HOST-PATHOGEN INTERPLAY: A STUDY OF FACTORS APPLICABLE TO THE INFECTION EFFICACY OF *METARHIZIUM ANISOPLIAE* TO WIREWORMS (*AGRIOTES SPP.*) L.

by

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Abstract

Wireworms have been a pest of agriculture crops in Canada for nearly 80 years. More recently the lack of pesticide control options have caused their threat to be serious. The insect pathogen *Metarhizium anisopliae* (Metch) Sorokin has been tested as a biocontrol for wireworm resulting in variable efficacy in both the field and laboratory. A series of tests were performed to determine the role of the wireworm immune system in this variability. All the major features of the invertebrate immune system were investigated before and after exposure to the fungus. Changes in hemocyte ratios and in the hemolymph protein concentration indicated that adverse health effects had occurred from the treatments, but otherwise no immune response was detected. Experiments were designed to test for interactions between combination treatments of *M. anisopliae* and three reduced risk pesticides; essential oil blend, halofenozide, and spinosad. A synergistic interaction was identified between spinosad and the fungus such that transmission of the disease was improved, and the total mortality was 2X greater than predicted when spinosad was at 3 ppm, and the fungus was at 10^2 conidia per gram sand. These results suggest that more sensitive tests are required to identify the immunological mechanisms that mediate the interplay between *M. anisopliae* and wireworms. This study has shown that combination treatments can be effective in improving transmission of a fungal disease, and that high mortality can be achieved without the use of traditional pesticides. Based on these results, it is suggested that field trials be tested to determine if a similar interaction would occur.

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Dedication

To: DAVID MICHAEL BRAUL

The first true scientist I ever met, and someone who's contribution to my life will never be forgotten.

Co-authorship statement

Chapter 2 was co-authored by Judith Myers, J.Todd Kabaluk, and Mark S. Goettel. The experiments were designed to address the inherent variability in the wireworm's susceptibility to *Metarhizium anisopliae* that was originally observed by M.S Goettel and J.T. Kabaluk. For this chapter, the design and planning of tests, selection of treatments, bioassays, data analysis, and rough manuscript preparation were completed by J.D. Ericsson. All authors provided numerous editing comments that greatly improved the manuscript.

Sincerely,

Jerry D. Ericsson

Chapter 1: General Introduction

Wireworms as an agroeconomic problem

Nearly 400 species of Elateridae occur in Canada, and with the exception of several species, most are harmless to agriculture (Vernon and Pats, 1997). *Agriotes obscurus* is a European elaterid known to have been present since 1950, but may have been introduced as early as 1900 with the distribution of contaminated ballast soil (Vernon, 1998). 'Wireworm' is the common name given to the larval stage of the adult 'click beetle,' the latter known for both its ability to snap its thorax to right itself when overturned, and the 'click' sound that is produced by this action. Although the adults pose few problems, the larvae are major threats to agriculture because of their polyphagous and wasteful feeding, their vigor and adaptability, and their long development time (several years). Wireworm feeding damage can be severe, and affects root crops and field vegetables of high economic value throughout the season. For example, if wireworm feeding occurs early in the season in potato crops, the damage results in a harvest of deformed, knobby and unmarketable potatoes. If feeding occurs late in the season, wireworm will bore holes into the flesh and cause the tuber to rot (Vernon, personal communication). In 1994, British Columbian farmers from the Surrey and Delta regions suffered losses of between $500,000 and $800,000 due to the European wireworm (Vernon, 1998). On a global scale, the effects of this pest have been reported in Italy, Belgium, Switzerland, Germany, France and Spain (Furlan, 2004), Australia (Sampson, 2003), the U.K. (Parker, 2001), the U.S.A (LaGasa, 2000), and Russia (Kalbanov, 1975; Yatsynin, 2001). The crops susceptible to wireworm damage represent a large portion of

crops in the lower mainland of British Columbia, and because food graders have zero tolerance for blemishes, a significant portion of the local market is under threat. In British Columbia the list of affected crops is increasing, and currently includes corn, potatoes, strawberries, carrots, beets, lettuce, one case of pine seedlings (UBC Totem farm), and larvae have even been found feeding on a bird carcass (Vernon, 1998).

Biology and ecology of the elaterids. Wireworm larval development lasts from three to six years depending on species, temperature and moisture conditions (Vernon,1998; Kabanov,1975). During development, wireworms vary in their mobility within the soil profile based on moisture, temperature, and developmental stage (Furlan, 1998). Furlan (1998) reported that *Agriotes* larval development involves 12-14 instars, which are defined by periods of mandible hardening (10% of total time), feeding (20% of total time), and in a sedentary state called 'pre-molting' (70%). Although the feeding period is a relatively short portion of the life stages, the wireworm's wasteful feeding accentuates its ability to inflict crop damage. Wireworms are attracted to CO_2 produced by germinating seeds and growing root systems (Doane et al., 1975), but they can also maintain preferences for specific host plants (Hemerik et al., 2003). Upon arrival at the host plant, they feed on the succulent root tissues or the crop itself by macerating the tissues with their short mandibles, by regurgitating digestive enzymes, and by drinking the resulting semi-digested pulp (Brian, 1947). The mixture is sucked through a dense oral filter consisting of branched hairs that are capable of excluding particles larger than 3 microns (Eidt, 1959). Within the soil profile, wireworm are normally found at depths

of 5-20cm, but have been found as deep as 1m during mild drought conditions (personal observation). Of the many species of elaterids, the two dominant species at the UBC study site, *A. obscurus* and *A. lineatus* are very difficult to distinguish as larvae (Vernon, 1998). After four to five years of development, wireworms build small capsules in the soil, pupate, overwinter and emerge as adults in the spring of the next year.

The adults mate once and lay 150-200 eggs (Furlan, 1996). On average, *Agriotes* females live longer than males, and have a mean survival time of 74 days with ranges from 64 to 84 days depending on the host plant. Males live an average of 59 days, with ranges from 46 to 75 days (Brian, 1947). The population levels of wireworms in the soil is difficult to determine, thus indirect estimates are performed through the capture of the adult beetles either through pitfall or pheromone trapping. The distribution of *Agriotes* species was found to vary throughout southwestern British Columbia with *A. lineatus* occurring more frequently on the coastal regions, and *A. obscurus* occurring more frequently further inland (Vernon and Pats, 1997). *Agriotes* species have also been found to exist in spatially defined patches within a field (Brian,1947), and this suggests that the beetle's dispersal is low, or preference for the location is high. However, since Brian (1947) showed that the host plant affects the fecundity and longevity of adult elaterids, and other studies have shown that above ground effects occur in the host plant after wireworm root feeding (Wackers and Bezemer, 2003), a relationship may exist between larval food preference and adult survival and oviposition behavior.

Wireworm management options

History of wireworm management. A historical summary of government research by Agriculture Canada describes a number of efforts in wireworm control made over the past 80 years by dozens of scientists. However it wasn't until 1946, when W.B. Fox tested the organochlorine gammexane as a wheat seed treatment that a viable control was found. Fox found that 70 grams active ingredient per hectare reduced wireworm damage to undetectable levels (Harding, 1986). This management success and other similar findings spawned the development of numerous organochlorine products that were used liberally for years as seed treatments, as sprays, and in granular form. Due to the environmental persistence of these chemicals, pesticides such as aldrin and heptachlor could control wireworms for up to 10 years (Vernon, 1998). The widespread use of aldrin and dieldrin caused both *Agriotes obscurus* and *A. lineatus* to develop resistance on some farms in France (Coulon,1965). Given their ecological toxicity, many of the organochlorine products were withdrawn, and replaced with a number of organophosphate, and carbamate chemicals over the years.

Currently, there are few registered chemical controls for wireworms in Canada (Vernon, 1998). Both the USA and Europe have active ingredients registered for regular use, with the most common being carbamates, organophosphates, and the phenylpyrazoles (Kuhar et al., 2003). A 1998 study demonstrated that wheat trap crops could be used to lure wireworm away from a crop, and if combined with an insecticide, could cause wireworm mortality and significant crop protection (Vernon et al., 2003). Cultural methods such as crop rotation, changing the planting or harvest times to avoid peak wireworm activity, and

utilization of mating interruption can all be part of a wireworm management program (Vernon, 1998; Vernon, 2004). However, if these options are not suitable or successful the manager is left with few options.

Biological control and variances in susceptibility. Biological control of wireworm has had mixed results, despite the reports of susceptibility to nematodes, and the fungi *Beauvaria bassiana, Metarhizium anisopliae,* and once from *Entomophthora elateridiphaga* in Switzerland (Parker, 2001). Of these agents, the most promising and widely tested on elaterids is the fungus *Metarhizium anisopliae* (Rockwood, 1950; Fox and Jaques, 1958; Fox, 1961; Zacharuk and Tinline, 1968; McCauley and Zacharuk, 1968; Kabaluk et al., 2001; Kabaluk et al., 2005).

Metarhizium anisopliae, is a filamentous facultative fungus in the family Hypocreales and order Clavicipitaceae, and is a true fungi that has no known sexual phase. It is capable of biotrophic and chemotrophic feeding as indicated by its pathogenic and saprophytic lifecycles. It grows as a white, dense mycelial mesh in culture, and produces long chains of hydrophobic, asexual, naked spores called conidia aerially from the substrate (Goettel and Inglis, 1997). The conidia are the infectious propagules that are sensitive to UV radiation (Rangel et al., 2004), and can remain viable in soil for several years under normal conditions.

The *M. anisopliae* treatment protocol involves inoculation of the soil with conidia to a density of $10^5 - 10^6$ conidia per gram of soil by way of soil drench (Ericsson et al., 2006), or by various soil amendment methods in conjunction with cropping (Kabaluk et al., 2005). As the wireworm moves through the treated soil towards the growing root system, the conidia attach to the insect cuticle, and

produce specialized penetration hyphae called appresorium that puncture the cuticle (Madelin, 1966; St.Leger, 1991). Appresorial penetration occurs via a combination of mechanical pressure and enzymatic degradation, and once inside, the fungus begins proliferating via yeast-like budding of hyphal bodies (Goettel, 2000). The wireworm ultimately dies from a combination of fungal toxins, starvation, and organ failure (Madelin, 1966), and then the remaining host-tissues are digested by the growing fungal cells and mycelia (Zacharuk, 1971). As the disease progresses, the wireworm cadaver increases in stiffness until mycelium emerges from the inter-segmental regions of the wireworm and produce millions of olive-green, asexual conidia.

This latter description represents the typical progression of the fungal disease in wireworm during laboratory trials, but numerous bioassays have identified that wireworm maintain a degree of resistance or tolerance to infection that results in variable survival times. These resistant features can manifest themselves as both behavioral traits (pre-mature ecdysis), or as immunological peculiarities (induced effects) within wild populations that can render the treatments ineffective. Although behavioral evasion may be occurring in the field, the lab results suggest that emphasis should be directed towards interpretation of host-pathogen interplay at the physiological level. For example, a study cited by Madelin (1966) showed that only 15% of larvae presented symptoms of *M. anisopliae,* when nearly 50% of the hundreds of larvae examined contained fungus. This evidence of cryptic fungal infections was thought to be common, and suggests that the pathological interplay between elaterids and *M. anisopliae* may be more complex than previously thought.

