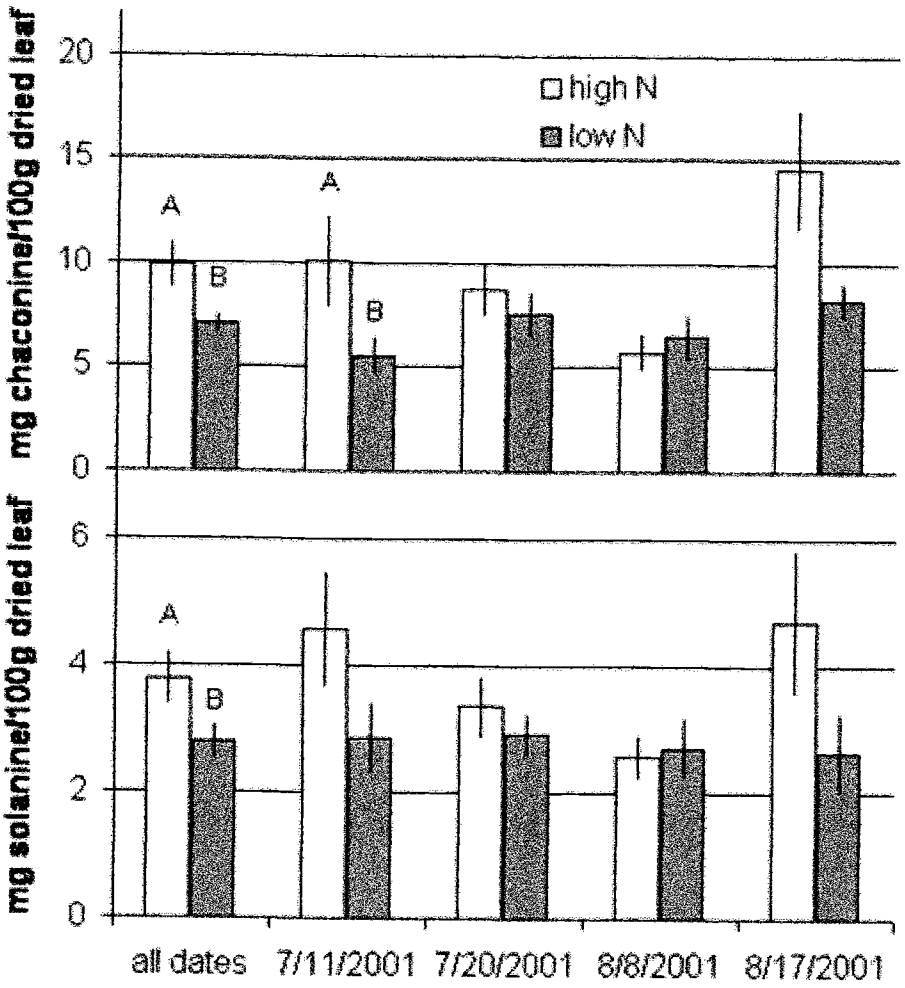

Figure 3.1. Alkaloid quantities in dried potato foliage collected in July and August 2001. Mean±SE alkaloids per 100g dried foliage are indicated. Different letters over the bars on each date indicate statistically significant differences at the α=0.05 level. Bars without letters did not differ between the treatments. Data were natural-log transformed for analysis, and back-transformed for graphical presentation.

CPB in beat sheet samples: There was a significant reduction in adult CPB populations in plots with nematode application compared with untreated plots by July 20 (2-way ANOVA F=4.76, df=1, 31; *P*=0.04), three weeks after nematode application (Figure 3.2). On August 8, CPB populations were *higher* in nematode-

treated plots for first instars (F=12.87, df=1, 28; *P*=0.001), second instars (F=14.21, df=1, 28; *P*=0.0008), and CPB third instars (F=5.03, df=1, 28, *P*=0.03). First and second instar populations were about 50% higher in nematode-treated plots than in control plots. No other CPB beat sheet samples differed between nematode-treated and control plots. Fertilizer level did not affect CPB population counts, although a trend late in the season suggested that higher nitrogen had the potential to increase larval populations (August 17 third instars: H=3.26, df=1, *P*=0.06; August 17 fourth instars: H=3.41, df=1, *P*=0.06). No interactions at the α=0.05 level existed between nematode treatment and fertilizer treatment in the CPB count data.

CPB in soil samples: More CPB prepupae and pupae died in plots treated with nematodes (F=8.97, df=1, 15; *P*=0.01) than in control plots (Figure 3.3) from the 13,700 cm^3 soil samples collected August 24. Nematodes killed about 87% of the CPB prepupae in treated plots. A total of 104 dead prepupae were dissected, and no reproducing nematodes were found. Fertilizer had no effect on numbers of dead prepupal and pupal CPB (F=0.56, df=1, 15; *P*=0.47). There was no difference in numbers of live CPBs found due to either nematode application (F=1.58. df=1, 15; *P*=0.23) or fertilizer levels (F=0.22, df=1, 15; *P*=0.65).

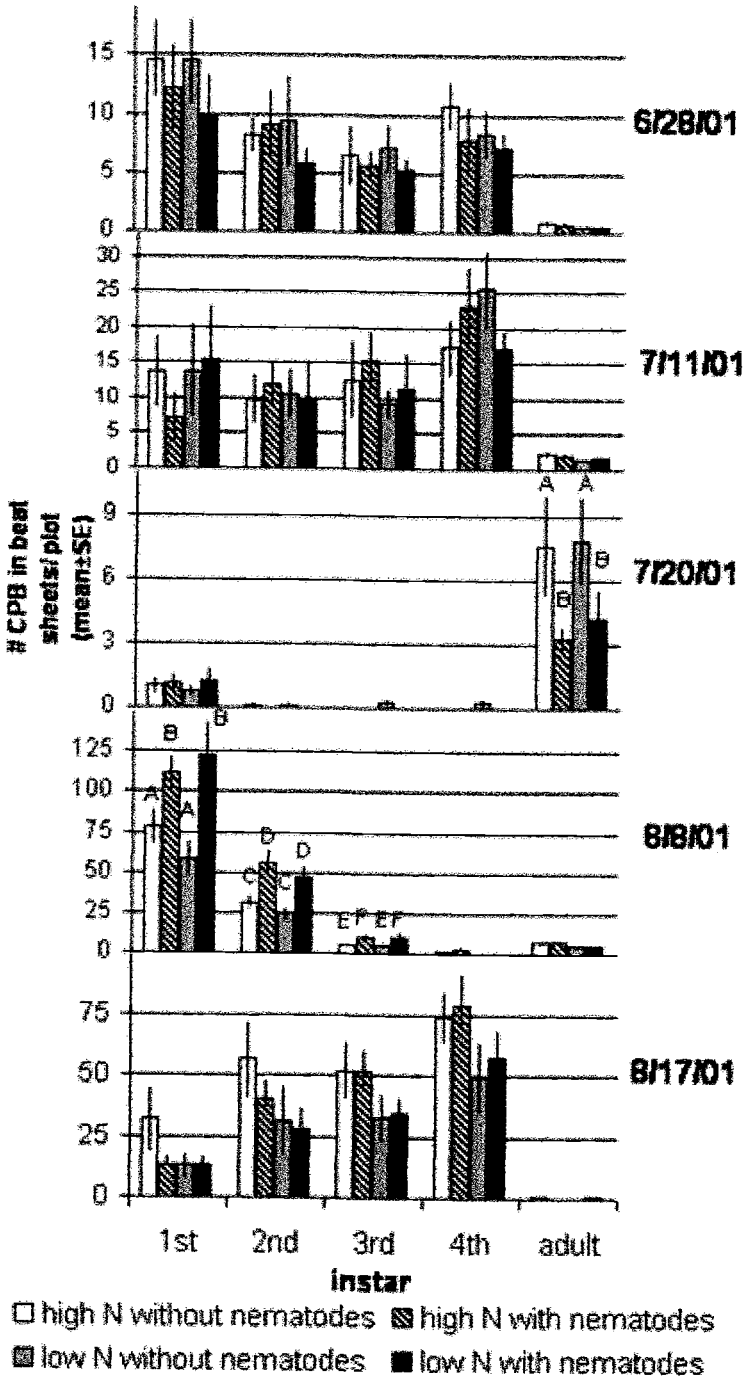

Figure 3.2. CPB populations in beat sheet samples. Four beat sheet samples, each with ten beats/sheet, were taken in each plot on each date. The average and SE of beetle counts per plots are indicated. Bars on graphs with different letters are significantly different at α=0.05.

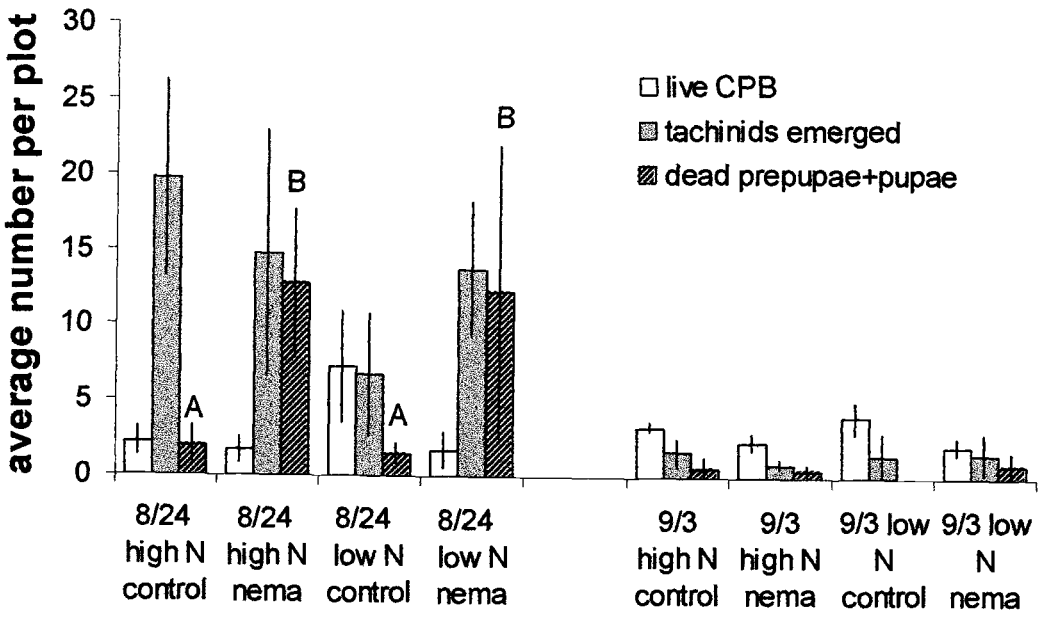

Figure 3.3. Effects of nematodes and fertilizer on the number of live CPB, tachinid parasitization, and the number of dead prepupal and pupal CPB found in 13,700 cm^3 soil samples. Error bars indicate the standard error. Bars with different letters were significantly different at α=0.05. Unlabeled bars indicate the results did not vary among treatments.

Adult CPB numbers in soil samples collected September 3 did not vary by nematode treatment (F=3.61, df=1, 20; *P*=0.07) or fertilizer treatment (F=0.00, df=1, 20; *P*=0.95) (Figure 3.3). The number of dead CPB prepupae and pupae was not affected by nematodes (H=0.35, df=1, *P*=0.60) or fertilizer (H=0.15, df=1, *P*=0.72). The fifteen dead prepupae from nematode-treated plots were dissected, and no nematodes were found.

CPB in bucket samples: Nematode application on June 28 killed most of the CPB prepupae in the bucket samples, leaving only 2.5% of beetles alive in the

low-nitrogen plots and 12.8% alive in the high-nitrogen plots (Figure 3.4). In the low and high-fertilizer plots not treated with nematodes, 44% and 36%, respectively, of the prepupae survived to adulthood. The nematode treatments caused a large difference in survivorship (H=14.77, df=1, P=0.001), but the difference in fertilizer levels did not affect CPB survival (H=0.4, df=1, P=0.45). There was no interaction between CPB survivorship due to nematode application and fertilizer level. None of the 72 dead beetle prepupae and pupae from treated plots dissected in the laboratory contained nematodes.

The nematode application on August 17 killed a significant number of CPB in buckets (H=13.23, df=1, P=0.001) (Figure 3.4). Fertilizer had no effect on CPB survival, either with or without nematodes (H=0.029, df=1, P=0.85). No nematodes were found or reproduced in the 112 dead CPB prepupae and pupae dissected in the lab.

Predators in beat sheets: No negative effects of the nematode applications were seen on any non-target insect beat sheet counts. More heteropteran predators were found in the nematode-treated plots than in plots without nematodes on August 8 (F=4.66, df=1, 28; P=0.04). Numbers of heteropteran predators on August 8 paralleled the numbers of CPB first- and second-instars in a simple linear regression (R^2=0.15, P=0.04). Other predators (spiders, hemerobiids, and coccinellids) were more dense in the nematode-treated plots on July 11 than in the untreated control plots (F=5.16, df=1, 31; P=0.031). Non-target arthropod numbers

did not vary significantly between nematode-treated and untreated plots on other dates.