Furthermore, given the prevalence of cryptic infections, wild collected wireworm may respond differently to treatments depending on if they are harboring fungal cells or not. Whether a cryptic infection makes the wireworm more or less susceptible to *M. anisopliae* remains to be determined, but this phenomenon may explain some of the variability observed in many of the studies cited.

Thesis theme and objectives.

Given the ecological, fiscal, and logistical problems associated with wireworm management, the development of alternative treatment options is highly desirable. Biological control has shown much promise for elaterid management, but unexplained differences in susceptibility amongst distinct species as well as within conspecific groups have resulted in poor crop protection. Therefore, the purpose of this thesis is to evaluate the physiological aspects of the wireworm's immune response to the biological control agent *M. anisopliae* and the factors that contribute to the widely reported variability in susceptibility. The information obtained about the immune system will contribute to identifying methods for improving the transmission of the biological agent. The two main objectives of this thesis are, (1) to determine the physiological changes that occur in the wireworm upon exposure to *M. anisopliae,* and (2) to determine if treatment combinations utilizing both *M. anisopliae* and biorational insecticides can be developed that provide reasonable pest control with reduced ecological impact.

General Materials and Methods

Pheromone trapping. *Agriotes lineatus* and *A. obscurus* were monitored using pheromone traps to catch the adult males (Vernon, 2004). Eight traps were placed at the UBC farm, and four at the Totem fields, with half the traps baited for *A. obscurus* and half for *A. lineatus.* The total trap catches were pooled for two seasons and the percentage of each species was determined, and was assumed to be correlated with the larval population in the soil. The UBC populations were estimated to be 88% *A. lineatus* and 12% *A. obscurus*, and this is consistent with recent elaterid distribution maps indicating the dominance of *A. lineatus* in coastal regions of British Columbia (Vernon and Pats, 1997).

Collection, characterization, and storage of wireworm populations. Larvae were collected from several fields during a study to compare variability in susceptibility and phenoloxidase activity, but the vast majority of wireworms in this study were collected from the UBC farm. Wireworms were collected from Boardman, Oregon (*Ctenicera pruinina*), Agassiz (*Agriotes obscurus*), Pender Island (*A. lineatus*) and Vancouver (*A. lineatus*), British Columbia by sifting clumps of sod and soil in infested fields. *Ctenicera pruinina* were collected from soil and stored in sterile vermiculite for legal transport into Canada. Upon arrival in Canada, wireworms were transferred to clean soil and were maintained with wheat seeds (*Triticum aestivum).* After collection of the *Agriotes* species, wireworms were immediately placed into 40L plastic bins filled with field soil. These bins were kept at 7° Celsius until larvae were used in the assays, and wheat seed was periodically provided.

Wireworm selection. Larvae were categorized in three ways before experimentation, by length, mass, and by feeding status. In some of the earlier assays, length and mass were used as the only criteria for wireworm selection, but it became apparent that developmental stage was important. Without rearing the insects, the only suitable method of synchronizing wireworm development was to separate the actively feeding wireworm from the quiescent pre-molting and/or molting larvae. Actively feeding larvae were identified first by warming the soil from 7°C to 20°C over a 24 hour period, then by baiting the wireworm with sections of potato. Four sections of potato (75-100 cm^3) were buried 2cm below the soil surface in each 40L bin to attract actively feeding larvae. From these wireworms, those within a 10 cm range of the potato bait and within a 19-24mm range in length were selected for experimentation. The average wireworm mass ranged from 32 to 42 mg, and the size range corresponded to larval instars 11 to 13 as previously described by Kabanov (1975).

Isolation of pathogen. Wireworms occasionally died from *M. anisopliae* infections despite no known exposure, and these strains of fungi were isolated for use in the bioassays. After the wireworms had died, the fungus was allowed to grow and sporulate on the cadavers under high humidity. A sample of conidia was collected from the cadaver by touching the conidial chains with a sterile inoculation loop. The conidia coated loop was mixed in 500µl of distilled H_2O (0.025% Triton X-100), and vortexed. Five 100µl aliquots were spread on 110mm petri plates of Saboraud dextrose agar supplemented with yeast extract (SDAY), and were grown at 28°C for 15 days. Single spore colonies identified as *M. anisopliae* were sampled

with a flamed loop and mixed into 500µl. Five more plates were prepared as above, but were allowed to grow for 21 days at 28°C, and these became the parent cultures for each bioassay.

Preparation of injection bioassays. *Metarhizium anisopliae* cultures were started in SDAY broth amended with trehalose and 0.025% Triton X-100 (recipe in appendix), and allowed to grow at 22°C while being shaken at 150 rpm in total darkness. These cultures were grown under the described conditions for three days, after which they were grown at 4 C, while being shaken at 150rpm, and kept under total darkness. (Note: a reduction in temperature was expected to stress the fungus and induce the changes necessary to induce blastospore production). These cultures are then filtered through sterile cotton batten and then washed three times with phosphate buffered saline (PBS, 0.2M, pH 7.0) and were collected between washes by centrifugation in a bench-top centrifuge (Eppendorff 5650, Switzerland), at 3000 rpm for 5 minutes. The resulting blastospores were re-suspended in a saline solution, and the resulting cell density was determined through enumeration with a hemocytometer. Wireworm injections were performed as described in appendix three.

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Chapter 2

Spinosad interacts synergistically with the insect pathogen *Metarhizium anisopliae* (Metch.) Sorokin against wireworms, *Agriotes lineatus* (Coleoptera: Elateridae).*

Introduction

Two exotic species of wireworms *Agriotes lineatus* (L.), and *A. obscurus* (L.) are established in British Columbia as significant crop pests, especially of potato and corn (Vernon and Pats, 1997; Vernon et al., 2001). The wireworm's threat to agriculture is due to several factors including their 3-6 year larval development, their polyphagous diet, their hardiness, and their difficulty to target in the subterranean environment. In Canada most pesticides previously used to control wireworm have been withdrawn from registration because of their ecological toxicity. With unchecked expansion of wireworm populations and few registered treatment options, alternate pest management strategies are needed.

Hyphomycetes are the most commonly encountered insect pathogens, and are described as facultative filamentous fungi that reproduce via asexual conidia on solid substrate, and via yeast-like hyphal bodies within a host (Goettel and Inglis 1997). *Metarhizium anisopliae*, is a ubiquitous hyphomycete found globally in most soils and is known to be pathogenic to a wide range of insects (Huxham et al, 1989). This wide host range is facilitated by many unique isolates of *M. anisopliae* that are able to express 'pathogen-related' macromolecules towards specific host insects (Zacharuk, 1981; St. Leger, 1987). Other differences in infection efficacy are due to

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factors related to the host's health, developmental stage, and particular ability to resist infection (Gillespie, 2000). Soil temperature, moisture, and exposure time to conidia have also been shown to be important contributors in causing mortality. *Metarhizium anisopliae* causes wireworm mortality in the laboratory, and inundative field applications have been associated with wireworm mycosis and reduced feeding damage in potato crops (Kabaluk et al., 2005). Despite the successful infections of wireworms, the potato crops sustained wireworm feeding damage.

Similar deficiencies with other control agents have led to the formulation of binary treatments of biological and physical controls with insecticides (Pachamuthu 2000, Furlong and Groden, 2001), nematodes (Ansari 2004), boric acid (Zurek 2002), diatomaceous earth (Akbar et al 2004), and other biological agents (Inglis et al 1997, Thomas et al 2003) or metabolites (Brousseau 1998) to synergize the total control potential. It is unclear why or how these combined treatments interact, but it is clear that the physiological effects caused by one agent increase the pathogenicity of the biological agent, and thereby cause higher or faster mortality. In fact, many of these combination treatments have interacted synergistically, and have caused significantly higher mortality than predicted assuming only additive effects of each treatment. These studies have also indicated that combination formulations use less of each active ingredient than would be required if they were to be applied individually.

The spinosyns are the first in a new class of pesticides called naturalytes, compounds that are produced by living organisms. The spinosyn molecules are large, complex, ringed structures called macrolides, and are neurotoxic through the

inhibition of nicotinal acetylcholine receptors, and gamma amino butyric acid (GABA) receptors by means of a novel mode of action (Salgado, 1998; Wilson, 2003). Spinosyns are produced by the actinomycete *Saccharopolyspora spinosa,* and are known to be active against many noxious pests, as well as some parasitoids and pollinators, but are otherwise harmless to most non target organisms (Wilson, 2003).

Here we report tests of the interaction of *M. anisopliae* and spinosad in causing wireworm mortality by exposing the wireworms to different dose combinations, and found that a metabolite from an unrelated soil microbe improves the pathogenicity of an entomopathogenic fungus.

Materials and Methods

Wireworm collection and selection. Wireworms were collected from land with a 30-year history of pesticide free management (UBC Farm, Vancouver, British Columbia, Canada). The species composition, determined through pheromone trap catches of adult males as found to be two dominant species *Agriotes lineatus* and *A. obscurus* with an estimated prevalence of 88% and 12% respectively. Following collection, wireworms were transferred to 27 liter plastic tubs, kept at 7°C in the soil, and periodically fed wheat seed (*Triticum aestivum*). Eight hours before the bioassay, sections of potato were buried near the soil surface to attract actively feeding larvae. From these, wireworms within a 10 cm range of the potato bait, and within a 19-24mm range in length were selected for experimentation. The average wireworm mass ranged from 32 to 42 mg, and the size range corresponded to larval instars 11 to 13 as previously described by Kabanov, 1975.

Preliminary bioassay. Three biorational insecticides were combined with *M. anisopliae* to determine if the combination had a synergistic effect on wireworm mortality. A commercial essential oil blend of thyme and clove oil (EcoSmart Technologies, Franklin, TN, USA), an insect growth regulator halofenozide (DOW Agrosciences, Indianapolis, IN, USA), and a commercial blend of spinosyn's A and D (DOW Agrosciences, Indianapolis, IN, USA) were applied at both high and low label rates with one rate of *M. anisopliae* (10^6 conidia per gram sand). All experimental units were prepared as described in the synergy bioassay below. The high and low rate of essential oils (47 and 19 ppm a.i.), and halofenozide (12 and 6 ppm a.i.) were applied by way of treated corn-cob granules, whereas spinosad (3 and 1.5 ppm a.i.) was applied as a soil drench.

Synergy bioassay. Industrial sand (Target Products Ltd. Burnaby, British Columbia, Canada), and course sands of varying particle size (0.1-0.4mm diameter) were rinsed ten times, heat sterilized, and equivalent amounts of each were thoroughly mixed together. To each 35ml plastic cup, 31.5g of the sand mixture was added and subsequently hydrated to 7% moisture wt/wt. All treatments were applied as a soil drench that was included in the total moisture calculation such that each experimental unit received equivalent hydration. Each wireworm was weighed to verify uniformity of size, and then placed in 35ml plastic cups filled with the sand mixture. In applying the treatments, 1ml of spinosad solution (0, 45, 90, and 180ppm a.i.) was added to each cup, and then 1ml of *M. anisopliae* suspension was added to each corresponding treatment. Both treatments were mixed into the sand with a glass rod, and sterile tap water was added to adjust the moisture to 7%.

Fitted lids were applied to the plastic cups, and five cups from each treatment combination were randomized in a covered plastic tray lined with moistened paper towels to maintain humidity. Three replicate trays were prepared. Each tray was covered with aluminum foil to maintain darkness, and all trays were kept at 22°C for duration of trial, and the trial was repeated twice.

Metarhizium anisopliae soil drench: An isolate from a local cadaver was grown in 10cm petri plates on Sabaroud dextrose agar amended with yeast (SDAY) culture media at 28°C for 18 days. Conidia were washed with 25ml dH_2O amended with 0.0025% Triton X-100. The resulting suspension was kept in total darkness at room temperature until application the same day. After enumeration with an Improved Neubauer hemocytometer, stock suspensions were adjusted to 10^6 and 10^4 conidia/ml, and one milliliter of either conidia drench was added to the plastic cup to yield a concentration of 3.2×10^4 or 10^2 conidia/ gram sand respectively. For the control, sterile tap water with 0.0025% Triton X-100 was used. Germination of the conidia was determined 20 hours after spreading on the SDAY media.

Spinosad soil drench: The pest control product Tracer™ (Dow Agrochemicals, Indianapolis, IN, USA), a commercial formulation of spinosyns A and D, is 44.5% active ingredient (a.i) in aqueous suspension. Three stock solutions were prepared containing 10, 20, and 40 µl of commercial spinosad in 100ml distilled water (45, 90, and 180 ppm). One ml of each solution was added to corresponding plastic cups to yield a final sand concentration of 1.5, 3 and 6 ppm (a.i.). Sterile tap water was used for control treatment.

Binary treatment compatibility. Because studies have shown that insecticides can interfere with growth and sporulation of *M. anisopliae* (Li and Holdom, 1994), we determined if spinosad was fungicidal against *M. anisopliae* on artificial media. A 4mm sterile filter paper disc saturated with ten ml of 0, 45, 90, and 180ppm (a.i) spinosad solution was assigned to one of four quadrants of a SDAY culture *of M. anisopliae.* Culture plates were replicated nine times. Germination was evaluated after 20 hours, colony growth was monitored every three days by measuring the zone of inhibition around the treated paper disc, and total sporulation was evaluated 16 days after preparation. All cultures were incubated at 27°C under continuous darkness.

Antifeedant effect of treatments. On each evaluation date, the amount of feeding that had occurred on each wheat seed, and the proportion of wireworms feeding in each plastic tray were recorded. If the wheat seed had been consumed, another seed was added to the bioassay cup. The percentage of individuals feeding at each sampling date was averaged over two experiments, and the treatment means were subject to ANOVA.

Experimental Design and Statistical Analysis. For the preliminary assay each of the 18 treatment combinations, two *M. anisopliae* levels (0, 10^6 conidia/g sand), three biorational insecticide levels (0, low, and high), and three biorational pesticides were arranged in a complete random design. There were fifteen replicate cups for each treatment combination and ten repeated measurements of the percent mortality were taken over the 60 day trial. Expected mortality was generated by the formula described below, and observed mortality of the binary treatments were

compared by way of a chi square comparison. For the synergy bioassay, each of the 12 treatment combinations, three *M. anisopliae* levels (0, 10^2, 10^4 conidia per gram sand), and four spinosad^{TM} levels (0, 1.5, 3, and 6 ppm a.i) were replicated 15 times per trial with 10 repeated measurements of proportion mortality, and feeding status over 60 days. For synergy effects expected mortality (E) was generated from the following formula, $E = O_{Spin} + O_{Met}\,(\,1 - O_{Spin}\,)$, where E is the expected mortality, and O_{Spin} and O_{Met} represent the observed mortalities due to treatments of pure spinosad and pure *M. anisopliae* respectively. This formula has been used in similar studies by Trisyono (1999), and Hummelbrunner and Isman (2001). The predicted (E) effects of spinosad and *M. anisopliae* treatments were compared to the observed (O) mortality of the binary treatments with the following formula, $\chi_2 = \{(O - E)^2\} / E$. Since the speed of kill was dependant on the level of *M. anisopliae,* chi-square comparisons were performed at 20 days in the 10^4 conidia per gram treatments, and at 50 days in the 10^2 conidia per gram treatments. All statistical analysis was performed with JMP IN v5.1 statistical software package (SAS Institute, 2005).

Results

Preliminary bioassay. Halofenozide and thyme/clove oil interacted additively with *M. anisopliae* (Appendix 3), while the spinosad interacted synergistically. Binary treatments produced significantly higher mortality than expected at a spinosad concentrations of 3 ppm (df =1, $\chi_2 = 48.0$), and at 1.5 ppm (df=1, $\chi_2 = 85.5$). These synergistic results led to the design of a more sensitive factorial experiment with increased levels of spinosad and fungus. Although the

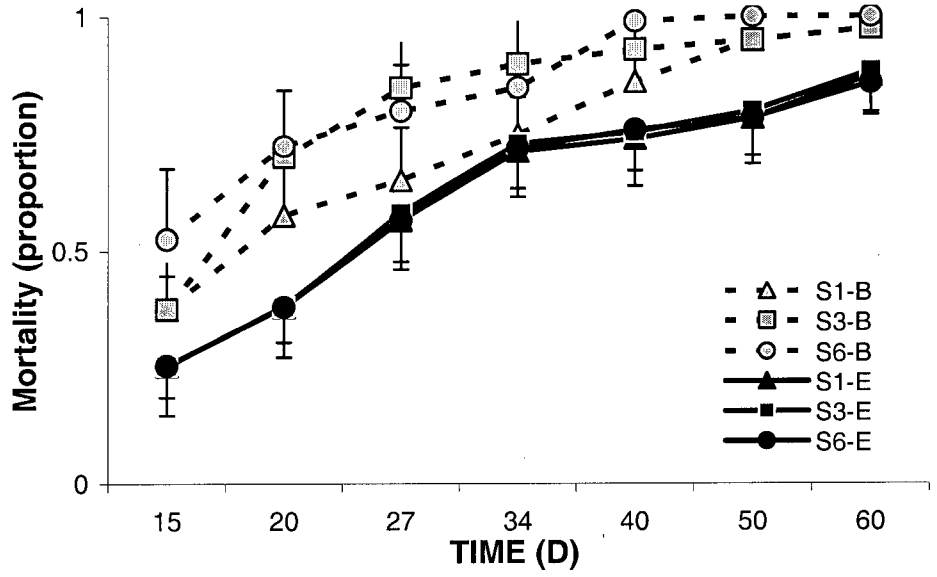
other pesticides were not synergistic with *M. anisopliae,* their potential to control wireworm is currently being evaluated.

Synergy bioassay. *Metarhizium anisopliae* levels of 10^2 and 10^4 were deemed low to medium based on data from previous trials (Kabaluk et al 2005). When acting independently, conidia at 10^2/g sand caused low levels of mortality over the 60 day trial, whereas conidia at 10^4 /g caused high levels of mortality, but acted slowly. Spinosad treatments caused changes in larval behavior consistent with neurological poisoning, but only caused a low level of mortality when applied alone. When spinosad and *M. anisopliae* were applied together, mortality was significantly higher than the expected value of their combined effect, indicating synergy. Based on individual treatment levels, the greatest synergistic effect occurred when 10^2 conidia per gram sand were used with 6 ppm spinosad (Table 2.1).

Table 2.1. The mortality effects of combinations of spinosad and *M. anisopliae* at 50 days (10^2 conidia /g), on wireworm mortality and at 20 days (10^4 conidia/g). Average mortality is shown with standard deviation in parentheses. Tabular chi-square value is 3.84, with df = 1, and $\alpha = 0.05$. Calculated values larger than the tabular value are considered synergistic.

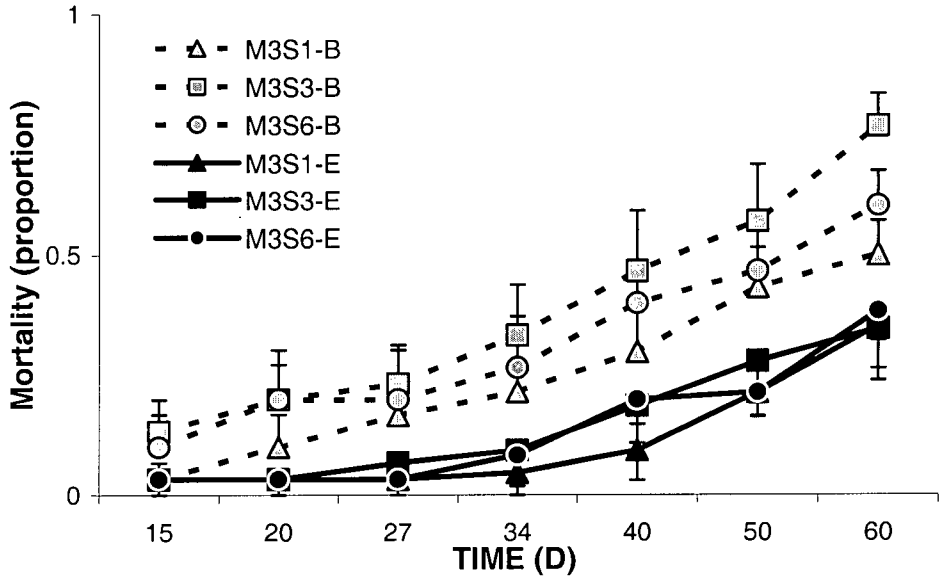
Dosage		Larval mortality, %					
		Fungi	Spinosad	Mixture		χ_2	Effect
Fungi (spores/g)	Spinosad (ppm)			Expected	Observed		
10^2	1.5	7.5 (3.5)	15.0 (7.1)	21.5 (9.2)	37.5 (24.7)	11.91 *	SYNERGY
10^2	3	7.5 (3.5)	22.5 (25.7)	28.0 (25.5)	60.0 (14.1)	36.57 *	SYNERGY
10^2	6	7.5 (3.5)	15.0 (7.1)	21.5 (9.2)	57.5 (31.8)	60.27 *	SYNERGY
* 50 days after treatment, n=30							
10^4	1.5	35.0 (7.1)	5.0 (7.1)	38.0 (5.7)	57.5 (31.7)	10.01 **	SYNERGY
10^4	3	35.0 (7.1)	5.0 (7.1)	38.0 (5.7)	70.0 (28.2)	26.95 **	SYNERGY
10^4	6	35.0 (7.1)	5.0 (7.1)	38.0 (5.7)	73.0 (37.8)	32.24 **	SYNERGY
** 20 days after treatment, n=30							

Figure 2.1 High fungal concentration. Binary treatment combinations of spinosad at 0, 1.5 (triangle), 3 (square) and 6 (circle) parts-per-million (ppm) soil concentration with *M. anisopliae* at 10^4 conidia/gram sand. The percentage mortality was recorded over time (n=30), and SE shown. Observed mortality from binary (B) treatment (grey) compared to expected mortality (E) treatment (black).



Similar mortality was achieved at this dose as that in the levels of fungi two orders of magnitude higher without spinosad (Fig 2.2).

Figure 2.2 Low fungal concentration. Binary treatment combinations of spinosad at 1.5 (triangle), 3 (square), and 6 (circle) ppm soil concentration and *M. anisopliae* at 10^2 conidia/gram sand. The percentage dying was recorded over time (n=30), and SE is shown. Observed mortality from binary (B) treatment (gray) was compared to expected mortality (E) treatment.



In the 10^4 conidia per gram treatments, mortality was consistent in all treatments, but progressed faster than predicted when spinosad was added at all treatment levels. Despite the variability in mortality, the binary treatments consistently caused higher mortality than the predicted values of additive effects.