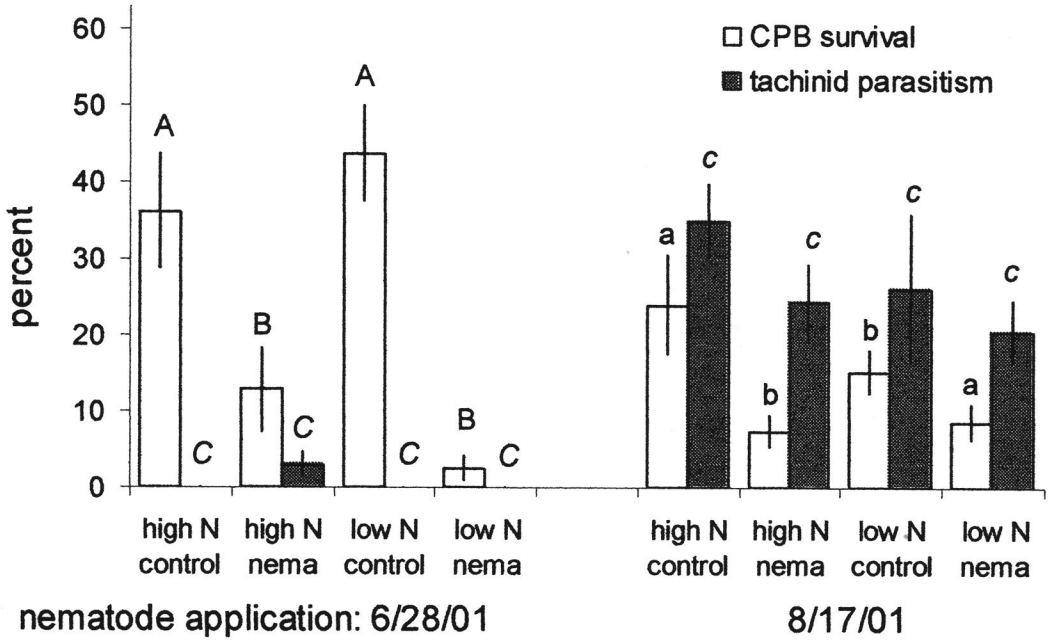

Figure 3.4. Effects of *Heterorhabditis marelatus* application on CPB survival and percent tachinid infection of CPB in buried buckets. Different letters over the CPB survival rates indicate significant differences at the $\alpha<0.05$ level. Error bars indicate the standard error. Survival was not compared between the two dates. No differences were found between treatments for parasitism rates.

Tachinid CPB parasites in soil samples: Approximately 56% of the CPB prepupae were parasitized by the tachinid *M. doryphorae* on August 24, averaged over all the treatments. Parasitism ranged from 44% in the low-nitrogen control plots to 82% in the high-nitrogen control plots (Figure 3.3), with about 50% of beetles parasitized by the tachinids in both sets of the nematode-treatment plots.

Number of beetles parasitized by tachinids was not affected by nematodes (F=0.04, df=1, 15; *P*=0.84).

About 28% of beetles found in all treatments on September 3 were parasitized by tachinids. The number of tachinids attacking CPB was not affected by nematodes (H=1.03, df=1, *P*=0.30). One of the 20 tachinid pupae developing in a CPB treated with nematodes had nematodes developing inside, which either killed the tachinid or which entered after it had died in the CPB of other causes.

Tachinids in bucket samples: Only 2 out of 80 emerged CPB in the high-nitrogen nematode-application plot were parasitized by tachinids on the June 28th sampling (Figure 3.4). No other tachinids emerged from the bucket samples from the first nematode application.

Tachinid parasitism ranged from 21-35% in bucket samples after the second nematode application (Figure 3.4). Parasitism rate not was affected by nematodes (F=0.16, df=1, 31; *P*=0.69).

DISCUSSION

This study is the first to examine CPB control by the nematode *H. marelatus* in field plots. Colorado potato beetle populations were greatly reduced by the application of *H. marelatus* just prior to the time the fourth instars drop to the ground to become prepupae. However, no nematodes were found to reproduce in CPB.

Foliar alkaloids: The potato plant alkaloids chaconine and solanine were successfully increased in foliage by addition of high levels of nitrogen fertilizer. Mondy and Munshi (1990) established that nitrogen fertilizer levels paralleled tuber alkaloid increases in Russet Burbank potatoes, and Friedman and Dao (1992) indicated that tuber alkaloid levels parallel foliar levels. Therefore, the addition of nitrogen fertilizer would be expected to increase foliar alkaloid levels, as established here. The increased alkaloids, however, did not affect nematode reproduction. Solanine and chaconine are acetylcholinesterase inhibitors (Roddick, 1989), and can disrupt cell membranes (Roddick et al., 1988). Preliminary laboratory studies suggested CPB could be negatively affected by high levels of nitrogen application to their host plants, which led to an increase in number of beetles supporting nematode reproduction (Armer, unpublished). No nematodes reproduced in any of the 303 treated beetles that were dissected, regardless of plant alkaloid level, indicating the elevated alkaloids did not encourage nematode reproduction. Alkaloids in growth-chamber grown plants in preliminary studies were three to four times more concentrated than those in field-grown plants (Armer, unpublished), which may have accounted for the different responses of CPB to nitrogen and nematode applications.

Nematode reduction of CPB populations: Numbers of adult CPB were reduced 50% in nematode-treated plots (Figure 3.2), and the number of dead prepupae found in the treated soil was at least three to four times higher than that

found in untreated plots (Figures 3.3 and 3.4). The lower apparent CPB death rate due to nematodes in the beat sheet samples than in the soil samples probably was due to adult CPB movement onto plants in treated plots. Most CPB that were late fourth instars and prepupae when nematodes were applied on June 28 did not emerge from the soil until about July 12-16. Thus, the July 20 beat sheet samples would be the first to indicate the effects of the nematode application. The beetles which emerged as adults in untreated (control) plots after the July 11th sampling had up to 8 days to move into the nematode-treated plots where there was little competition for food and oviposition sites. Although time constraints prohibited daily sampling, such precise monitoring may have clarified the origin of CPB adults in the nematode-treatment plots. However, daily sampling may have disturbed the plots and helped to transport beetles between plots. The combination of beat sheet sampling with other techniques therefore probably best indicated the effects of *H. marelatus* on CPB populations.

Nematode efficacy diminished by about one half over the ten-day period between the two soil sampling dates in late August and early September. Plants were entirely defoliated by the end of August, and late-developing beetles would be stressed by an inadequate food supply during their final larval stages. Larvae do not readily move long distances, and so could not travel to other fields to find adequate food. Beetles that had not pupated by the September 3 sampling probably had a high mortality rate due to starvation, which would obscure any effects of nematode parasitism on mortality. Additionally, many of the nematodes may have died

because of high temperatures associated with lack of ground cover, since all the foliage had been eaten or wilted from the plants.

CPB population recovery: The higher populations of first, second, and third stage CPB in the nematode-treated plots than in the control plots on August 8 was notable because just 18 days earlier, on July 20, CPB adult populations in nematode-treated plots were half those in control plots. Adult populations on August 8 were equal between nematode treatments and control plots. This suggests that adults in the control plots may have dispersed to the nematode plots after nematode application. Unlike larval CPB, the adults can easily disperse between fields (Alyokhin and Ferro, 1999) to find new food and oviposition sites. The adults in areas with lower beetle densities may have laid more eggs, or more early instars may have survived in sites with low densities of feeding beetles due to lack of competition. Lucas et al. (1995) found that CPB larvae developing under low population densities were larger and stored more lipids as adults than larvae living under higher densities, suggesting high densities increase competition for top-quality food. Beetle feeding may induce secondary plant compounds that lower the nutritive value of the potato leaves, such as low levels of polyphenol oxidase (Constabel and Ryan, 1998), which could cause the inverse density-dependence seen here.

Although there were no long-lasting differences between nematode-treated and untreated plots in terms of CPB numbers in beat sheet samples, it is clear that

H. marelatus was effective in killing beetle prepupae. Studies of prepupae in buckets and soil sampling indicated that nearly all CPB were killed by *H. marelatus*. The lack of differences in beat sheet beetle counts between plots apparently was due to adult movement between plots. Unless the nematodes kill nearly all the beetles in a large area, the beetle population can rapidly increase in treated areas through immigration and reproduction.

Beneficial arthropods: Potential negative effects on non-target arthropods are clearly a concern for the introduction of a stable, breeding population of parasitic nematodes. Several species of insect-parasitic nematodes caused significant mortality in immature carabid and staphylinid beetles, but not in adult predators (Georgis et al., 1991). Kaya and Gaugler (1993) noted that nematodes often had far narrower host ranges in the field than in the laboratory. Ground-dwelling arthropods, such as carabids and some spiders, were not monitored in this study unless they happened to be on the plants and detected in beat sheet counts. Although these organisms could be extensively exposed to the nematodes in the soil, these arthropods are highly mobile and move readily between plots and so cannot be monitored accurately in relatively small field plots. Direct non-target effects of *H. marelatus*, especially on beneficial organisms, would need to be tested thoroughly before application in an environment that would allow long-term nematode survival and reproduction.

Indirect effects on predators: Nematode applications may indirectly affect non-target organisms such as the range of predators found in potato fields. Numbers of heteropteran predators were higher in the nematode-treated plots on August 8. Probably the presence of more early-instar CPB in the nematode-treated plots attracted predators to those plots or improved survival of the predators. Predator numbers were never lower in nematode-treated plots than in control plots, suggesting the nematodes have no negative effects on foliage-inhabiting predator populations in potato fields. The heteropteran predators can feed on plants to supplement their prey diet, and predator numbers were higher on plants in high-nitrogen plots on August 8 (1-way ANOVA: $F=5.73$, $df=1, 27$, $p=0.02$). Any possible negative effects of plant-feeding by the heteropteran predators of increased alkaloids in the plants with high nitrogen fertilizer may have been negated by the benefits of high nitrogen availability.

Direct nematode effects on parasites: The parasitic tachinid *M. doryphorae* larviposits a tiny first instar in 2nd and 3rd stage CPB larvae (Tamaki et al., 1982). The fly larva remains inactive until the last-instar host ceases feeding and burrows into the soil, at which time the tachinid begins to feed inside the host. The fly usually kills the beetle before the host molts into the pupal stage. Therefore, both the tachinid and the nematode are active parasites of the prepupal CPB, and could potentially compete for nutrients. However, the tachinid parasitism rates and emergence rates did not differ between insects from nematode-treated and

control plots. The nematodes appear not to negatively affect the tachinid. The tachinid may secrete an antibiotic substance which halts the growth of the nematodes' symbiotic bacteria, or the tachinid may be able to ingest adequate nutrients from the beetle that has bacteria growing in it. The lack of negative influence of the nematodes on the tachinid, however, is correlative and has not been tested directly.

Potential for *H. marelatus* control of CPB: The use of elevated potato plant alkaloids to stress CPB prepupae did not allow increased nematode reproduction in the field. *H. marelatus* reproduced in less than 1% of CPB in the laboratory, and in none of the 303 CPB hosts collected from the field. The nematode clearly cannot sustain high populations through reproduction in potato fields, as there appear to be no appropriate hosts. Research that clarifies why the nematodes do not reproduce in CPB while reproducing at high levels in the waxworm, *G. mellonella*, could lead to increased nematode recycling in the field. These nematodes may survive at most for a few weeks under the hot soil conditions found in the Columbia Basin potato-growing area, and so cannot provide control for more than one generation of CPB. Mass-production of *H. marelatus* through inexpensive fermentation has not been successfully developed. Rearing nematodes in waxworms, as is done for research purposes, is prohibitively expensive for producing nematodes for large-scale agricultural pest control. Until a technique is discovered that allows *H. marelatus* to reproduce in potato fields, or to be mass-

produced in an inexpensive manner, this nematode will not be useful for CPB control in large potato fields. However, *H. marelatus* does kill a high percentage of CPB prepupae, and may be useful for CPB control by home gardeners and small-scale organic producers in regions where only one or two generations of CPB develop per year.

ACKNOWLEDGEMENTS

We could not have completed this research without the time and labor of Phil Rogers, Tim Weinke, Phil Hector, Don Horneck, and assistance and patience of other researchers and staff members at the Hermiston Agricultural Research and Extension Center; many thanks to all these individuals! Additional thanks go to Sujaya Rao and Russell Ingham for helpful comments on earlier drafts of this paper. This research has been supported by EPA-STAR Graduate Fellowship #U915727 and an Oregon State University Agricultural Research Foundation grant.