Feeding behavior assay: Over 60 days, wireworm behaviour alternated between periods of intense feeding and periods of inactivity. Sometimes larvae fed only on the embryos of the germinated seed, and other times they consumed the entire seed and all shoot and root growth. The average percent of feeding damage that was recorded decreased as both *M. anisopliae* concentration and spinosad concentration increased (Figs 2.3 and 2.4).

Figure 2.3 The percentage of wireworms feeding after fungal treatment. *Metarhizium anisopliae* was found to have a significant antifeedant effect (F = 12.163; df = 2, 93; p <0.0001). Means with the same letter are not significantly different (Tukeys HSD, α=0.05).

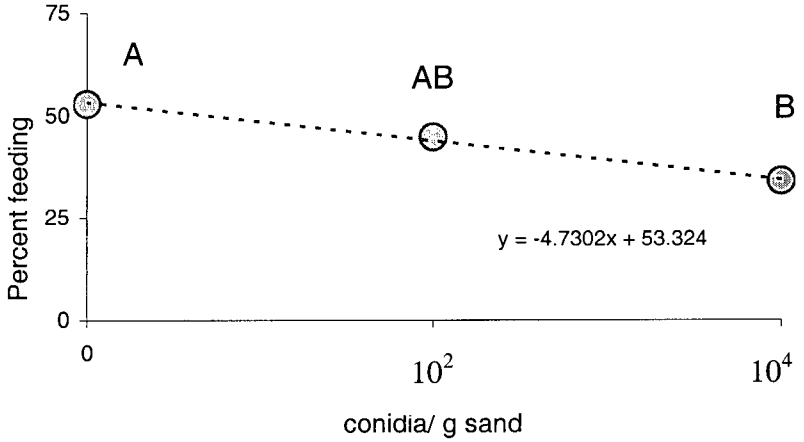
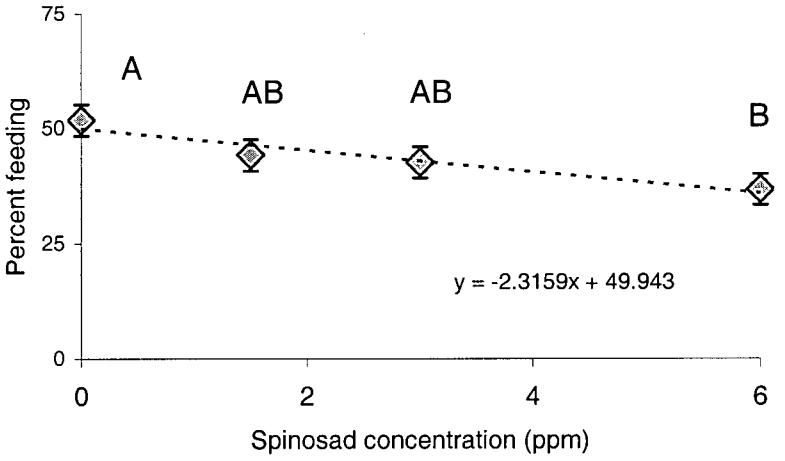


Figure 2.4 The percentage of wireworms feeding after spinosad treatment. A significant antifeedant effect was found (F =3.3639; df = 3, 92; p= 0.0220). Means with the same letter are not significantly different (Tukeys HSD, α=0.05).



Toxicological effects of spinosad. Toxic effects such as nervous tremors, serpentine contortions, and discoloration of cuticle occurred 5 days after spinosad application and continued at varying intensities until most larvae recovered.

Fungicidal interactions between agents. In SDAY culture spinosad did not appear to affect growth and sporulation of *M. anisopliae*, even when spinosad levels reached 180 ppm. Germination percentage was found to exceed 95% in all treatments. No differences in colony growth were observed, whereas sporulation occurred at 16 days in all treatments, and appeared uniform in color and abundance in all quadrants of the Petri plate.

Discussion and Conclusions

We found that a combined treatment of spinosad and *M. anisopliae* caused significantly higher mortality than either treatment alone. This suggests that low-levels of a reduced risk pesticide can be combined with a biological agent to provide control of wireworms without reliance on traditional pesticide strategies.

The mechanism for the observed interaction between spinosyns and an entomopathogenic fungus is unclear. Perhaps when the wireworm becomes infected, several resistance pathways are activated including cellular and humeral immune mechanisms to destroy the pathogen, as well as detoxification pathways to clear the metabolites produced by the fungus. *Metarhizium anisopliae* is known to produce toxins that interrupt the metabolism and immune function of an insect (Suzuki et al., 1971; Zacharuk, 1981), and these molecules would have to be cleared by the insect's detoxification system to prolong the insect's survival after infection. It is known that as an infection proceeds in the hemocoel, a hyphomycete proliferates as yeast like cells called hyphal bodies (Goettel and Inglis, 1997). Therefore, it is reasonable to expect that hemolymph toxin levels will increase as the

population of fungal cells grows until eventually the level of toxins causes the death of the insect. If death of an insect is dependant on the type and rate of toxins produced by the insect pathogen, then a compound that inhibits any of the insect's detoxification pathways would increase the speed of kill. Spinosyns are neurotoxins that are known to act by way of a novel allosteric mechanism, but they may also alter the wireworm's sensitivity to fungal toxins, or the wireworm's immune response to *M. anisopliae.*

A positive side affect of the treatments is that both spinosad and *M. anisopliae* treatments reduced larval feeding, however the average proportion of individuals feeding was never less than 50% of the control. Although intermittent feeding behavior is typical of *Agriotes* wireworm species (Furlan, 1998), it is also known that both immune responses (Rolff and Siva-Jothy, 2003), and detoxification processes (Ahmad et al., 1987) are biochemically expensive to sustain. Thus a growing larva may rely on nutritional inputs to facilitate the response against *M. anisopliae,* for detoxification of the xenobiotic molecules, or both, and thus must feed.

It is known that wireworm need 3-6 years to develop and that most infested fields consist of larval populations of mixed ages and stages of development (Kabanov, 1975). Other studies have shown that wireworm infested fields often have co-established elaterid species (Vernon, 1998), that are known to differ in their susceptibility to *M. anisopliae* (Zacharuk and Tinline, 1968). Thus a viable biological control treatment has to be able to overcome abiotic factors as well as immunological and behavioral differences associated with the mixed population.

Since *M. anisopliae* is known to gradually degrade in the soil, the inclusion of periodic spinosad treatments could extend the total control period provided by one application of the fungus by improving transmission when inoculum levels were low. This laboratory study suggests that field trials should be carried out to determine if combined treatments of spinosad and *M. anisopliae* are practical and effective.

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Chapter 3

Evaluation of host-pathogen interaction of *Agriotes* sp wireworm and *Metarhizium anisopliae* Metchnikoff♠

Introduction

One barrier to the successful biological control of a pest, is host resistance. Host resistance manifests itself as variability in susceptibility to disease and has been reported within and among several species of wireworm (Elateridae), (Tinline and Zacharuk, 1960) as well as in numerous studies with other insect families. In 1950, Rockwood studied the infection of wireworm (*Agriotes obscurus)* by *Metarhizium anisopliae*, and found that a 3-4 month incubation period was required to cause an overall mortality range of 49-72% (Rockwood, 1951). Another study demonstrated that several wireworm genera collected in Canada were susceptible to *M. anisopliae* at all life stages (Zacharuk and Tinline, 1968). These latter studies of heavy fungal inoculation of *Ctenicera aeripennis* found a 60% morality after 97 days and for *Limonius calilfornicus* 80% mortality occurred after 42 days. No explanation was proposed for the observed variability in mortality levels and time to death except that cuticle structure and chemistry were most likely involved. *Metarhizium anisopliae* infection was also reported to occur naturally in *Agriotes obscurus* wireworm (Fox, 1961), yet large variances in susceptibility suggested that interactions with the host varied or strains of the fungus differed in virulence.

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More recent studies have shown that *M. anisopliae* has a wide host range, and many features specific to the host determine what triggers germination (Vey et al., 1982; St Leger et al., 1994). Hundreds of isolates of *M. anisopliae* have been identified that maintain differences in host specificity and in optimum temperature (McCammon and Rath, 1994) and nutrient requirements (Li and Holdom, 1995).

In insects, resistance to pathogens can manifest itself through changes in behavior as well as in physiology. Both strategies represent important mechanisms for survival of insects, and a complete understanding of the host-pathogen relationship includes knowledge about the behavioral features of host resistance. Here I focused on the physiological mechanism involved in resistance to pathogens.

The insect immune system is robust, and effective at warding off the many pathogens and opportunistic microbes encountered daily (Gillespie and Kanost, 1997). The strength of this immune response varies with the health and vigor of the insect and to genetically based resistance mechanisms. As an example, *Galleria mellonella*, the wax moth, are commonly used in studies of insect pathology because they are susceptible to many entomopathogens, including *M. anisopliae*. This inherent susceptibility is likely to stem from their existence in a sterile honeycomb, where they are protected from exposure to pathogens. Conversely, soil-dwelling insects, such as the elaterids are able to sustain *M. anisopliae* disease for months, sometimes only succumbing to particularly virulent isolates. Whether the difference in resistance is due to the different environments they inhabit remains to be determined, but it is reasonable to expect physiological differences amongst the immune system functions of elaterids and wax moth larvae. The reason for this

comparison is two-fold, first to illustrate that there is variance in susceptibility to pathogens amongst insect orders, and secondly to highlight that most of the literature on insect immunity is biased towards lepidopteran hosts that require far shorter incubation periods than elaterids to cause mortality. Although diversity in immunity strategies exists, common traits are shared by all invertebrates (Gillespie and Kanost, 1997). These common traits can occur as humeral enzymes with detoxifying or defensive properties, or as induced peptides with antimicrobial activity, or as cellular traits via the adaptive population of hemocytes.

The enzyme phenoloxidase (PO) and its pathway derivatives are a key feature of the insect's response to wounding and pathogenic challenge (Soderhall and Smith, 1986). PO has been shown to be involved in cuticle fortification and sclerotization (Ashida and Brey, 1995), as well as blood clotting, nodulation, and encapsulation in a number of insect orders (Gillespie, 1997; Soderhall, 1998). Despite this prevalence, PO is expressed in different amounts, by different mechanisms, and may be controlled by one to nine genes depending on the species (Cerenius and Soderhall, 2004). Melanization in response to wounding is observed in wireworms. Dark melanin spots form near cuticle segments, at injection sites and near suspected points of fungal entry (personal observation). Other studies have reported similar indicators of melanization after fungal exposure (Gillespie, 2000; Serebrov, 2001).

Insects are repeatedly exposed to toxic compounds that include insecticides, defensive phytochemicals produced by host plants, and toxins produced by invading pathogens (Bernays and Chapman, 1994). Detoxification of these substances is a

crucial function in organisms, such as insects with an open circulatory system because stable hemolymph chemistry is required for optimal growth. Many enzymes are involved in detoxification, but a group of most interest is the esterases because of their role in detoxifying destruxins (Pedras et al 2002), a major toxic metabolite of *M. anisopliae.* Since wireworms can sustain fungal infections for 30-100 days depending on the host species (McCauley and Zacharuk, 1968) and are therefore most likely exposed to an increasing amount of destruxins, it is possible that esterase activity contributes to the overall host resistance to infection.

Unlike vertebrates, insects do not rely on antibody-mediated immunity, but instead maintain a diverse spectrum of powerful antimicrobial peptides that maintain variable specificity towards microbial encounters (Lowenberger, 2001). A spectrum of antimicrobial molecules is often expressed after biochemical recognition of the foreign agent, and can be selective against fungi or bacteria (Lemaitre et al., 1997). These molecules are produced by epithelial tissues, and are mostly produced by the fat body. During an invasive infection such as with *M. anisopliae*, antimicrobial molecules are expected to occur in the hemolymph. Proteome assessment of the hemolymph has been performed on a number of insect species, and nearly 200 peptides that have displayed antimicrobial activity against microbes *in vivo* and *in vitro* have been purified and characterized (SwissProt database). These peptides vary in structure, mode of action, and size, but are produced rapidly upon induction following infection (Lowenberger, 2001).

A second dimension of host resistance lies with the diverse population of hemocytes that maintain a hemolymph environment devoid of foreign cells and

debris. In some insect orders, specialized hemocytes store phenoloxidase to facilitate the encapsulation mechanism or delivery of enzyme to wounded areas (Ashida, 1995). Much debate surrounds the nomenclature of hemocytes because they are named for both form and function, but currently nine types of hemocytes have been described with no single insect species having all cell types (Drif and Brehelin, 1993). These cells can vary in type and proportion with the age and stage of the insect (Lavine and Strand, 2002). A focus of this study was to characterize the cell types occurring in wireworm larvae through phase contrast microscopy. Other studies have shown that fungal treatments can alter both cell to cell interactions such as phagocytosis (Bandani, 2005), and the hemocyte load and composition (Gillespie, 2000), but no studies have been performed on elaterids.

The most common invertebrate immunity themes described above were investigated to identify key components of the *M. anisopliae* and wireworm interaction.

Materials and Methods:

Insecticidal treatment of wireworms. The synergistic treatments that caused the highest mortality in chapter two were used for all the immunity assays, unless specified otherwise. Wireworms were selected based on their feeding status, length and mass after warming the soil and baiting with potato sections. Each wireworm was placed in a 110mm plastic petri plate filled with 50 grams of the sand mixture described in chapter two. *Metarhizium anisopliae* conidia were applied as a drench to achieve a final sand concentration of 10^4 conidia/g. Spinosad was applied as a

soil drench to cause a final sand concentration of 3ppm (a.i/g sand). Moisture was maintained at 7%, and parafilm was used to seal the petri plate after one wireworm was introduced to treatment plate. Treatments included a spinosad, a *M. anisopliae,* a combined treatment and a control. Fifteen replicate wireworms were used for each trial, and the trial was repeated once. Hemolymph was sampled at 7, 14, and 21 days after treatment, and was analyzed as described below.

Phenoloxidase studies. Several experiments were designed to test the effects of fungal exposure on systemic prophenoloxidase (proPO) activity within the hemolymph of wild collected elaterid larvae. For hemolymph sampling, each wireworm was placed on the injection apparatus (described in Appendix 2) and a medial pro-leg was clipped with an extra-fine, Vannas-style, iris spring scissor. After cutting, a small droplet of hemolymph is produced at the wound site and 1.5 µL was removed with a pipette and placed into 60µL of ice-cold Dulbecco's phosphate buffered saline (DPBS, 0.2M, pH 7.2). This preparation was then mixed and immediately frozen at –25°C for 24 hours to rupture hemocytes. For the spectrometer assay, each well from a 96-well plate was loaded with 50 µL hemolymph-buffer sample and 50 µL of absolute methanol was added to activate the prophenoloxidase. Immediately 150 µL of 15mM dopamine hydrochloride substrate (SIGMA) in DPBS, was added to each sample well. All samples were simultaneously mixed by the spectrometer for 5 seconds and optical density readings were taken every 20 seconds at a wavelength of 492 nm, for a total of 180 measurements, using a SpectroMAX 190 (Molecular Devices, Sunnyvale, CA, USA).

The rate of product formation was calculated by the SpectroSOFT version 1.3 software, and hemolymph protein concentration was used as a covariate to standardize the treatment effect. The mean treatment effects were compared by ANOVA.

Esterase studies. The esterase studies involved the analysis of enzyme activity systemically through kinetic assays of hemolymph, as well as locally through histological staining of fatbody and hemolymph smears.

Kinetic assays. Larvae were mechanically held under a dissecting microscope such that their prolegs were exposed. One medial proleg was excised, and hemolymph was collected onto parafilm. Two µl of each hemolymph sample were added to 60 µl chilled Dulbecco's phosphate buffered saline (DPBS, 0.2M, pH 7.2) in a 96-well plate. Each treatment well was subsequently flushed with the pipette three times to ensure the uniformity of the mixture. A 10 µl aliquot of the hemolymph-DPBS mixture was set aside for determination of protein concentration as described below under Hemolymph Proteome Assessment. The 100mM naphthyl acetate substrate solution was prepared by adding 18.9 mg/ml α-napthyl acetate, with 6.0 mg/ml RR Fast Blue salts to DPBS. The mixture was pre-heated for 5 minutes in a 37°C water bath before use in the enzyme reaction. To each remaining 45 µl hemolymph-PBS mixture, 200µl of 37°C α-naphthyl acetate substrate solution was added and was immediately analyzed spectrophotometrically in a SpectaMax 190, 96-well plate reading spectrometer for 20 minutes. The spectrometer temperature was maintained at 37°C, and absorbance readings were taken every 30 seconds at a wavelength of 450nm, for the duration of the assay.

Tissue specific esterase assays. Larvae were mechanically affixed under a dissecting microscope such that their prolegs were exposed. An incision was made along the frontal plane of the lateral side of the proleg-plate, and hemolymph and fat body tissues were collected onto parafilm. A glass cover-slip was used to delicately drag the tissue droplet across a microscope slide to form a uniform smear. Slides were allowed to air dry, then were processed with an esterase kit (Sigma kit #2, 90A1) originally formulated to identify vertebrate blood cell enzyme activity. Dried smears were fixed for 10 minutes in a methanol-citrate-acetone fixative by pooling 100 µl of fixative onto slides. Slide smears were then rinsed with deionized water to remove any excess fixative. One capsule of *alpha*-napthyl acetate was dissolved in two ml ethylene glycol monomethyl ether, and one capsule of RR fast blue salts was dissolved in 50ml pre-warmed TRIZMAL buffer solution (pH 7.6). Then both solutions were mixed together to make the final solution, which was transferred to the Coplin container. Fixed slides were immersed in solution for 30 minutes (under darkness), and then were rinsed for three minutes with deionized water. No counter stain procedure was performed, and slides were mounted with water and scoring of esterase intensity was done under phase contrast microscopy. A positive test was indicated by the accumulation of black staining in or around the cells. Scoring was performed by identifying a field-of-view containing tissue, and then by counting the numbers of esterase (+) cells and esterase (-) cells. Slide mounts were documented and photographed.

Hemolymph proteome assessment. The protein concentration of hemolymph was used as a covariate in all enzyme assays to standardize the rate of product formation due to the treatment. The protein concentration was also analyzed separately to determine if non-specific systemic effects of a treatment could be detected in wireworm hemolymph samples. Both the total protein concentration and peptide complexity were analyzed after various treatments.

Protein concentration protocol: Total hemolymph protein concentration was determined using methods developed by Bradford, (1979). A bovine-serum albumin (BSA) standard was prepared to calculate the amount of protein found in each sample-well. Thus enzyme activity is reported as activity per mg protein. A 10 µl aliquot of hemolymph-buffer mixture was pipetted into reaction well, and 200 µl of BIORAD Protein Assay Kit #2 dye (4:1) was added. The reaction was allowed to proceed for 5 minutes before an end-point absorbance reading was taken at a wavelength of 595nm. Absorbance readings from experimental samples were compared to absorbance readings from standardized samples of BSA.

Peptide complexity. Matrix assisted laser desorption ionization time of flight (MALDI – TOF) mass spectrometry was performed on hemolymph samples after exposure to treatments. Hemolymph sampling was performed as described in general methods section. Saturated matrix solutions of sinapinic acid (used with larger peptides) and alpha-cyano-hydrocinnamic acid (used with smaller peptides) were prepared immediately before use. Two µl of hemolymph were diluted in 100 µl 0.1% trifluoroacetic (TFA) acid (SIGMA). Five µl of hemolymph-TFA mixture was combined with 5 µl of either matrix solution, mixed thoroughly with a pipette, and

spotted onto the MALDI -TOF sample plate (Applied Biosystems) by way of the dried droplet (Karas-Hillenkamp) method. After the sample was combined with the matrix solution and flushed with a pipette tip to mix, a one microliter aliquot was spotted onto a stainless steel mass spec sample stage. The sample was allowed to evaporate, leaving a dry film of protein on the stage. After laser ionization, molecules fly towards a sensor that measures the related mass. Spectra are produced that indicate the presence of molecules of a given mass, and the relative abundance of each. Specific machine settings for each analysis such as laser intensity, number of laser desorption shots, specific matrix used, and signal intensity are all reported on the mass spectrometer output, and these settings often varied with each sample. A mixture of low molecular weight peptide standards (Invitrogen) were used to calibrate sample readings.

Characterization of fungal metabolites. Equivalent volumes of a 25% strength solution of Bandoni-malt yeast peptone (MYP) broth, potato dextrose broth (PDB), and soy trypticase broth (STB) were tested for their ability to promote metabolite production by *M. anisopliae* (recipe in appendix 3). Cultures were grown for 14 days at 22°C while being shaken at 150 rpm. Entire cultures were freeze dried for 72 hours, and resulting extract was dissolved in 100ml 99% methanol. Mixture was filtered through paper filter paper, then syringe filtered with a 0.2 micron pore size, before solvent was evaporated under vacuum. Crude extracts were dissolved in methanol before MALDI-TOF mass spectrometer analysis was performed.

Hemocyte studies. Hemolymph sampling was performed as described in the general methods section, and a series of experiments were designed to quantify the changes in hemocyte characteristics at both systemic and local scales. Changes in total hemocyte count (THC) and hemocyte variety were enumerated by way of hemocytometer counts, whereas cell to cell interactions were documented and photographed under phase contrast microscopy.

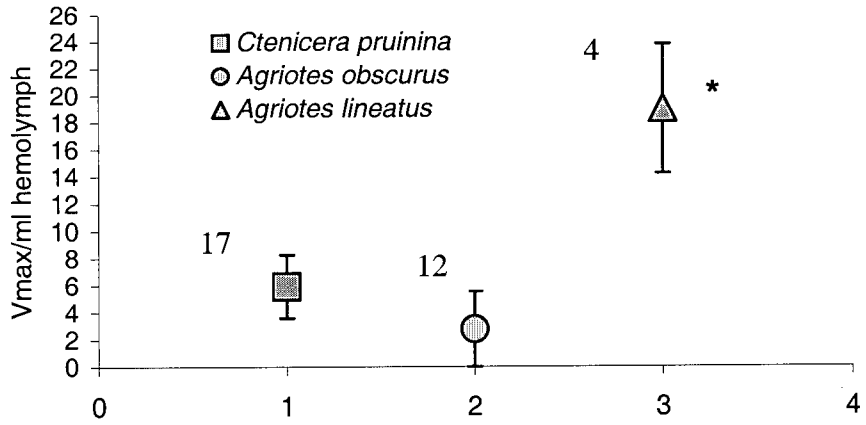
Changes in hemocyte number and ratio. After bleeding the wireworm, 1.5 µl of hemolymph was added to 10 µl anticoagulant buffer (0.392g NaOH, 0.853g NaCl, 0.632g EDTA, 1.206g citric acid, 100ml ddH_2O), and was flushed through the pipette onto parafilm five times to mix thoroughly. A 10 µl aliquot of the mixture was added to an Improved Neubauer hemocytometer, and each hemocyte was counted within the 25 cell-area, for a total observed volume of one µl (0.01cm * 0.01cm * 0.01cm= $1.0 * 10^{-6}$) per sample. Variation in total hemocyte count, and proportion of each hemocyte type was compared with ANOVA. When appropriate, fungal cell densities were recorded, as were other changes in hemolymph quality, such as sepsis or other molting phenomenon. Sepsis was noted if bacteria were found in the hemolymph sample, but no quantification of bacterial loads was performed.