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CHAPTER 4: ANTI-PATHOGEN DEFENSES IN COLORADO POTATO BEETLE HEMOLYMPH

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For submission to Journal of Invertebrate Pathology

ABSTRACT

The nematode *Heterorhabditis marelatus* and its symbiotic bacteria, *Photorhabdus luminescens*, successfully attack and kill Colorado potato beetle (CPB) prepupae in laboratory and field trials, but the nematode rarely reproduces in the beetle. In this study we examined possible effects of the CPB immune system, potential hemolymph toxins, and lack of nutrients in hemolymph, on development of the nematode and its bacteria. In vivo nematode infection of CPB prepupae and waxworm (*Galleria mellonella*) larvae (highly susceptible positive control), and subsequent dissections of the infected insects indicated that only 6% of CPB supported nematode reproduction, but 89% of *G. mellonella* had reproducing nematodes. Encapsulation of nematodes was lower in CPB (1.6%) than in *G. mellonella* (12%), suggesting that the immune system did not play a significant role in limiting nematode reproduction in the CPB. This was verified using hanging drop assays, in which nematodes did not reproduce in CPB hemolymph, despite the same encapsulation rates in CPB and *G. mellonella* hemolymph drops. A proteinaceous toxin caused 40-50% of samples of *P. luminescens* to switch from the

primary form that produces antibiotics and nutrients for the nematodes' development, to a secondary form that does not benefit the development and reproduction of the nematodes. The protein was denatured by heating 10 minutes at 60°C, and SDS-PAGE gels indicated the toxin to be about 58kD. The protein was isolated and bioassayed, and was found to negatively affect the *P. luminescens* in hanging drops, although the isolated protein did not cause the bacteria to switch from the primary to the secondary form. The addition of lipids in the form of olive oil to heated CPB hemolymph allowed nematodes to reproduce in 17% of hanging drops, in contrast to the lack of reproduction in unheated hemolymph without oil. The lipids present in CPB hemolymph may not be available in a form which the bacteria or nematodes are able to utilize. Providing olive oil to CPB hanging drops that were not heated also allowed limited nematode reproduction, indicating the toxic protein affected only the bacteria, and not the nematode directly. These data suggest a combined role of the toxic protein and a lack of lipids in limiting the CPB-bacteria-nematode interaction. These factors could have an impact on biological control of the CPB with a variety of pathogens and other agents attempting to utilize the CPB as a nutritive source.

KEY WORDS: biological control, hanging drop, *Leptinotarsa decemlineata*, *Photorhabdus luminescens*, Heterorhabditidae, *Heterorhabditis marelatus*, entomopathogenic nematodes, Chrysomelidae, chrysomelid

INTRODUCTION

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), is a significant pest of potatoes and related solanaceous crops throughout North America and large parts of Europe (Casagrande, 1987; Hare, 1990; Hough-Goldstein et al., 1993; Ferro, 1994). A potential biological control agent for CPB is the heterorhabditid nematode, *Heterorhabditis marelatus* Liu & Berry (Liu and Berry, 1996). This nematode kills nearly 100% of CPB prepupae when applied to the soil at 50/cm^2 (Berry et al., 1997). However, preliminary studies indicated that the nematode reproduces in less than 1% of CPB that are killed. Unless the nematodes can reproduce in the host in the field, season-long CPB control would require prohibitively expensive repeated applications of the nematode. Determining what factors limit reproduction of the nematode and its symbiotic bacteria could provide insights into CPB defensive mechanisms that may also affect other biological control agents.

The complex life cycle of *H. marelatus* and its symbiotic bacteria, *Photorhabdus luminescens* (may be *L. temperata*, see Liu et al., 2001), could be affected at several points during CPB infection. An intricate sequence of events occurs in an ideal host, such as the waxworm, *Galleria mellonella* L. (Lepidoptera: Pyralidae). The nematode persists outside the insect host as a third-stage infective juvenile (IJ) that actively seeks hosts in the soil. The IJ enters a host through the mouth, anus, or spiracles, and then pushes into the host's hemocoel (Akhurst, 1980) and halts the host's immune system (Boemare, 1997). The IJ releases the symbiotic

bacteria *P. luminescens* that is carried in the nematode's foregut (Boemare and Akhurst, 1988). The bacteria reproduce, obtaining nutrients from the host tissues, and kill the host through bacterial septicemia (Akhurst, 1980). The bacteria also produce an antibiotic to inhibit the growth of competing pathogens (Akhurst, 1982) that could enter through the wound left by the nematode's entry or from the host's gut once the insect's tissues begin to degrade. The nematode feeds on the bacterial/host tissue slurry (Akhurst, 1994), maturing and reproducing through two or three generations within one host. When the available nutrients drop and the bacterial/nematode waste products increase, the bacteria are triggered to develop to a secondary form which differs significantly from the primary form which is vectored by the nematode. In contrast to the primary form, the secondary form of *P. luminescens* does not provide as many nutrients for *H. marelatus*, and does not produce the antibiotics that inhibit growth of other fungi and bacteria (Akhurst, 1980). The switch to the secondary form of the bacteria signals the nematodes to develop to the IJ stage. Neither the nematode nor the bacteria can complete their life cycle without the other; the bacteria needs the nematode as a vector to find an appropriate host, and the nematode requires an intact host killed by bacterial septicemia in order to feed on the by-products of bacterial digestion of host tissues. Any host-related factor which affects bacterial growth, antibiotic production, or nematode development could stop the nematode from successfully reproducing in the host.

When *H. marelatus* attacks the CPB, the nematode successfully kills the host but rarely develops, suggesting that the nematode, its symbiotic bacteria, or both are inhibited after entry into the host's hemocoel. Factors in CPB hemolymph affecting the nematode and its symbiotic bacteria could include the host immune response, the presence of toxic compounds, or a simple lack of nutrients necessary for successful development. Insects have an array of immune responses to invading organisms, including phagocytosis of single-celled organisms such as bacteria (Gotz and Boman, 1985). Larger organisms such as nematodes can be encapsulated by either intact (Gotz and Boman, 1985) or ruptured hemocytes that spread over the invader (Poinar et al., 1968), binding the intruder so it cannot ingest nutrients or reproduce. In addition to possible immune inhibition of the pathogens, the nematode and bacteria may be affected by a variety of toxic compounds in the CPB. The beetle may sequester glycoalkaloids, membrane-lytic neural inhibitors (Carman et al., 1986) from solanaceous host plants. Leptinotarsin, another neuro-active toxin, has been identified (Hsiao and Fraenkel, 1969) and isolated (Hsiao 1978) from CPB hemolymph. Leptinotarsin could affect either the *P. luminescens* or the *H. marelatus*. Limited nutrient availability also could inhibit bacterial or nematode growth. A source of amino acids, a variety of vitamins, and lipids are required for nematode development (Dutky et al., 1967). The symbiotic bacteria normally digest host tissues to release amino acids, lipids, and other nutrients for the nematode. If the nutrients are not available in a form that the bacteria or nematode can utilize, the nematode will not complete development.

In vivo and in vitro studies were conducted to examine the complex interaction between the nematode, its symbiotic bacteria, and the CPB to determine potential factors affecting reproduction of the nematode in the CPB. Specific questions addressed were: 1. Does the CPB immune system affect nematodes to a greater extent than the immune system of the ideal host *G. mellonella*? Both in-vivo and in-vitro studies were completed to examine the effects of the immune cellular response on *H. marelatus* in CPB. 2. Are there toxins in CPB hemolymph which affect bacterial form and/or nematode development and reproduction? 3. Can supplementation of lipids to the CPB hemolymph increase nematode development and reproduction rates in vitro? The in-vitro studies examined the role of each factor listed above on development and reproduction of both the bacteria and the nematode in the CPB.

MATERIALS AND METHODS

Plants and insect colonies: Russet Burbank potato plants were grown in 15 cm diameter pots with 2 g 16:16:16 (N:P:K) fertilizer per pot, and were watered three times per week. Plants were placed in cages with CPB when the plants were 8-10 nodes tall. Beetle colonies were initiated and supplemented with CPB adults and eggs collected in the field in Hermiston, OR, and from a laboratory colony in Michigan. Beetles were maintained in same-age cohorts and were fed live plants ad libitum. Both plants and beetles were reared in a walk-in growth chamber at 16:8

L:D at about 35°C:25°C, with 400 watt high-pressure sodium lamps and plant-grow broad-spectrum fluorescent bulbs providing the lighting. Beetle prepupae used in the experiments were removed from the soil after they had ceased feeding and buried themselves prior to pupation.

Waxworm (*G. mellonella*) larvae served as a positive control, as they are highly susceptible to attack by *H. marelatus*, and readily support nematode reproduction. Sixth-instar *G. mellonella* purchased from Vanderhorst Wholesale Inc., St. Mary, OH were stored unfed up to 2 weeks at 13°C until use.

Hemolymph collection and inoculation: All experiments other than dissections of nematode-infected CPB prepupae and waxworms were performed in hanging drops of CPB hemolymph. The hanging drop technique allows close observation of bacteria and nematodes developing in a single drop of host hemolymph. Insects were rinsed in 95% ethanol for 30 seconds to surface sterilize them. Hemolymph was collected by snipping a small lateral hole in the insect's abdomen and drawing the hemolymph into a sterile capillary tube. A drop of hemolymph (approx. 5-8 µl) was dripped on a glass microscope slide, which was placed in a plastic 90 mm petri plate lined with filter paper dampened with 1 ml sterile distilled water. Additional water was added as needed to maintain high humidity in the petri plates throughout the experiment. An hour after collecting the hemolymph, isolated primary form *P. luminescens* in Luria-Bertani broth (FisherBiotech, Fair Lawn, NJ) was inoculated into the hemolymph drops. The

bacterial broth was diluted so 150,000 cells in 3 μl of broth could be inoculated into each hemolymph drop. After bacterial inoculation, each slide was inverted and supported by two toothpicks lying perpendicularly on the filter paper, allowing the hemolymph drop to hang from the slide. In order to ensure that all the bacteria was in the primary phase when inoculated into the hemolymph, bacterial broth was inoculated onto NBTA agar plates (which distinguish bacterial form by colony color) and examined after 48 hours for the presence of any secondary phase colonies (Woodring and Kaya, 1988) (agar from Difco Laboratories, Detroit, MI; NBTA stains from Sigma Chemical Co., St. Louis, MO). Each petri dish was sealed with Parafilm, and held in a growth chamber at 25°C.

The petri plates were opened at 48, 168 and 288 hours post-inoculation, and a dab of hemolymph from each slide was streaked onto NBTA agar to determine if the growing *P. luminescens* were in the primary or secondary form. A few agar assay plates contained bacteria in both the primary and secondary forms at the same time, and so they were scored as having half of the bacteria in each form. The rest of the plates had just one bacterial form growing at any one time, and were recorded accordingly.

Preliminary time-course studies of bacterial development in CPB hemolymph indicated that the bacteria would switch form within 12 hours in most CPB hemolymph drops, and then the bacterial form would remain constant until about 130 hours post-inoculation, at which point more bacteria would begin to switch to the secondary form. Most *G. mellonella* hemolymph drops supported the

bacteria in the primary form until 144 hours after inoculation. Therefore, examination of the drops at 48, 168, and 288 hours post-inoculation would allow determination of bacterial form during the periods most likely to indicate changes.

Two *H. marelatus* IJs were added to each hanging drop two days after application of *P. luminescens*. Nematodes were not placed into the hanging drops immediately because the sterility of the nematodes could not be guaranteed. Nematodes surface-sterilized by rinsing in Hyamine detergent solution were unable to release live bacteria into the hemolymph; nematodes may have ingested some of the detergent, killing the bacteria carried in their gut. Nematodes that were not surface sterilized prior to inoculation into fresh hemolymph often carried bacteria on the cuticle which competed with the *P. luminescens* in the hanging drop. When *P. luminescens* was inoculated into hemolymph 24-48 hours prior to nematode introduction, the bacteria had the opportunity to develop a growth of pure primary form symbiotic bacteria that produced antibiotics to inhibit competing bacteria that might be carried on the surface of the nematode's cuticle.

The nematodes were monitored for development or death at 168 and 288 hours after initial set-up of the hemolymph drops. The hanging drops retained immune system activity for at least 144 hours in the form of encapsulation (host hemocytes binding and bursting on the nematode), melanization (ruptured hemocytes infused with melanin), and cellular clumping (intact hemocytes bound in clumps to the nematode).

Dissection of in-vivo nematode-infected insects: Dissections determined if the CPB immune system affects nematodes in-vivo to a greater extent than the immune system of the ideal host *G. mellonella*. Nematode encapsulation rates in CPB prepupae and *G. mellonella* larvae were compared to determine if *H. marelatus* development was significantly limited in either species by the cellular immune response in-vivo. Clean fine-grit sand was autoclaved after moistening to 10% weight/volume with distilled water. The damp sand was placed in 90 mm sterile Petri plates and loosely packed. Infective juvenile *H. marelatus* that had been stored at 4°C up to two weeks after collection from *G. mellonella* larvae, were applied at 50 nematodes/cm^2 in 1.4 ml tap water. Control dishes of sand were treated with 1.4 ml tap water. Prepupal CPB from the laboratory colony were placed on top of the nematode- or water-treated sand, and were allowed to dig into the sand. Up to ten CPB prepupae were placed in each dish, along with four *G. mellonella* larvae. A total of 182 CPB prepupae and 72 *G. mellonella* were treated with nematodes, and 42 CPB and 42 *G. mellonella* were in the water control treatment, in four trials replicated over time. Two weeks after placing the insects on the sand, the insects were dissected under a dissecting scope to determine how many CPB and *G. mellonella* had encapsulated and melanized nematodes. Results were combined as either 'encapsulated' or 'unencapsulated' because no encapsulated but unmelanized nematodes were found. The number of insects supporting nematode reproduction was also recorded.