Results

Phenoloxidase Studies: The first experiment examined the baseline activity found in larvae collected from three locations, Oregon, Agassiz, and UBC. Great variability in enzyme activity occurred both within and among species groups, with some individuals testing highly positive and some individuals not responding at all. The

UBC population, *Agriotes lineatus* dominant had the highest PO activity and the lowest number of larvae responding (4/25). The Oregon population, *Ctenicera pruinina*, had the median level of PO activity and the highest proportion of larvae responding of all species groups (17/25). The Agassiz population, *Agriotes obscurus* dominant had the lowest PO activity and the median number of larvae responding (12/25). (Figure 2.1).

Figure 3.1. Baseline phenoloxidase activity in three species of wireworms for responding individuals with no known exposure to pathogens. Means are shown with standard error, and significant differences were found (F = 4.35; df= 2, 30; p= 0.022) as indicated by ' * '. Samples with effects < 0.2 vmax/ml hemolymph were deemed non-responsive and were excluded from analysis.

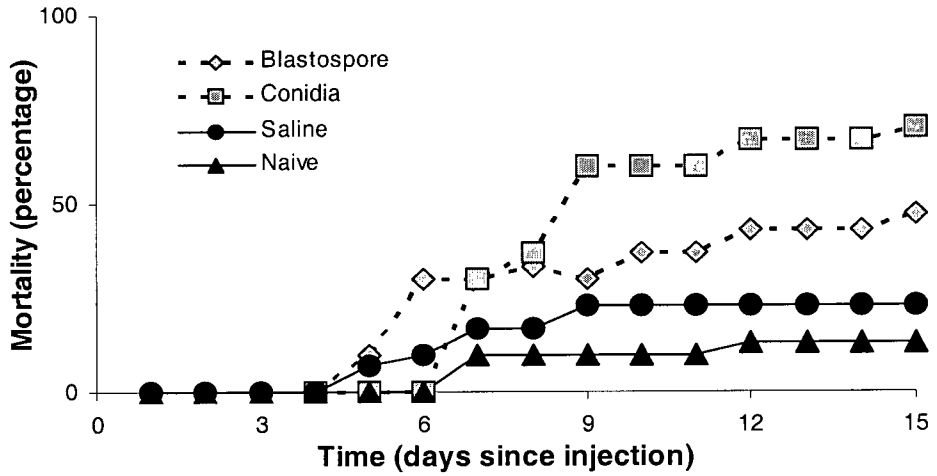


Therefore, the significantly higher PO levels found in *A. lineatus* wireworm may be an artifact of the low number that responded.

The second series of experiments involved testing different methods of handling the hemolymph samples to reduce variability in PO enzyme activity, and involved the development of standardized infection doses, as well as post-infection handling techniques. Standardization of dosage was tested by way of blastospore or conidial injections (protocol in appendix 1), whereas the effects of freezing, sonication and methanol activation were developed to reduce variability in enzyme

activation. Injections of blastospores, and conidia caused fatal mycosis in wireworms to occur within one week of the injection (Figure 3.2).

Figure 3.2 The effects of injecting different *M. anisopliae* phenotypes on wireworm mortality. Conidia and blastospore injections were performed at 1.68 x 10^7 and 5.46 x 10^5 cells /ml respectively. Wireworm were bled for PO analysis at 4 days, thus a low level of mortality was due to secondary infection.



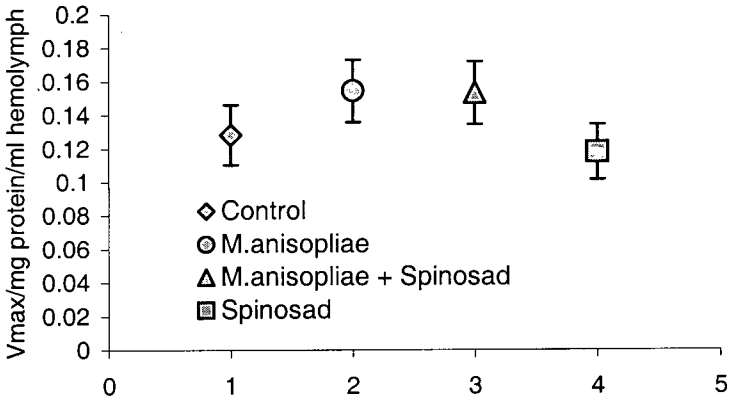
Despite the successful infection rate and the wireworm's presentation of symptoms, no differences in PO activity were found. At the beginning of the experiment, the control treatments had the highest activity (probably an artifact), but no difference was found in any of the other samples.

Based on visual assessment of reaction wells, a mixture containing pooled hemolymph and an equivalent amount of methanol changed color faster and more intensely than mixtures without methanol. Since methanol activation improved the consistency and vigor of the assay (Goldworthy et al., 2002), it was used for all subsequent reactions.

The final series of experiments was designed to test the effects of incubation time on the PO activity after both injection and natural infection with fungal pathogen. Both short (hours), and long term (21 days) timelines were investigated

to determine if there was an inducible component to the wireworm's PO cascade. No significant PO effect was detected at any timescale, or with any treatment from the hemolymph samples (Figure 3.3).

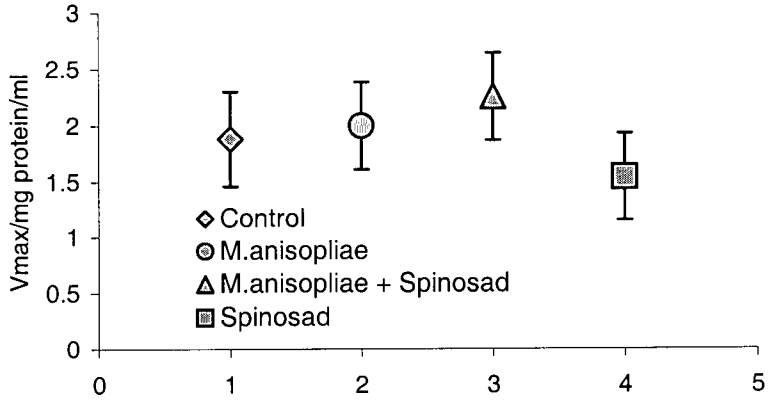
Figure 3.3 Phenoloxidase activity in hemolymph after exposure to insecticidal treatments. Means are shown with standard error, and no significant differences were found (F = 1.07, df = 3,44, p = 0.373).



Esterase Assays: Of the many detoxification enzyme families, the esterases are responsible for detoxification of many molecules encountered by an insect, and are entirely responsible for detoxification of destruxins, a suite of toxins produced my *M. anisopliae*. These experiments were designed to test for activity of systemic esterases within the hemolymph as well as the localized effects within the fat body.

Kinetic assays. The first experiment tested the esterase activity of fresh hemolymph samples after the larvae had been exposed to a fungus, spinosad, and combination treatment of spinosad and fungus. No significant differences in esterase activity were found in any of the treatments over the 14 days of the trial (Figure 3.4).

Figure 3.4 The changes in hemolymph esterase activity after insecticidal treatments. Means are shown with standard error. No significant differences were found (F = 0.587, df = 3,23, p = 0.63).



The esterase activity was moderately variable, and the absence of differences from the control suggested that esterase was either not involved in the host defense, or that destruxin production was not produced as abundantly *in vivo,* as previously thought.

Tissue smears analysis. An additional experiment was designed to test the differential esterase activity in two tissue types by way of stained tissue sample analysis. Differences were found between the spinosad and control treatments such that intensity of esterase activity was higher after spinosad treatment. The spinosad treatments caused esterase activity to increase in the fat body and in both hemocyte types that were monitored (Table 3.1).

Table 3.1 Relative esterase response by tissue or cell type. The number of cells, plasmatocyte (PLAS) or granular cells (GRAN) that tested positive for esterase. The fat body (F.B.) score represents the cumulative intensity of background staining in all fields of view.

	Control			Spinosad		
	PLAS	GRAN	F.B.	PLAS	GRAN	F.B.
EST (+)	1	6	10	7	73	18
EST (-)	15	38	3	15	11	4
Totals	16	44	13	22	84	22

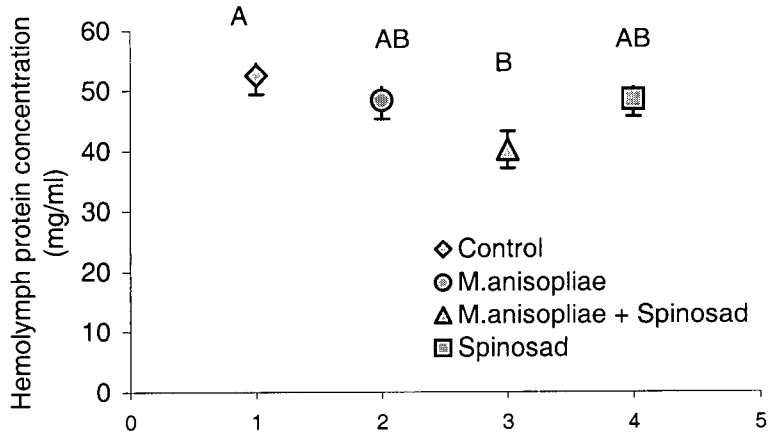
More granular cells were positive for esterase activity than plasmatocytes, and displayed esterase activity even in the samples from control wireworm. Plasmatocytes only rarely tested positive in the control treatments, but were observed displaying increased esterase activity after exposure to spinosad. The most esterase activity appeared in the fat body in control and in treated individuals. It was most pronounced after a spinosad treatment.

Hemolymph proteome assessment: Attempts were made to characterize the myriad of proteins and peptides present in the hemolymph by way of measurements of protein concentration and composition.

In the first experiments, total protein concentration was measured as a cofactor for the enzyme assays, and was also analyzed separately to determine hemolymph quality. The protein concentration varied with the treatments and in general decreased with the severity of treatment (combination vs. individual) and length of exposure time to insecticidal agents. Combination treatments caused additive reductions in protein concentration, thus suggesting a loss of protein stores due to detoxification of xenobiotics, or to the facilitation of an immune response.

Even with the presence of proliferating fungi that exceeded 16 thousand cells/ µl hemolymph only hours before death, blood samples contained lower protein concentrations than uninfected controls (Figure 3.5).

Figure 3.5 The hemolymph protein concentration in wireworms after exposure to *M. anisopliae* and spinosad insecticidal treatments. ANOVA found differences amongst the means (F = 2.84; df = 3,71; p = 0.044). Means are shown with standard error, and were separated by Tukeys HSD test (*alpha* = 0.05). Values with the same letter are not significantly different.