Hanging drop immune tests: Hanging drop experiments determined if the CPB immune system affects nematodes in-vitro to a greater extent than the immune system of the ideal host *G. mellonella*. Hanging drops of both CPB prepupal and *G. mellonella* larval hemolymph were inoculated with isolated primary-form *P. luminescens* in Luria-Bertani broth. At 48 hours post-inoculation, two IJ *H. marelatus* were applied to each hanging drop, after one of two treatments. The nematodes were either rinsed 30 seconds in 0.01% Hyamine detergent solution or in sterile distilled water. Previous studies (Armer, unpublished) indicated that the Hyamine rinse increased the rate of the immune response of encapsulation. The Hyamine solution probably destroyed part or all of the nematodes' surface coat protein (Wang and Gaugler, 1999) that protects the nematode from detection and response by the host immune system. A total of 11 CPB hanging drops received Hyamine-rinsed IJs, 11 CPB drops received water-rinsed IJs, and 11 *G. mellonella* drops had Hyamine-rinsed IJs, and 9 *G. mellonella* drops had water-rinsed IJs, in two replicates. Nematode development was monitored under a dissecting microscope at 168 and 288 hours. Rates of nematode reproduction were noted at 288 hours. The three possible immune responses of encapsulation, melanization, and cell clumping were also noted, and results were combined as either 'encapsulated' or 'not encapsulated.' Numbers of nematodes encapsulated vs. non-encapsulated were compared by a χ^2-analysis of a 4x2 contingency table (Glantz, 1997), a non-parametric comparison of counts which is equivalent to an ANOVA. The analysis determined if insect host (CPB vs. *G. mellonella*) or nematode rinse

method (water vs. Hyamine solution) affected the numbers of nematodes encapsulated. A comparison of encapsulation rates between the host insects were made by subdividing the contingency table and adjusting with the Bonferroni adjustment for multiple tests (Glantz, 1997); this method is similar to the LSD analysis performed to isolate treatment effects in a multi-way ANOVA. Percentages of hanging drops supporting nematode reproduction, based on Hyamine vs. water rinses and with insect species combined, were also compared by subdividing the contingency table and adjusting the data with the Bonferroni correction factor.

Protein toxicity hanging drops: CPB hemolymph hanging drop studies examined whether there are toxins which affect bacterial form and/or nematode development and reproduction. In preliminary attempts to inactivate CPB immune enzymes by heating hemolymph, we found evidence of a substance in the CPB hemolymph that appeared to negatively affect *P. luminescens*. We speculated the substance was one or more proteins because of inactivation (denaturation) by heating at 60°C for five minutes. Here we describe tests to analyze effects of the protein(s) on the nematodes and bacteria, and the possible identity of the protein(s).

Hemolymph was collected from CPB prepupae and *G. mellonella* larvae, and was placed in 0.5 ml snap-cap vials. Half of the vials were placed in a 60°C water bath for ten minutes, and the other half were held at room temperature. To determine if the toxicity persisted when the protein concentrations were reduced by

dilution with *G. mellonella* hemolymph, samples from CPB and *G. mellonella* were also combined in 1:1 mixes and either heated or maintained at room temperature. After allowing the heated hemolymph to reach room temperature in the vials, it was applied in drops to slides, and was inoculated with *P. luminescens* as above. The other half of the hemolymph was not heated prior to bacterial inoculation, as a control treatment. The bacterial form was determined at 48, 168, and 288 hours, and nematode survival and reproduction was recorded at 288 hours. A total of 11 slides was set up for each treatment (CPB or *G. mellonella* hemolymph, or CPB+*G. mellonella* hemolymph, heated or room temperature) in three replicates over time.

Bacterial form at 48 hours was compared for the three hemolymph types (CPB and *G. mellonella* individually and mixed) and two temperatures via a χ^2 analysis of a 6x2 contingency table. Individual differences were then determined for the three hemolymph types (including the mixture) and the two temperatures via separate contingency tables with a Bonferroni adjustment. Nematode survival and reproduction were also compared via a χ^2 analysis of a 6x2 contingency table, and again individually to compare hemolymph type and temperature affects. Percent of nematode reproduction was calculated only for those nematodes not encapsulated by the immune system. Heating the hemolymph did not denature enzymes necessary to raise an immune response, as many of the nematodes added to heat-treated CPB and *G. mellonella* hemolymph were encapsulated and melanized.

Toxic protein identification via SDS-PAGE gels: SDS-PAGE gels separate proteins based on molecular weight of a denatured protein. Hemolymph from prepupal CPBs was collected, and half from each individual was heated ten minutes at 60°C, and half was immediately frozen at -20°C. The heated hemolymph was stored at -20°C immediately after removal from the water bath. Samples of heated and unheated hemolymph were run at 2.5 µl per column on a 10% agarose SDS-PAGE gel with a 3.94% agarose stacking gel. The gels were stained with coomassie blue stain to mark the proteins. A total of four SDS-PAGE gels were run to examine hemolymph from eight CPB and four *G. mellonella*, both heated and unheated. Ten microliters of a rainbow marker was run to provide an approximation of sizes of proteins. Each gel was run at 110 volts for 20 minutes, and then 200 v for 100 minutes at room temperature. Proteins that were denatured by heating ten minutes at 60°C were identified by size on the SDS-PAGE gels.

Toxic protein identification via native PAGE gels and protein extraction: SDS-PAGE sample preparation involves destruction of the proteins' secondary, tertiary, and quaternary structures, and active proteins cannot be retrieved from SDS-PAGE gels. Therefore, in an attempt to isolate active proteins, native (non-denaturing without SDS) PAGE gels were run to separate active proteins by approximate size and charge. Hemolymph was again collected from CPB, and half was heated at 60°C, and the other half was immediately stored at -20°C. The hemolymph samples were run on 8% agarose gel in a 5°C cold room,

beginning at 40v gradually increasing to end at 150v over 5.5 hours. In the first two native gels, samples of 3 µl were run in each column, and the entire gel was stained. In the other two native gels, hemolymph samples of 3µl were run in the first four columns, and the same samples were repeated with 12 µl each in the last four of the ten columns on the gel. After running, the gel was placed on a piece of glass, and the gel cut in half between the first five and the last five columns. The first half of the gel was stained to determine the approximate location of proteins that differed before and after heating CPB hemolymph at 60°C for ten minutes. The other half of the gel was stored at -2°C until band excision. The first of the two frozen, unstained gels which had intact proteins was lined up with the corresponding stained gel, and the four bands which appeared in the unheated hemolymph but not in the heated hemolymph were excised and saved from the frozen gel. Regions of gel that aligned with these four bands in the heated hemolymph columns were also collected and extracted as a control. These four denaturable bands were thus collected from two unheated CPB hemolymph columns and two from heated columns. Each of the sixteen gel bands was ground with a plastic pestle in a power drill in 210 µl of Luria-Bertani bacterial culture broth for 15 seconds in 1 ml snap-top tubes held on ice. The gel/broth mixture was then sonicated five seconds with a Branson Sonifier 150, again on ice. The tubes were then spun 15 minutes at 13°C at 3000 rpm to separate the gel particles from the broth/protein suspension. The other of the frozen, unstained gels was cut into

columns, and then into nine bands the length of the columns. The nine sections of the four bands (total of 36 gel pieces) were ground in Luria-Bertani broth as above.

The first of the two native gels with bands excised had only those bands removed that corresponded to bands which appeared as denatured in the stained gel. Those four band extracts from two unheated samples of CPB hemolymph were run on a 10% agarose SDS-PAGE gel to determine the size of the proteins. This step verified whether the four bands on the native gel corresponded to the denaturable bands previously identified on the SDS-PAGE gels.

Protein bioassay: Protein suspended in Luria-Bertani broth was bioassayed to determine if the protein caused *P. luminescens* to switch from primary to secondary form in hanging drops of *G. mellonella* hemolymph. Both the four sets of four narrow band extracts corresponding to the denaturable protein, and the four sets of nine broad band extracts corresponding to the entire protein column, were bioassayed. Seven microliters of the broth-protein extract was placed in 3 µl of *G. mellonella* hemolymph with 3 µl Luria-Bertani broth with approximately 150,000 cells of primary form *P. luminescens* on an inverted glass slide. A dab of hemolymph was streaked on NBTA nutrient agar at 24 and 48 hours to determine bacterial form. The hanging drops were discarded after 48 hours. Additionally, 7 µl of protein extract was mixed with 3µl Luria-Bertani broth with primary form *P. luminescens*, and these drops were also sampled at 24 and 48 hours for bacterial growth irregularities.

Lipid supplementation: Hanging drop studies determined if supplementation of lipids to the CPB hemolymph could increase nematode development and reproduction rates in vitro. Preliminary experiments established that nematodes could not grow in nutrient broth without lipids, and here we test the effects of lipid addition to CPB hemolymph to determine if the presence of added lipids will allow nematode reproduction. Hemolymph from prepupal CPBs was supplemented with highly purified olive oil (Sigma) as a lipid source. The hemolymph was heated at 60°C for ten minutes to inactivate the toxic protein. The hemolymph was inoculated with *P. luminescens* as above. Forty-eight hours after bacterial inoculation, two IJs rinsed in sterile distilled water were added at the same time as 3 µl of 0.2 µm-filtered olive oil to the hanging drops. Twenty four CPB hanging drops were tested with oil, 21 without oil, 20 *G. mellonella* drops were tested with oil, and 19 drops were tested without addition of oil, in a total of four replicates over time. The hanging drops were monitored at 48, 168, and 288 hours for bacterial form, and at 288 hours for nematode survival, reproduction, and size of nematodes. Chi-squared analyses of 4x2 contingency tables examined differences between hemolymph type and presence or absence of olive oil in affecting bacterial form at 48 hours, nematode survival, and nematode reproduction. Bonferroni's adjustment was made for 2x2 comparisons of hemolymph type and of presence/absence of oil. Nematode reproduction in CPB hemolymph with and without olive oil was calculated separately via a χ^2 analysis of a 2x2 contingency table. The sizes of grown nematodes (as measured on an ocular micrometer on a

dissecting scope) were square-root transformed and compared between hemolymph types via a one-way ANOVA.

This series of experiments indicates that addition of lipids may benefit nematodes. We also tested the addition of olive oil to hanging drops of CPB hemolymph that had not been heated, to determine if the toxic protein examined earlier affected *H. marelatus* directly. Although this experiment focuses on the toxic protein, the study could not be done until the beneficial effects of lipids on the nematodes were determined. A total of 24 drops of unheated and 24 drops of heated hemolymph were provided with olive oil as above. The number of hanging drops supporting nematode reproduction was compared between the two treatments via a χ^2 analysis of a 2x2 contingency table.

RESULTS

Dissection of in-vivo nematode-infected insects to see differences in immune responses in CPB and *G. mellonella*: Ninety-eight percent of the 182 CPB prepupae treated with nematodes died, and 6% of the dead CPB supported nematode development. All of the 72 nematode-treated *G. mellonella* died, and 89% supported nematode development. Thirty-three percent of the 42 water-treated CPB died; none of the 42 water-treated *G. mellonella* died.

Only 1.6% of the CPB had encapsulated nematodes. Nematodes that had not been encapsulated were found in 2.8% of the dead CPB. Almost 12% of the *G.*

mellonella had some encapsulated nematodes in addition to the nematodes that did develop successfully. No dead, unencapsulated nematodes were seen in the *G. mellonella*, although they could have been hidden amongst the live nematodes.

The adult reproductive nematodes found inside CPB were visibly smaller than those in *G. mellonella*. Not only were the adults smaller, but they produced as few as 20 offspring before dying, whereas several hundred nematodes were produced by nematodes which matured in *G. mellonella* larvae.

Hanging drop immune tests: CPB hemolymph tended to encapsulate fewer nematodes than the *G. mellonella* hemolymph in the combined Hyamine-rinse and water-rinse treatments, although the differences were not statistically significant (χ^2 =4.69, df=1, p=0.09) (Figure 4.1). The Hyamine rinse caused a clear increase in the percent of nematodes encapsulated over the water rinse (χ^2=18.69, df=1, p<0.005). Of the 22 Hyamine-rinsed nematodes placed in 11 CPB hanging drops, nine nematodes were encapsulated. Fifteen Hyamine-rinsed nematodes were encapsulated in the *G. mellonella* hemolymph. Twenty-two water-rinsed nematodes were placed in CPB hanging drops, and only one of those nematodes was encapsulated. Three out of 18 water-rinsed nematodes placed in *G. mellonella* hanging drops were encapsulated.