In the second experiment, changes in protein and peptide composition were analyzed by way of a MALDI TOF mass spectrometer. Changes in peptide complexity occurred after exposure to fungal pathogen, and these changes indicated that an induced response had occurred. Molecules with masses equal to or highly similar to masses of known antimicrobial peptides were identified in hemolymph samples. The SWISSPROT database has detailed listings for anti-microbial peptides produced by insects, and provides precise masses for comparison. The masses generated by MALDI-TOF require sequence data from a purified peptide source to confirm their identity, but do provide a precise portrayal of the hemolymph complexity. Of the 11 hemolymph peptides that were identified by mass, six are known to have specific antifungal activity. This evidence suggests that an inducible

response to the fungi, its metabolites, or both has occurred in the wireworm (Table 3.2).

Table 3.2 Description of peptides identified in hemolymph from wireworms infected with *M. anisopliae*. Positive controls of saline injected and naïve wireworm were included, and peaks with similar mass were removed to identify only the peaks unique to the fungal infection.

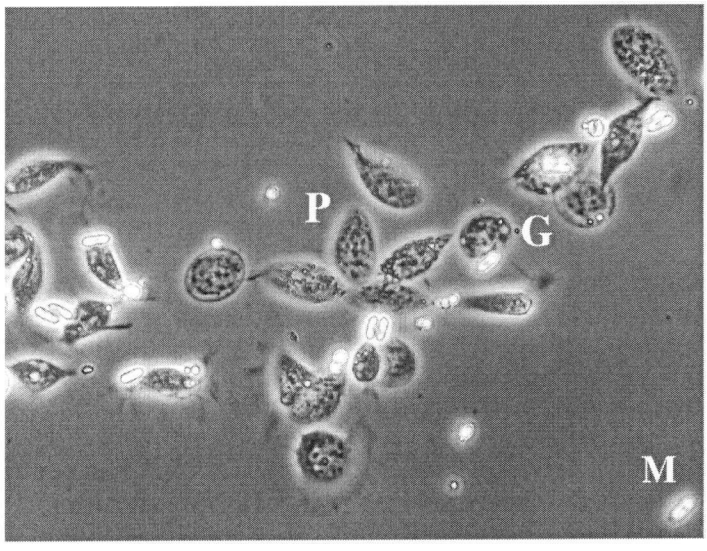
Observed mass (Da.)	Name	Function	Observed mass (Da.)	Name	Function
3167.09	Ponericin G4	antifungal	8152.61	Coleoptericin	Anti-gram(-) bacteria
3399.02	Ponericin G3	- antibacterial - insecticidal	8455.82	LCR77 precursor	antifungal
3614.99	Cecropin B	antibacterial	10577.99	Tenecin-3 precursor	Antifungal
5627.50	Metchnikowin	antifungal	13665.79	Gloverin	Anti-gram(-) bacteria
6947.28	Megourin 2 or 3	fungi	15226.96	Growth blocking peptide (GBP)	Prevent metamorph osis
7049.96	Drosocin precursor	antifungal			

Hemocyte studies. The previous studies were designed to test the effects of a pathogen on biochemical aspects of the humeral fraction of the hemolymph. The morphologically diverse cellular fraction of hemocytes was studied as another indicator of the wireworms immune response.

Characterization of hemocyte populations was required to identify cells of interest for the future insecticidal stress bioassays, as well as to establish the 'normal' range found in wild and unchallenged insects. There is great debate about the classification of hemocytes because they are given special names based on structural features or based on functional features. Cells were categorized based on these descriptions. Phase contrast microscopy doesn't allow absolute confirmation of each hemocyte subdivision, however it sufficient to monitor the total hemocyte

count relative to the two dominant cell types, granular cells, and plasmatocytes (Figure 3.6).

Figure 3.6 Typical hemocyte populations in wireworm, with M. anisopliae conidia (M). Spindle shaped plasmatocytes (P) and spherical granular (G) cells dominate the hemocyte cell types.



After treatment with spinosad, *M. anisopliae*, and combination treatments, the total hemocyte counts were determined. Treatment type, duration of exposure, and mass of larvae were not related to the total hemocyte count ($F=0.295$; $df=6, 179$; $p=0.9388$). The hemocyte load averaged 11.12×10^6 cells/ ml (SE+/- 3.57×10^5, n= 189), and was maintained until late establishment of fungal infection.

Usually hemolymph from recently deceased larvae contained no free flowing cells of wireworm or fungal origin, however other larvae sustained heavy loads of fungi in their hemolymph before death. Fourteen days after exposure to a high dose of *M. anisopliae* contaminated sand, fungal cell densities in the hemolymph reached 3.25×10^6 cells/ml in the more diseased of the two larvae, whereas at 21 days fungal cell densities reached a maximum of 16.5×10^6 cells/ml in one of the two larvae observed.

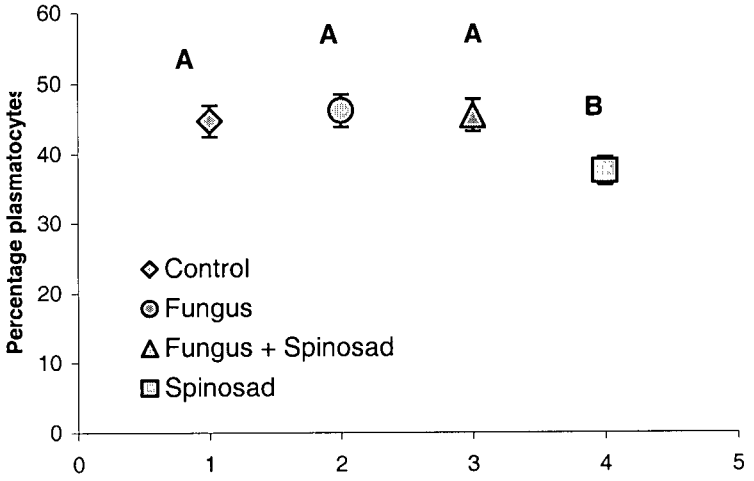
Despite the stable hemocyte loads found in the wireworm, differences in the population of hemocytes were detected after exposure to four treatments; spinosad, *M. anisopliae*, combined, and control. The percent of plasmatocytes was significantly lower after spinosad treatment than both the fungus-alone and combined treatments (Table 3.4).

Table 3.4 ANOVA results from the percentage of plasmatocytes. Four treatments spinosad, fungus, spinosad + fungus, and control were compared. A total of 45 wireworm per treatment combination were tested.

Source	Degrees freedom	Fisher	p-value
Model	8, 179	5.36	< 0.0001
Treatment	3	4.88	0.0027
Exposure time	2	22.87	< 0.0001
Time x Treatment	3	1.47	0.2253

The hemocyte population ratios fluxuated non-significantly depending on the treatments, and gradually increased in magnitude until significant differences occurred (Figure 3.7).

Figure 3.7 The average percentage of plasmatocytes found in hemolymph after each treatment. Means represent average of plasmatocytes over 21 day trial, and average of two trials with total of 45 larvae per trial per treatment. Standard error of mean is shown, and significant differences indicated by different letter (Tukeys HSD, $\alpha = 0.05$).

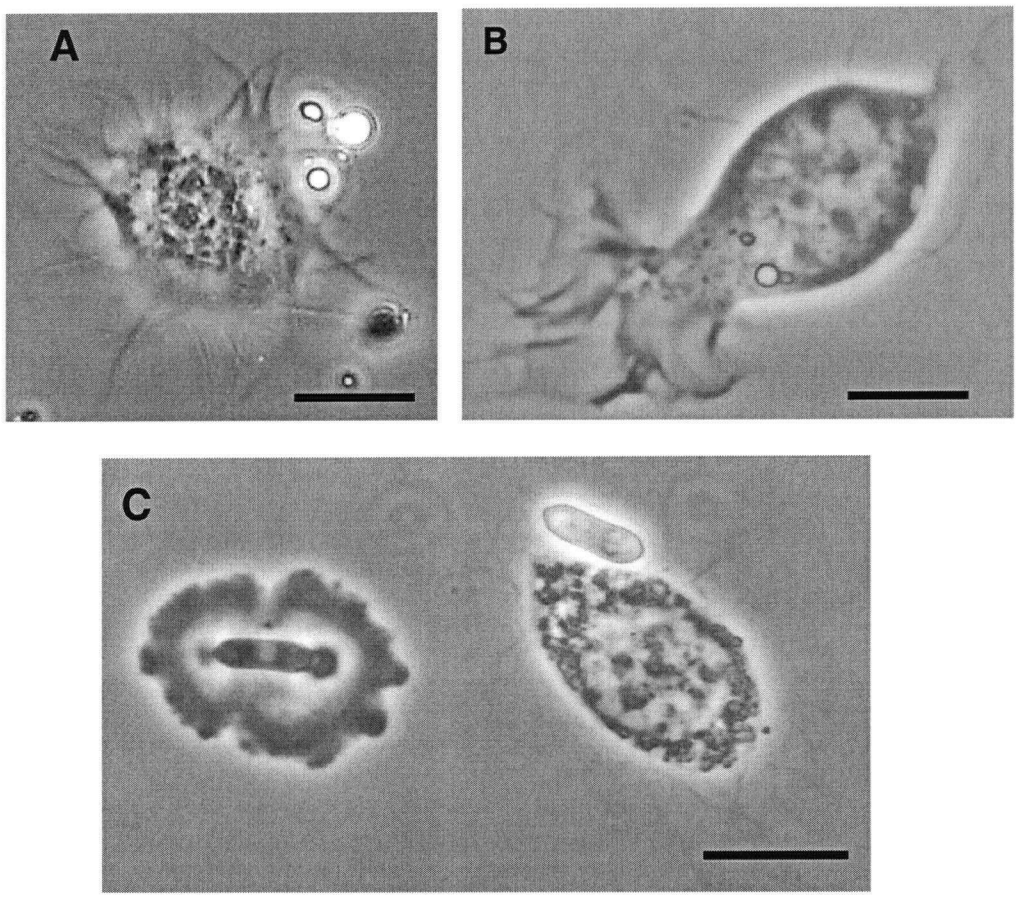


These trends in cell-type ratio indicated that a response to spinosad is apparent, whereas a response to the fungus is less obvious. In the combined treatments, the effects of the fungus dominated the response, and obscured the response to spinosad. This suggests that one mechanism of fungal establishment in a larva is to retard the differentiation rather than the proliferation of hemocytes, such that the cellular response is muted and unable to adapt to the increasing presence of the fungus. Otherwise, the change in cell-type ratio may be due to the indirect effects caused by the presence of a fungus in the hemolymph, such as reductions in protein and sugar stores, dissolved oxygen, or other features maintained by homeostasis.