Encapsulation, encouraged by rinsing nematodes in Hyamine solution, reduced reproduction significantly (χ^2=7.81, df=1, p<0.01). *G. mellonella* hanging

drops supported reproduction by 9% of Hyamine-rinsed nematodes (Figure 4.1), and by 89% of water-rinsed nematodes. No nematodes reproduced in CPB hemolymph, regardless of rinse treatment. Encapsulation reduced reproduction by limiting nematode movement and probably by halting feeding. When capsules were gently broken open at the end of the experiment, many of the nematodes were still alive, and some nematodes survived more than 150 hours within the capsules.

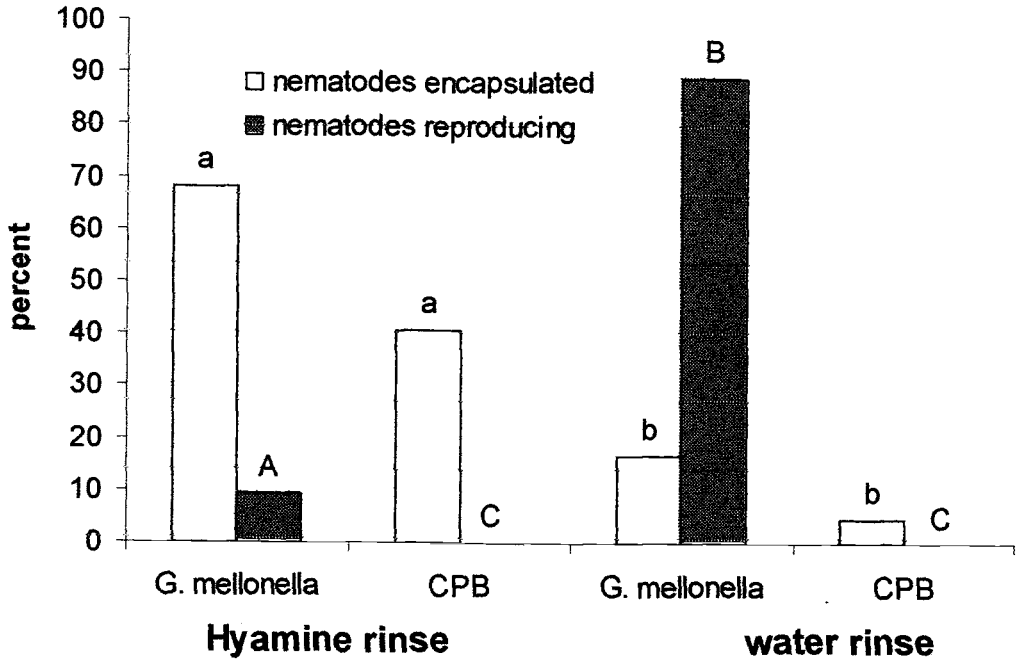

Figure 4.1. Hanging drop comparison of the importance of immune encapsulation responses on limiting nematode reproduction in *Galleria mellonella* vs. CPB hemolymph. The Hyamine rinse makes the nematodes highly susceptible to encapsulation, whereas the water rinse rarely triggers an immune response. Letters over the percent of nematodes encapsulated indicate differences significant at the $p<0.05$ level, as indicated by χ^2 analysis of a 4x2 contingency table and separation of individual means. Lower-case letters refer to the percent nematodes encapsulated, and upper-case letters refer to nematode reproduction.

Protein toxicity hanging drops: Heating the hemolymph significantly reduced the percent of hanging drops with bacteria in the secondary form at 48 hours compared with unheated hemolymph (Figure 4.2) (χ^2=12.64, 1 df, $p<0.003$). Only 64% of the unheated CPB hemolymph hanging drops supported the primary form of the bacteria at 48 hours. In comparison, heating the hemolymph allowed 100% of the *P. luminescens* to remain in the primary form in CPB hanging drops at 48 hours post-inoculation (Figure 4.2). A 50% dilution of CPB hemolymph with *G. mellonella* hemolymph did not alter bacterial switching rates. The bacteria switched to the secondary form in 50% of the unheated mixed-hemolymph hanging drops by 48 hours. Heating the mixed-hemolymph drops allowed the bacteria to remain in the primary form in 100% of the drops. There was no difference in bacterial form between hemolymph type (χ^2=2.11, 2 df, $p>0.05$).

Nematode survival was not affected by hemolymph heating or hemolymph type when all the treatments were examined (χ^2=6.67, 2 df, $p>0.05$) (Figure 4.2). Only 9-55% of the nematodes remained alive at the end of the experimental period in all the treatments, and 27-73% of the nematodes were encapsulated by the immune system responses of the hemolymph. Many of the capsules were also melanized, which strengthened the capsule so the enclosed organism was less likely to escape. Intact hemocyte cells clumped on, and restricted the movement of, 17-45% of the nematodes.

Nematode reproduction was affected by hemolymph type (χ^2=11.17, 2 df, $p<0.01$). No nematodes reproduced in CPB hemolymph, whereas more than 30% of the unencapsulated nematodes in *G. mellonella* hemolymph and in the CPB-*G. mellonella* mix reproduced. Heating the hemolymph improved nematode reproduction rates in the CPB-*G. mellonella* mix (χ^2=5.0, 1 df, $p<0.05$).

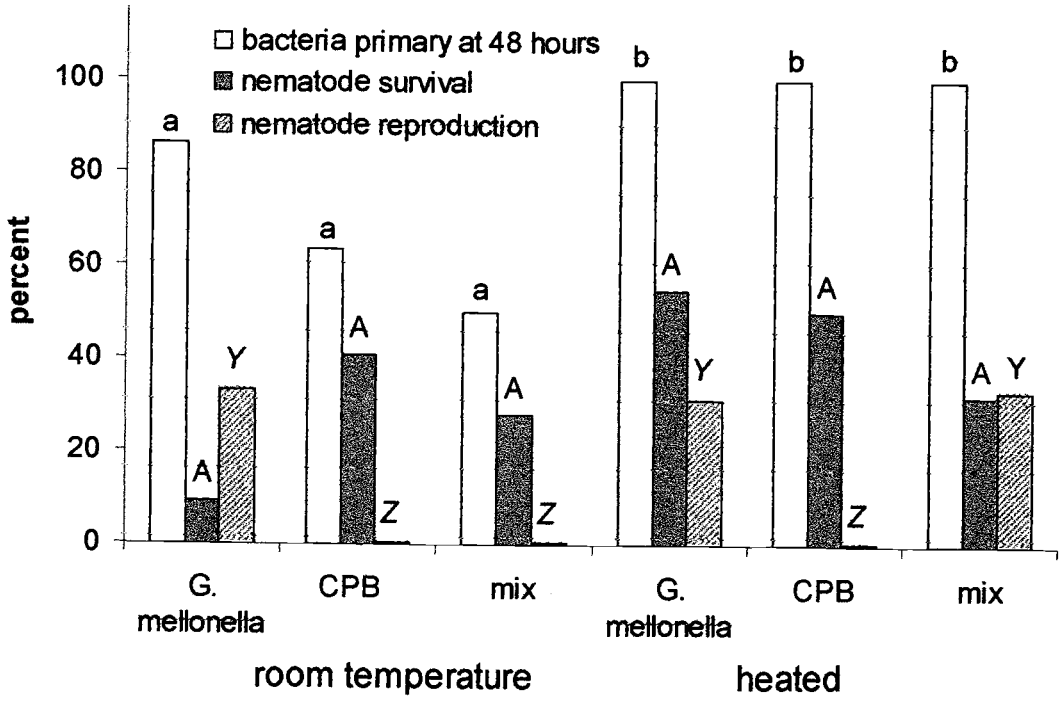

Figure 4.2. Percent of *Photorhabdus luminescens* in the primary form and percent *Heterorhabditis marelatus* survival and reproduction affected by heating host hemolymph at 60°C for 10 minutes. The mixed drops contained 1:1 CPB:*Galleria mellonella* hemolymph. Different letters above the columns indicate differences at the α=0.05 level, as determined by a χ^2 analysis of a 6x2 contingency table and separation of individual means. Lower-case letters refer only to differences in bacterial growth form, upper-case A's indicate there was no effect of temperature on nematode survival, and upper-case italicized Y's and Z's indicate differences in nematode reproduction in heated vs. unheated hemolymph.

Toxic protein identification via SDS-PAGE gels: Comparison of proteins from unheated vs. heated CPB hemolymph on SDS-PAGE gels indicated a protein of approximately 57-59kD was present in unheated hemolymph, but was either very faint or not visible in the heated hemolymph columns (Figure 4.3).

Toxic protein identification via native PAGE gels and protein extraction: The native PAGE gels indicated four close bands of mid-sized proteins were susceptible to denaturation at 60°C (Figure 4.4). When the four bands were excised and extracted in Luria-Bertani broth and then run on an SDS-PAGE gel, three of the four bands ran to the same location at about 58kD. The other band excised from the native PAGE gel separated into three bands of approximately 45, 58, and 65kD (Figure 4.5).

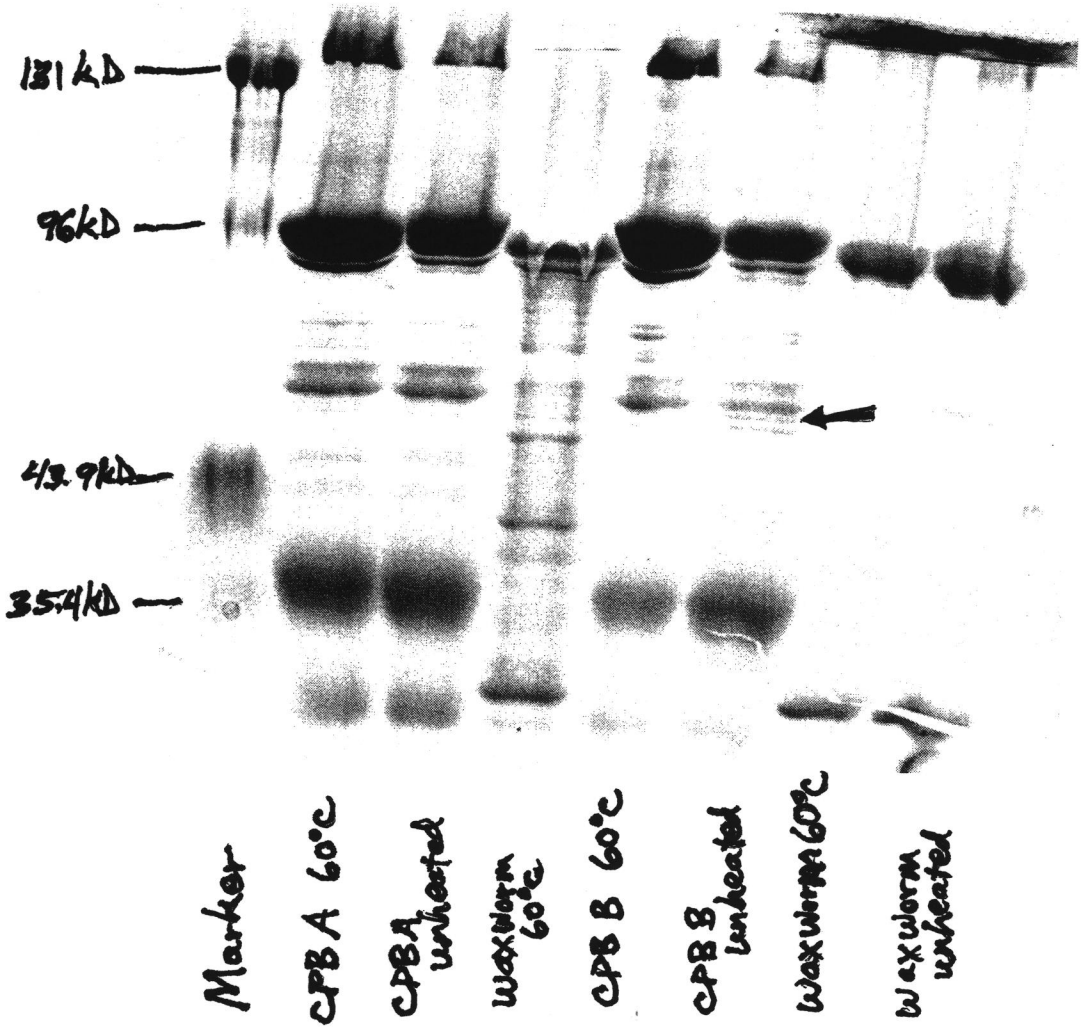

Figure 4.3. SDS-PAGE gel showing proteins from Colorado potato beetle and *Galleria mellonella* hemolymph (either unheated or heated at 60°C for 10 minutes). The type of hemolymph run in each column is labeled at the bottom of the columns, and the marker indicating approximate protein sizes is labeled at the left. The protein of interest is indicated with an arrow.

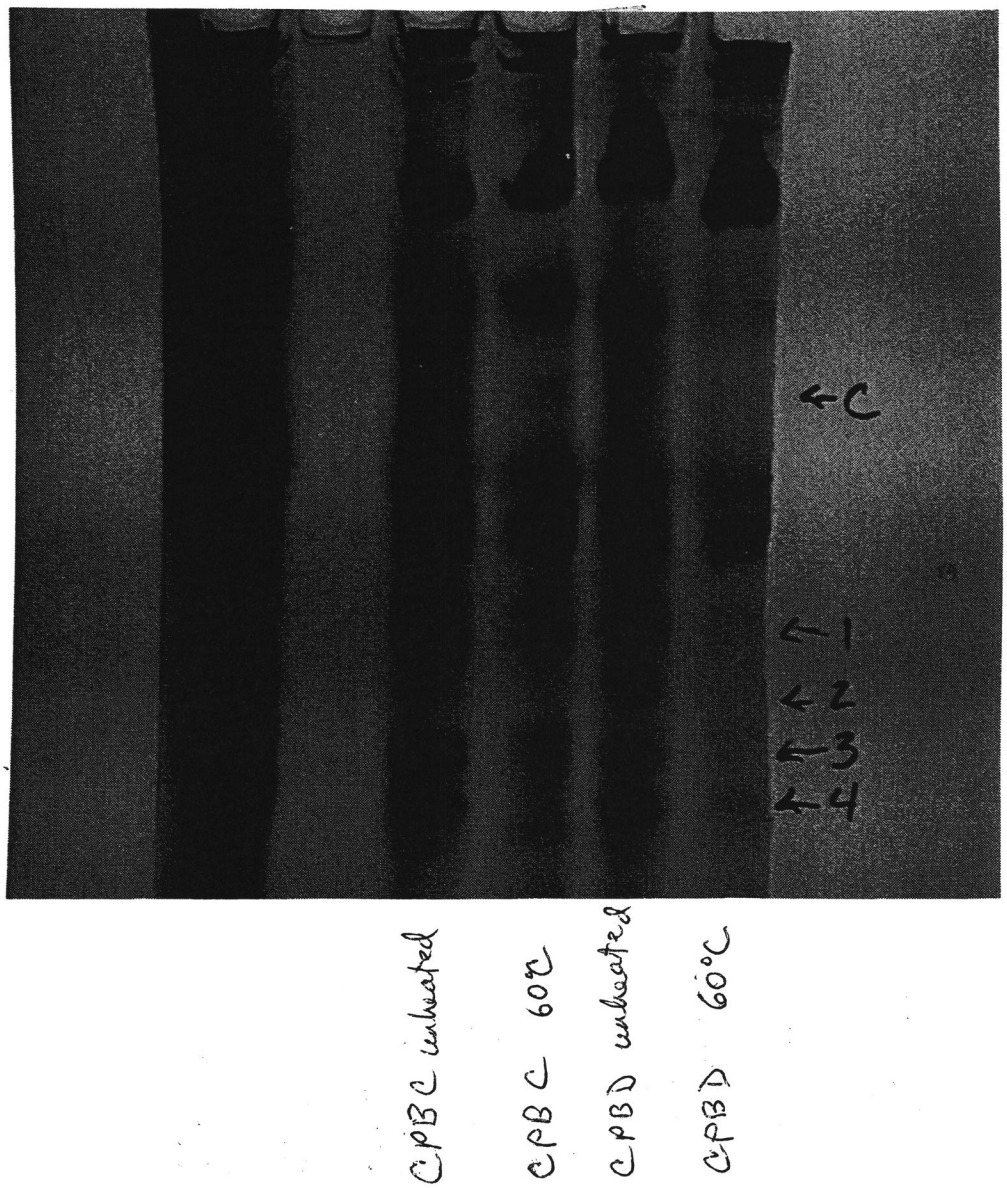

Figure 4.4. Native-PAGE gel showing Colorado potato beetle hemolymph (heated and unheated) from two prepupae. The four bands that differ between the heated and unheated hemolymph, and which were excised and extracted, are labeled with numbered arrows. These four protein extracts were bioassayed for activity against *Photorhabdus luminescens.*

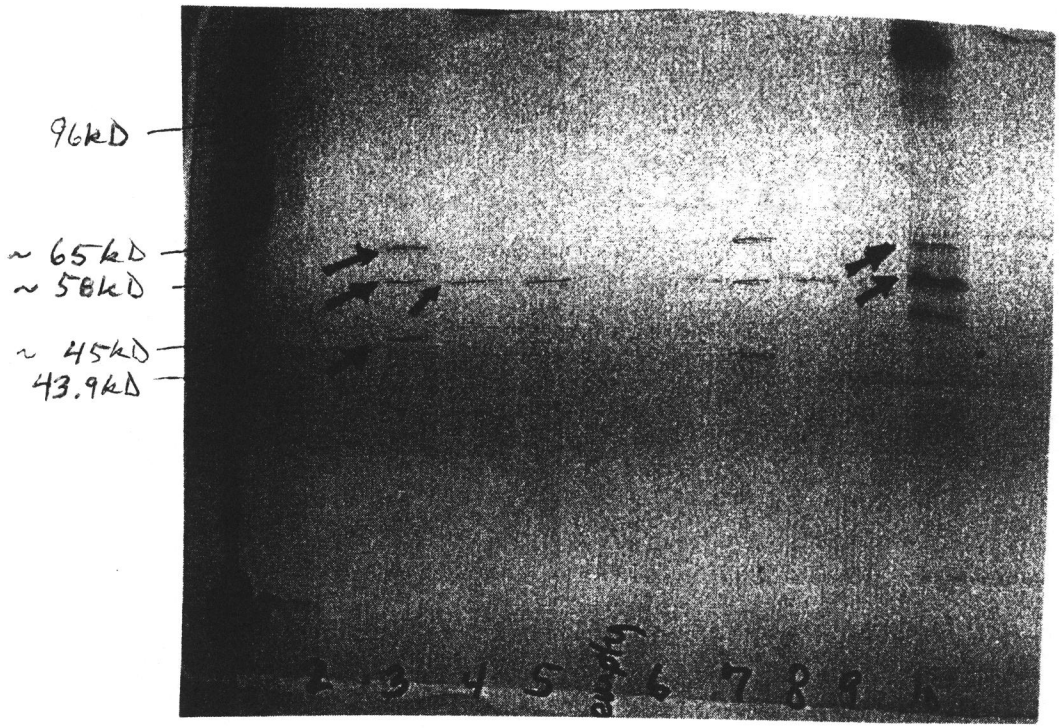

Figure 4.5. SDS-PAGE gel of the protein from the four bands excised from the gel in Figure 4.4. The marker on the left indicates the approximate size of each of the proteins. The right-hand column contains complete Colorado potato beetle hemolymph (unheated) which was run as a comparison.

Protein bioassay: The bioassay of the protein extracted the narrow bands from native PAGE gels indicated that none of the 8 extracts of protein from regions around 58kD caused *P. luminescens* to switch from primary to secondary form in *G. mellonella* hemolymph. Although the protein extracts did not cause a switch from the primary to the secondary form of *P. luminescens* in *G. mellonella* hemolymph, the extracts were related to a lack of bacterial growth in Luria-Bertani broth. Bacterial growth was inhibited by protein extracts from the broad bands of both heated and unheated CPB hemolymph in the 58kD region (arrows, Figure 4.6). Additionally, bacterial growth was delayed in 50% of *G. mellonella* hanging drops when active protein extracts from the unheated individual four bands of approximately 58kD were applied, but in none of the hanging drops with protein extracted from the bands from heated hemolymph.

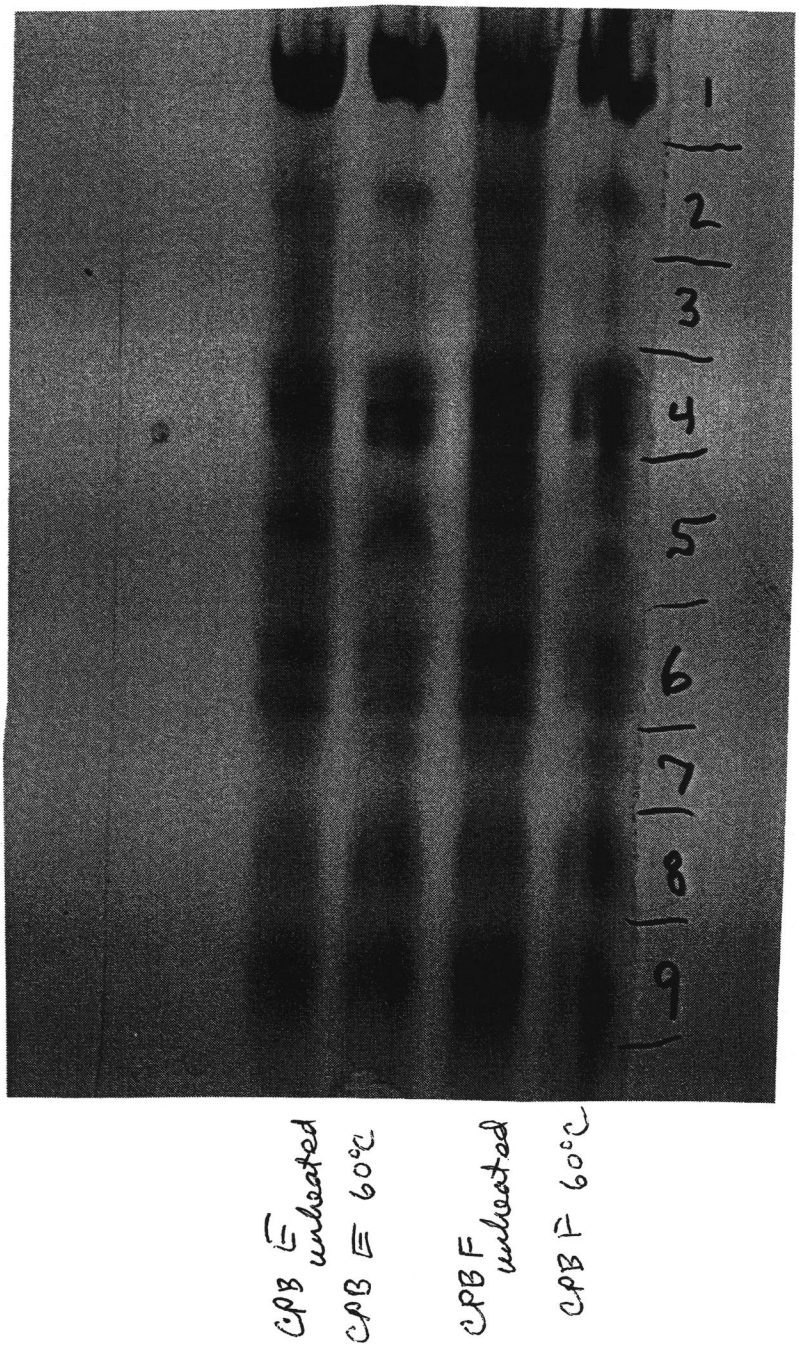

Figure 4.6. Native-PAGE gel showing Colorado potato beetle hemolymph (heated and unheated) from two prepupae, which was excised into nine bands per column and extracted. The extracts were bioassayed for activity against *Photorhabdus luminescens*.

Lipid supplementation to increase nematode reproduction in-vitro: The presence of olive oil allowed four nematodes to reproduce in 24 heated CPB hemolymph drops by 288 hours, vs. no reproduction in 21 drops without oil (χ^2=3.85, 1 df, p<0.05). Nematodes survived through 288 hours in higher numbers in *G. mellonella* than in CPB hemolymph (χ^2=11.01, 1 df, p<0.003) (Figure 4.7), and the presence of olive oil had no effect on nematode survival in *G. mellonella* hemolymph (χ^2=0.052, 1 df, p>0.05). Nematode reproduction was significantly higher in *G. mellonella* hemolymph than in CPB (χ^2=22.08, 1 df, p<0.003), and was not affected by the presence or absence of olive oil in *G. mellonella* hemolymph (χ^2=1.28, 1 df, p>0.05). Nematode size was not affected by the presence or absence of olive oil, and nematodes in *G. mellonella* hemolymph grew to be 1.6 ± 0.1 mm long (mean ± SE). These were larger (F=15.83, df=1, p<0.001) than those growing in CPB hemolymph, which only reached 1.13 ± 0.07 mm in length.

In the study of nematode development in heated vs. unheated CPB hemolymph, both with olive oil added, the toxic protein did not directly affect *H. marelatus*. An equal number of nematodes reproduced in CPB hemolymph that had been heated (16.7%) vs. unheated (12.5%) when both were provided with olive oil (χ^2=0.164, 1 df, p>0.5). Ninety-six percent of the heated CPB hemolymph drops had *P. luminescens* in the primary form at 48 hours, while only 58% of the unheated CPB hemolymph had the bacteria in the primary form. Only those hanging drops with bacteria in the primary form at 48 hours supported nematode reproduction, regardless of whether the hemolymph had been heated.

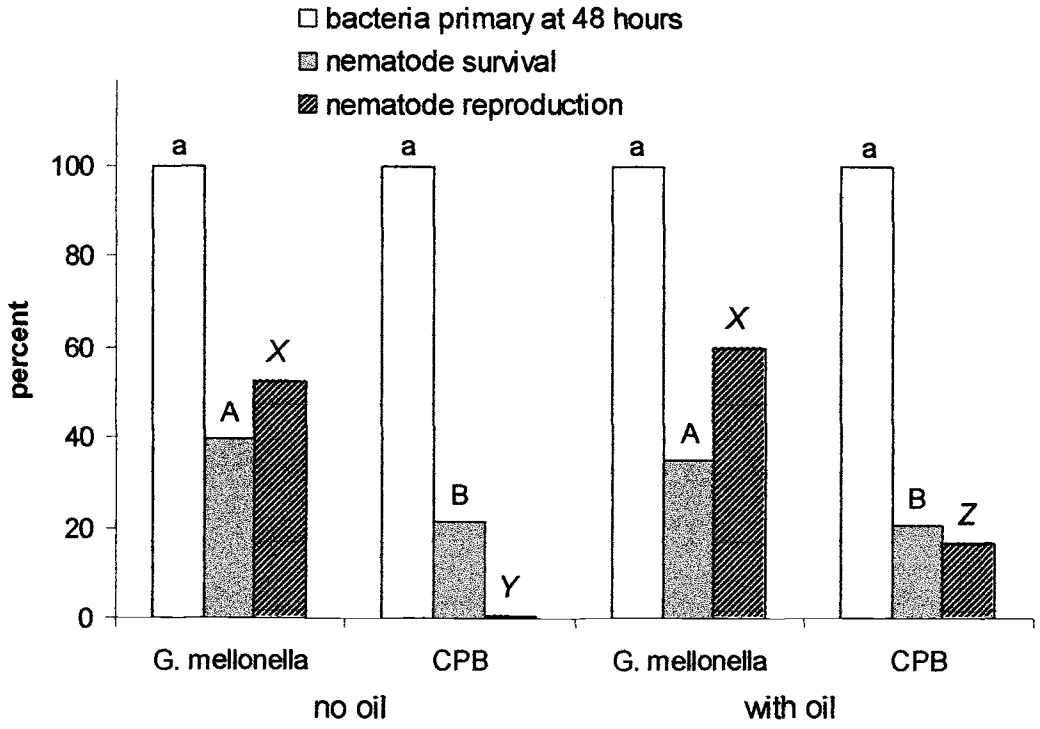

Figure 4.7. Effects on *Photorhabdus luminescens* form and *Heterorhabditis marelatus* survival and reproduction due to addition of 3 µl olive oil to heated hemolymph hanging drops. Different letters above the columns indicate differences at the α=0.05 level, as determined by a χ^2 analysis of a 4x2 contingency table and separation of individual means. Lower-case letters refer only to differences in bacterial growth form, upper-case A's and B's indicate the effect of temperature on nematode survival, and upper-case italicized *X*, *Y*, and *Z*'s indicate differences in nematode reproduction in heated vs. unheated hemolymph.

DISCUSSION

The nematode *H. marelatus* and its symbiotic bacteria, *P. luminescens*, provide nearly 100% CPB control in laboratory studies (Berry et al., 1997). However, the nematode does not reproduce in the beetle. Comparisons of bacterial

and nematode development in *G. mellonella* and CPB were made to gain insights into the limitations of CPB hemolymph for nematode reproduction.

The immune system of the CPB does not appear to be a significant factor limiting *H. marelatus* reproduction. Even though about seven times as many *G. mellonella* encapsulated nematodes than did CPB in whole-insect infections, *G. mellonella* supported far more nematode reproduction. Despite similar nematode encapsulation levels in hanging drops of hemolymph from the two species, nematodes reproduced well in *G. mellonella* hanging drops, but not at all in CPB hemolymph. The encapsulation response appeared to limit nematode reproduction in *G. mellonella* hanging drops, but some other factor appears to inhibit nematode reproduction in CPB. The percentage of nematodes that developed and reproduced in the CPB prepupae was far smaller and reproduced less than those in *G. mellonella*, suggesting other factors limit nematode growth in the CPB.

A variety of toxins in the CPB hemolymph could affect nematode development and reproduction. The beetle may store toxic alkaloids from the plants on which it feeds. The CPB feeds exclusively on plants in the family Solanaceae. These plants produce high levels of several toxic glycoalkaloids which are disruptive to cell membrane functioning (Roddick et al., 1988), as well as inhibiting acetylcholinesterase in the nervous system (Roddick, 1989). The glycoalkaloids are thought to provide protection for the plants against many herbivores and pathogens. Since the CPB feeds solely on plants containing these toxins, and because it has bright orange-and-black aposematic coloration, researchers have long thought it

sequesters alkaloids from its host plants to use in protection against its own natural enemies and pathogens (Hare and Andreadis, 1983; Costa and Gaugler, 1989). However, previous field studies (Armer, unpublished) indicate that increased foliar alkaloids have no effect on rates of nematode infection or reproduction in CPB preupae.

Although alkaloids that may be in the CPB do not affect *P. luminescens* or *H. marelatus*, there could be a protein in the CPB hemolymph with negative effects. The protein causes *P. luminescens* to switch to the secondary form in 40-50% of CPB hanging drops within 48 hours. Other researchers have identified a toxic protein, leptinotarsin, in the hemolymph of the CPB (Hsiao and Fraenkel, 1969; Hsiao, 1978). This protein has a molecular weight of about 57kD, and is heat-labile at 60°C (Hsiao, 1978). Leptinotarsin is toxic when injected into the hemocoel of several insect species, but does not appear to be toxic when ingested (Hsiao, 1978). No purpose has thus far been determined for leptinotarsin, but it may protect the CPB against a variety of pathogens. The toxic protein identified in our studies has the same size and heat lability as leptinotarsin. However, we were unable to isolate active protein, and leptinotarsin is not commercially available for testing, and hence we could not verify the identity of the toxic protein which caused *P. luminescens* to switch from primary to secondary form.

The primary-form bacteria did not switch to the secondary form in the protein extract bioassays. The proteins appeared to have some effect on the bacteria, in that the bacteria did not grow in Luria-Bertani broth drops with 58kD

protein extracts. Protein extracts from both unheated and heated hemolymph had activity in halting *P. luminescens* growth, perhaps because not all of the protein was denatured by heating. The stained native PAGE gels indicated some proteins remained in the 58kD region of the heated CPB hemolymph, supporting the idea that not all the protein is denatured by heating. The lack of activity in causing the bacteria to switch to the secondary form may have been due low concentrations of the toxin in the gel extracts. However, serial dilution hanging drops indicated that as little as 1.3% of CPB hemolymph mixed with 98.7% of *G. mellonella* hemolymph could cause *P. luminescens* to switch to the secondary form. The toxic protein appeared to have a high level of activity against *P. luminescens.* The protein from the gels may have been inactive because of incorrect conformation of the protein in the Luria-Bertani broth. Protein folding can be affected by the salts and other chemicals in the substrate, and the bacterial nutrient broth may not have had the appropriate balance of salts, pH, or other factors. If the protein was not folded correctly or maintained with the proper number of subunits, the protein would not have its normal activity in affecting bacterial growth form.

Heating CPB hemolymph to denature the toxic protein positively affected *P. luminescens*, but *H. marelatus* was still unable to reproduce in these heated CPB drops. It is possible, as indicated on the native PAGE gel, that not all of the protein was inactivated by heating. However, the nematodes not only grew and reproduced in high numbers in *G. mellonella* hemolymph, but also in heated CPB hemolymph diluted with *G. mellonella* hemolymph. Preliminary tests diluting CPB hemolymph

with Luria-Bertani broth, which supported bacterial growth but not nematode development, indicated that simple dilution of the CPB hemolymph did not allow nematode development. Therefore, some factor other than CPB toxicity appeared to affect the nematodes. Luria-Bertani broth does not provide a lipid source, whereas *G. mellonella* hemolymph does, and so we tested addition of lipids to CPB hemolymph to determine if lipid provision would allow nematode reproduction. Providing olive oil as a lipid source increased nematode reproduction from 0% to 17%. Therefore, CPB hemolymph appears to lack an appropriate lipid source for *H. marelatus*.

Abu Hatab and Gaugler (1999) tested a variety of lipids for adding to nematode artificial diet, and found that olive oil produced nematodes with the highest stored lipid levels. Preliminary tests (Armer, unpublished) with a variety of lipids indicated that adding salmon oil, olive oil, cod liver oil, or vitamin E (suspended in oil in a gelcap) each could supplement CPB hemolymph and allow nematode reproduction. Lipids in particular are difficult to access by organisms feeding in insects because the lipids are hydrophobic, and so are bound to proteins for movement in hemolymph (Downer, 1978). These lipoprotein complexes have several functions, including picking up free sterols from the midgut and delivering them as needed throughout the insect's body. However, in CPB, the lipoproteins may pick up glycoalkaloids, rather than, or in addition to, the sterols that the alkaloids closely resemble. The alkaloids would alter the accessibility of the lipids in the lipoprotein molecule for the nematodes. If alkaloids are sequestered by CPB

from the potato plants on which they feed, the alkaloids could directly affect the bacteria or nematodes attempting to obtain nutrients from the CPB.

Several researchers have found that some entomopathogenic nematodes can reproduce in the CPB. Toba et al. (1983) mentioned an unpublished study in which a steinernematid species developed in late-season CPB. Cantelo and Nickle (1992) examined CPB control with five nematode strains, including four steinernematids and one heterorhabditid. They briefly mentioned that nematodes reproduced in the beetles, but gave no information as to level of reproduction. There may be a difference between steinernematids and heterorhabditids as to ability to avoid the CPB's chemical defenses. The two nematode families have similar life styles due to convergent evolution, but are not phylogenetically closely related. Different genetic backgrounds may allow steinernematids and their symbiotic bacteria, *Xenorhabdus* spp., to develop more readily in CPB. We studied *Steinernema riobrave* development in CPB hemolymph (Armer, unpublished data), and found that the symbiotic bacteria from *S. riobrave* also often switches rapidly to the secondary form and that the nematode does not develop in CPB hemolymph. A broad survey of more steinernematid and heterorhabditid species, examining bacterial form-switching and nematode reproduction, might elucidate the causes for the variety of results from past researchers.

Nematode growth and reproduction appear to be halted at several points in the bacterial-nematode developmental pathway in CPB. Nearly all CPB placed in soil with *H. marelatus* die, so the nematodes are able to find and enter the host. A

toxic protein, perhaps leptinotarsin, causes *P. luminescens* to switch to the secondary form, which is not conducive to nematode growth. Even when the bacteria remain in the primary form, the nematodes are unable to utilize the nutrients provided by the bacteria both *in vivo* and in hanging drops. Most beetles infected *in vivo* become fragile sacs of watery fluid within a week after nematode inoculation. These beetles are filled with a variety of other bacteria. Either the *P. luminescens* was out-competed by other bacterial species due to slow growth in CPB, or it switched rapidly to the secondary form which does not produce antibiotics. The hanging drop data supports the second possibility: the symbiotic bacteria is released successfully, it switches rapidly to the secondary form in a large number of CPB prepupae, and so does not produce the antibiotics necessary to halt infection by other bacteria and fungi. In-vivo infections also support this idea, because the infective juveniles are encased in the cuticle of the previous stage which is retained around their current cuticle, and they lose the exterior cuticle when entering the host, so all foreign bacteria on the nematode is left outside the host on the shedded cuticle. Therefore, the nematode entry wound should be nearly sterile, and the only bacteria entering the host would enter after the host has died and the integument has begun to break down.

The secondary form of *P. luminescens* produces a variety of enzymes (Boemare and Akhurst 1988), although at reduced levels than the primary form, and so is able to kill a host, although it cannot produce the necessary milieu for nematode development and reproduction. Combining CPB and *G. mellonella*

hemolymph, and heating them to denature the leptinotarsin, creates an environment that allows nematode growth and reproduction. The *G. mellonella* hemolymph appears to provide nutrients that the CPB hemolymph lacks. Adding olive oil to CPB hemolymph provides nutrients which allow the nematodes to reproduce, although at a lower level than in the *G. mellonella*. This evidence suggests that lipids are not freely available in the CPB in a form which *P. luminescens* or *H. marelatus* can utilize. The possibility that alkaloids ingested by the beetles are affecting the nematodes has not yet been ruled out. Glycoalkaloids could affect CPB digestibility not by being directly toxic, but by binding to fat in the beetle and making the lipids unavailable to the nematode.

This study indicates the complexity found in pathogen-host relationships. The intricate interaction increases exponentially when two mutualistic organisms, here a nematode and its symbiotic bacteria, are affected by host chemistry. By using a step-by-step analysis of the nematode and bacterial infection process, we were able to determine factors inhibiting nematode reproduction. However, other host factors probably also affect the nematode in the CPB, as the nematodes did not reproduce as well in the heated CPB hemolymph with lipids as in the *G. mellonella* hemolymph. Each phase of the interaction needs to be understood in order to predict how the biological control agent will work on a specific host. It is important to examine inhibitory factors in the CPB, not just to determine why this nematode and symbiotic bacteria do not develop well in the CPB, but to determine features

which could affect a wide range of biological control agents with the potential to reduce CPB populations.

ACKNOWLEDGEMENTS

Many thanks to George Poinar for helpful and informative discussions, to Tae-Hwan Kim and Peter Gothro for assistance in learning techniques, and to Alison Moldenke for encouragement early in the process. This research has been supported by EPA-STAR Graduate Fellowship #U915727 and an Applied Insect Ecologists' Foundation Graduate Fellowship.

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CHAPTER 5: CONCLUSIONS

Entomopathogenic nematodes of many species provide varying levels of Colorado potato beetle control (Toba et al., 1983; Wright et al., 1987; Cantelo and Nickle, 1992; Nickle et al., 1994; Berry et al., 1997). Two species, *Heterorhabditis marelatus* Liu & Berry and *Steinernema riobrave* Cabanillas, Poinar & Raulston, provide extremely high levels of control, killing nearly 100% of the beetle population under laboratory conditions (Berry et al., 1997). Individual CPBs are well synchronized with others in their generation, and the nematode-susceptible CPB prepupae are present in discrete periods of approximately two to three weeks twice during the growing season. *S. riobrave* can survive longer periods than can *H. marelatus*, but both can live long enough to maintain high levels of CPB control during the beetles' susceptible period. Although *S. riobrave* survives a broader range of temperatures (4-37°C) than *H. marelatus* (4 to 31°C), *H. marelatus* can infect approximately twice as many hosts as does *S. riobrave* at 22°C. Thus, both species can live long enough at a wide range of temperatures to kill CPB in the field; however, because *H. marelatus* infects and kills twice as many hosts as does *S. riobrave*, the heterorhabditid would be the better choice for biological control of CPB.

As with any entomopathogenic nematode, the exposure of *H. marelatus* to extreme soil temperatures reduces the length of effectiveness. *H. marelatus* cannot survive long at extremely high temperatures, and so is best applied when soil is

cool. The CPB are only susceptible to nematode infection as prepupae, and this stage is often present in the field when temperatures are warmest. Having adequate foliar cover over the soil will help keep the soil cool and will increase *H. marelatus* longevity. The second generation of CPB larvae can remove much of the foliar cover of a potato field in north central Oregon before the larvae drop to the ground as prepupae. Therefore, using a combination of controls, such as the larval-killing bacterium *Bacillus thuringiensis* var. *tenebrionis* and/or the fungal pathogen *Beauveria bassiana*, in addition to *H. marelatus*, should reduce CPB feeding to levels where soil cover is complete and the nematode can provide maximal pest control.

Field studies indicated that *H. marelatus* reduces CPB populations. Adult CPBs collected in beat sheets were found at twice the density in untreated control plots as in the nematode-treated plots. Up to five times as many dead prepupae were found in soil samples from nematode-treated plots as in control plots. Non-target effects of the nematode appear to be minimal. *H. marelatus* does not negatively interfere with CPB parasitism by the native tachinid *Myiopharus doryphorae*. Equal numbers of tachinids emerged from prepupae collected from nematode-treated plots as untreated plots.

Although *H. marelatus* reduces CPB numbers in the field, the nematode does not reproduce in the beetle. Nematodes would have to be applied several times through the growing season in order to control the two generations of CPB found in north central Oregon. Preliminary laboratory studies suggested that potato

plant glycoalkaloids may have an effect on the nematodes by stressing the host CPB and making it less able to inhibit nematode development. Nitrogen fertilizer levels parallel glycoalkaloids levels in Russet Burbank tubers (Mondy and Munshi, 1990), and tuber alkaloid levels correspond to foliar alkaloid levels (Sanford et al., 1984). In the laboratory, CPB larvae were fed plants that were fertilized with zero, low, and high levels of nitrogen fertilizer. The prepupae were then inoculated with nematodes, and in some trials, the nematodes developed and reproduced in more of the beetles that fed on high-nitrogen plants. However, when the attempts at affecting CPB physiology via potato plant fertilization were tested in the field, the nematodes did not show any difference in reproductive response to beetles fed on plants with high or low levels of nitrogen fertilizer. The plants fertilized with high levels of nitrogen did have higher levels of the two primary potato glycoalkaloids, solanine and chaconine, but neither CPB densities nor the nematodes' ability to reproduce in the beetles were affected by the presence of high alkaloids in the beetles' diet.

In order to determine why *H. marelatus* does not reproduce in CPB prepupae, laboratory trials were conducted in which the nematodes and their symbiotic bacteria, *Photorhabdus luminescens*, were monitored in CPB hemolymph in vitro. Nematode and bacterial growth in CPB hemolymph were compared with growth in the hemolymph of the waxworm, *Galleria mellonella*, which is a highly susceptible host that supports high levels of nematode reproduction. No differences were seen in the immune responses of the CPB vs. waxworm hemolymph.

However, the bacteria *P. luminescens* did switch from the normal primary form to the secondary form in about half of the CPB samples, but in almost none of the waxworm hemolymph samples (Figure 5.1, step C). The primary form of the bacteria produces antibiotics to keep other bacteria and fungi from infecting the host and potentially competing with *P. luminescens* or *H. marelatus* for nutrients. The bacteria in the primary form also produces enzymes necessary for digesting the host to produce nutrients in a form that the nematodes can utilize. Bacteria in the secondary form do not produce antibiotics or digestive enzymes, and so the nematode cannot develop when only the secondary form is present. During attempts to deactivate immune enzymes by heating, it was found that heating CPB hemolymph to 60°C for ten minutes inactivated the component of CPB hemolymph which induced the *P. luminescens* switch to the secondary form. SDS-PAGE gel protein separations indicated the protein causing the switch from primary to secondary form was approximately 58kD, the size of the protein leptinotarsin that had previously been discovered in CPB hemolymph (Hsiao and Fraenkel, 1969).

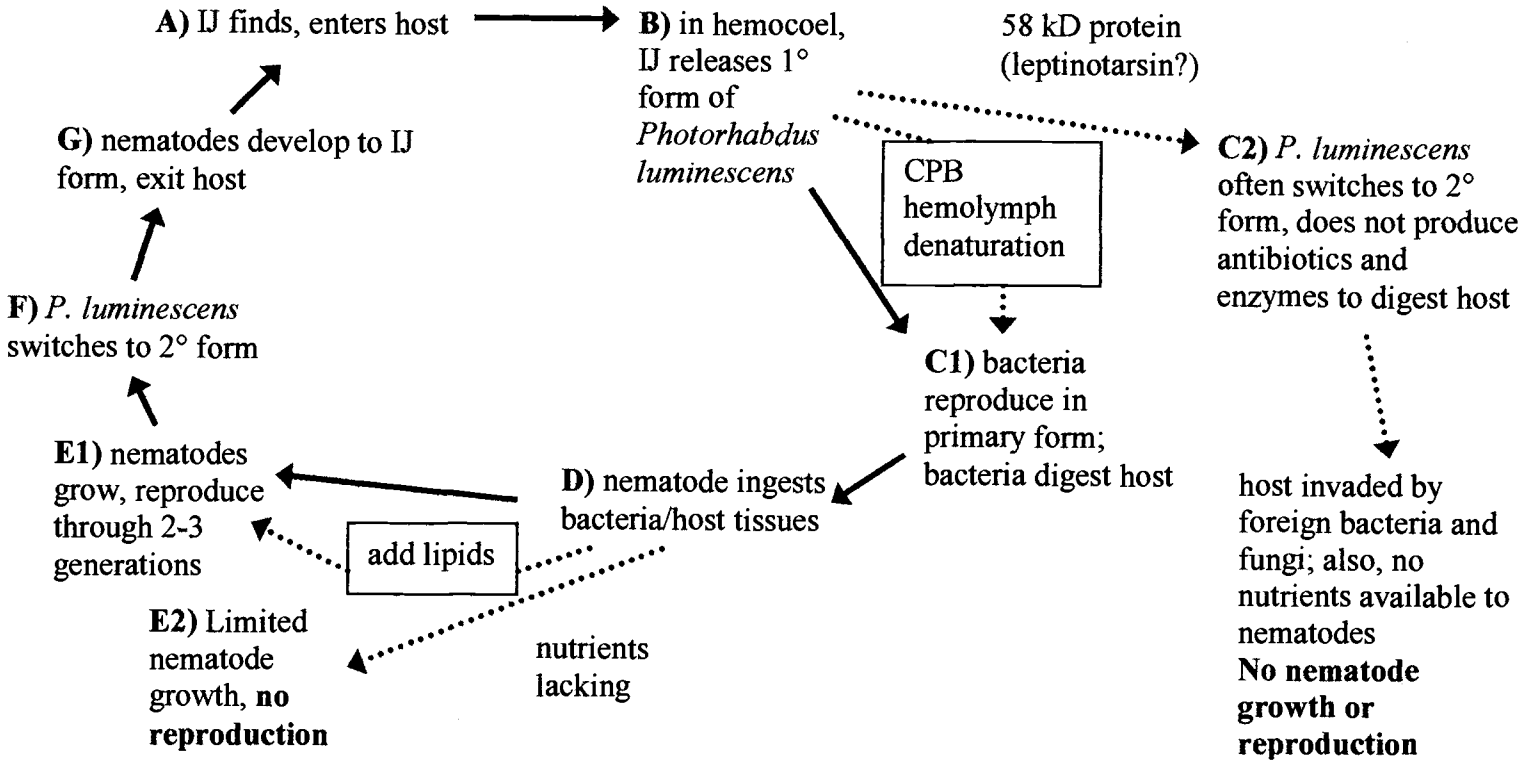

Figure 5.1. *Heterorhabditis marelatus* and *Photorhabdus luminescens* development and reproduction in a host. Solid arrows indicate the typical life cycle of the nematode and its symbiotic bacteria in a host which supports nematode reproduction. Dotted arrows indicate where the nematode life cycle is incomplete in Colorado potato beetles (CPB). Boxes on dotted arrows indicate remediation necessary to allow nematode reproduction in the Colorado potato beetle hemolymph. The typical life cycle in a host which supports nematode reproduction is based on information from the literature and from in-vitro studies in hemolymph hanging drops of the waxworm *Galleria mellonella*. The life cycle in CPB is based on in-vivo and in-vitro studies presented in previous chapters.

When the protein was inactivated by heat, the bacteria remained in the primary form, but the nematodes did not reproduce (Figure 5.1, step D). Lipids, in the form of olive oil as well as a variety of other oils, were added to the heated CPB hemolymph, and with a lipid source, *H. marelatus* could then reproduce. The nematodes did not grow as large, nor produce as many offspring, in CPB hemolymph as in waxworm hemolymph. Some other nutrient may have been limited or some slightly toxic compound which reduced the nematodes' growth may have been present. However, the presence of the toxic protein and the lack of lipids seem to be the two major components which inhibit *H. marelatus* growth and reproduction in CPB hemolymph. These factors probably cannot be manipulated by altering diet or environment of the CPB, and so *H. marelatus* will only be useful as a biological pesticide for CPB control, rather than as a long-term biological control agent that can coevolve with the CPB.

Mass-rearing the nematode is currently too expensive to make biological control with the nematode feasible on a large scale, but home gardeners could potentially use the nematode for CPB control in small plots. Small-scale nematode applications are simpler than large-scale uses because the nematodes must be stored in cool temperatures to ensure survival prior to application, and handling the nematodes in small batches may allow more rapid application than would mixing and applying the nematodes from a tank over a large field.

Although these studies indicate the limited potential of this nematode/bacterium complex for control the Colorado potato beetle, they do

provide useful information about the chemistry of the CPB's hemolymph. The 58kD proteinaceous toxin (may be leptinotarsin) clearly affects *P. luminescens* growth form, and hence affects *H. marelatus* reproduction in the beetle. This toxin could affect the reproduction of other pathogens, such as *B. bassiana* and *B. thuringiensis*, as well. Lipids supplement CPB hemolymph to support nematode maturation and reproduction, and it appears that some chemical interaction in the hemolymph is keeping the insect's lipids from being freely available. The CPB feeds only on solanaceous plants, which have high levels of glycoalkaloids with steroidal structures. The beetle may make use of these steroidal compounds as a nutritional sterol source. If the alkaloids are partially digested and used within the beetle nutritionally, *P. luminescens* and *H. marelatus* may not recognize the alkaloids as a lipid source or may not produce the enzymes necessary to digest and utilize the alkaloids. Further study could indicate if the CPB does indeed utilize the toxic glycoalkaloids from its host plants in a nutritive manner. Understanding the CPB's physiological defenses and avoidance of pathogen and parasite attack will help researchers develop more appropriate and more long-term controls for the Colorado potato beetle. Thus, the studies presented here indicate the limitations in the use of *H. marelatus* for CPB control, but also suggest possible topics of research which could lead to improved CPB control in the future.

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