Cell-to-cell interactions. The next series of experiments were designed to test the effects of fungal exposure on hemocyte morphology. Phase contrast microscopy showed changes in hemocyte morphology in response to contact with

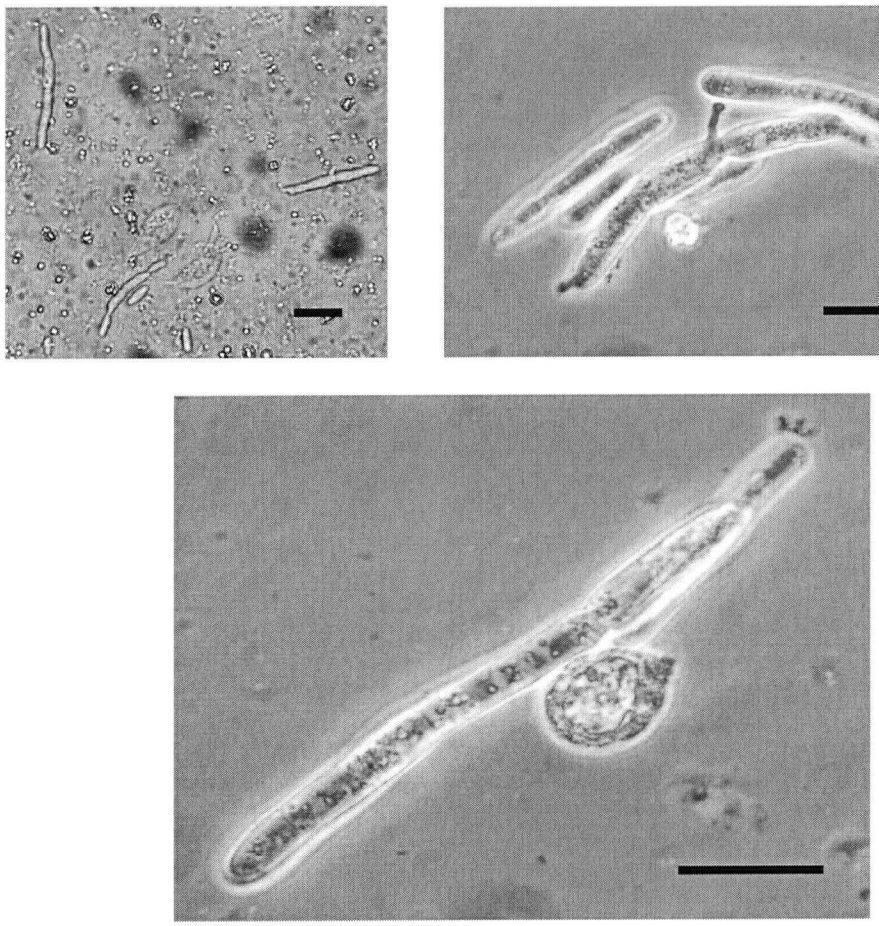
conidia *in vitro,* but not to hyphal bodies from a natural infection. Changes in the extracellular matrix structure such as the formation of sheets, the formation of filopodia (presumably actin-rich), as well as the nodulation of lipid-conidial complexes were observed in many hemolymph samples after mixing with conidial solutions. When mixed, a non-specific, hydrophobic interaction occurs between the conidia and the lipid bodies and fat body proper. Occasionally, these lipid-conidia complexes were observed to be encapsulated step-wise by multiple cell layers in vitro, but no such encapsulation was observed in hemolymph samples taken from naturally infected wireworm (Figure 3.8).

Figure 3.8 Changes in hemocyte morphology after exposure to *M. anisopliae* conidia. (A) filopodia formation in a granular cell, (B) sheet formation in plasmatocyte to form 'professional' phagocyte. (C) Conidia displayed non-specific attraction to acellular lipid bodies, while fibers are observed projecting from plasmatocyte. Each photo's scale bar represents 10 microns



It was very difficult to observe the cell-to-cell interactions during a natural infection because fungal cells are only apparent hours before death of the wireworm, and because the time required to kill a wireworm varies from 10 to 60 days under temperature and moisture controlled conditions (constant 22°C, 6% moisture wt/wt). Despite this inherent difficulty, *in vivo* cell-to-cell interactions were observed in numerous individuals, whereas the exponential phase of fungal proliferation, only observed hours before the host's death, was quantified in four wireworms, two each from day 14, and day 21. *In vivo*, no changes in morphology of any hemocytes occurred after repeated contact with hyphal bodies in various stages of development. Although conidia are small enough to be phagocytosed, the hyphal bodies are larger than the conidia, and are even much larger than the hemocytes (Figure 3.9).

Figure 3.9 Fungal cells in wireworm hemolymph at various stages in the infection. (A) Branched hyphal bodies near plasmatocytes. (B) Replicating hyphal bodies (note branching) (C) Multi-celled hyphal body adjacent to granular cell. Scale bar for each photo represents 25 microns. Note relative size of fungal cells to hemocytes in A and C from different wireworm.



Hyphal structures were observed to exist in multi-cell forms that ranged in cell number from one to five. During replication, fungal cells were observed budding from both poles of a primary hyphal body as well as laterally off branched 2-4 cell hyphal structures (Figure 3.9b). These differences in cell number are thought to be due to the observation of fungal cells at different stages of growth. As the infection proceeded, the number of free-flowing hyphal bodies increased until the density exceeded the mean hemocyte number maintained by healthy wireworm. At this point, the larvae were considered alive, in that they responded to mechanical stimulation with weak leg movement, and to bright light with mandible movement,

but they were very close to death. Finding wireworms at this stage was serendipitous, and allowed detailed analysis of late infection fungal growth that corroborated many observations from a similar study (McCauley and Zacharuk, 1968).

Characterization of fungal metabolites. Fungal metabolites resulting from growth of *M. anisopliae* in three growth media solutions were compared for their ability to induce effects on wireworm hemocytes. Differences in fungal growth were found among the solutions, such as pigment production, size of mycelial structure, and density of mycelial structures in a uniform volume of liquid media. These effects are most likely due to the differing ratios of nitrogen and carbon containing constituents in the growth media recipes however, after methanolic extraction of the metabolites and evaporation of solvent, differences in the yield of crude-extract were also found.

Methanolic extracts were analyzed by way of MALDI TOF mass spectrometry, as mentioned above, for the presence of peptides, and to quantify the complexity relative to destruxin sized molecules (Destruxin A = 577.71 Da. (Sigma); Destruxin E, 616 Da.). Several molecules were detected in the extracts that had highly similar masses as known destruxins (Table 3.2).

Table 3.5 Molecular masses of metabolites from three crude extractions. Masses are shown in Daltons, and represent the average mass detected from at least four replicate samples per methanolic extraction.

Media extraction	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
PDB	543.13	**566.37**	**583.46**	601.11	**617.13**
STB	551.87	693.38	783.74	806.04	822.67
B-MYP	543.10	**617.73**	694.81	779.64	875.56

From the PDB extraction, the masses at peak 2 and 3 were 98% and 99% similar to destruxin A whereas peak 5 was 99.82% similar to destruxin E. In the B-MYP extraction peak 2 was 99.72% similar to destruxin E. Since 35 destruxins have been identified to date (Pedras, 2002), it is possible that the other peaks are also destruxin molecules, or some other virulence peptides produced by the fungus. The presence of these molecules indicates that a number of low molecular weight peptides are produced by the fungus when grown *in vitro*, and that these metabolites may contribute to the immuno-modulation effects on the wireworm

Discussion

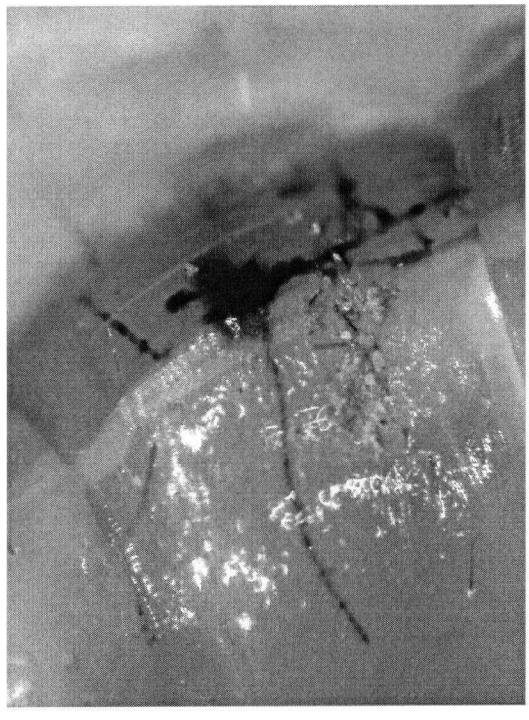
Studies of humeral immunity.

Enzyme activity. Species level differences in the susceptibility of wireworm larvae to *Metarhizium anisopliae* has been shown previously with five species of elaterids (Zacharuk and Tinline, 1968). In these studies, field collected insects maintained different susceptibilities to heavy inoculations of fungi. No efforts were made to control the dosage, but under these heavy inoculations the speed of kill differed and was thought to be due to inherent differences in cuticle structure and

chemistry. Another study that observed *M. anisopliae* in wireworm indicated that upon fungal penetration into the hemocoel, plasmatocytes aggregated near the fungi, but no phagocytosis was observed (McCauley and Zacharuk, 1968). Although quantification of the host resistance was not the purpose of the previously mentioned study, it is interesting that they observed a muted response to the fungi that was partly due to the size differences of the two cell types, but could also have been due to inhibition of recognition or response reactions. In this study it was found that results from most of the typical methods for measuring an immune response were not significantly different between infected and control individuals. PO activity in the hemolymph was variable, such that some individual wireworms were highly positive and others showed no activity. This variability did not appear to be associated with treatment effect, larval mass, or hemolymph protein concentration. The final PO experiment was designed to determine the length of time after exposure to fungus was associated with a PO response, and here I found no significant change in PO activity with any of the treatments, or on any of the sampling days. If the 'true' PO effect is weak, the significance of the effect may have been obscured by the inherent variability in the intensity of response and in the number of wireworm responding. This absence of a difference combined with the high mortality in the fungal treatments indicated that PO was either not a factor in the wireworms defence against the fungi, or was not detectable systemically in hemolymph samples using the methods described in this study.

Since melanized spots appeared on some individuals after exposure to fungi, and on all individuals at their injection wound sites it was surprising that a difference in PO activity among treatments was not found (Figure 3.10).

Figure 3.10 Injection wound melanization. Note that fractures in exoskeleton have been sutured with melanin.



Visible melanization was observed dozens of times after pro-leg removal, after micro injection, and occasionally after heavy attack by M. anisopliae (personal observation). Based on this evidence, melanin must maintain a role in wound response by acting as a repair polymer for exoskeletal or cuticle damage. Since PO effects cannot be detected consistently from samples of hemolymph, and visual signs of pigmentation are obvious, PO must be part of a local tissue response. The 21-day assay was long enough to cause high mortality in some of the treatments, and despite this successful infection rate, no changes in enzyme activity were detected.

The esterase studies indicated that there were differences in the tissue-specific activity of the fat body, and slight differences in the types of hemocytes that tested positive, but no differences in the systemic studies. This difference suggests that hemolymph analysis may not be appropriate in wireworm even though changes in hemolymph esterase activity after *M. anisopliae* infection were found in *Galleria mellonella* (Serebrov, 2001).

Hemolymph proteome assessment. During development, insects store proteins primarily in the fat body, but also in the hemolymph to drive the many biochemical processes of molting and pupation (Haunerland, 1996). In *Locusta migratoria,* reductions in total hemolymph protein levels were reported after infection with *M. anisopliae* (Ouedraogo, 2002). A study by McCauley and Zacharuk (1968) provided detailed descriptions of the pathology of the fungus and showed that tissue degradation only occurred during the penetration phase, and late in the infection. They showed that the fungi could maintain two cuticle penetration strategies depending on the elaterid; one that involved rapid penetration, the other that involved a stepwise infiltration. They also showed that tissue damage in the wireworm would be difficult to inflict, both mechanically and enzymatically as a free flowing hyphal body within a continuously circulating environment. Thus fungi are most likely reliant on free-nutrients from the hemolymph during establishment in the hemocoel. An additional effect of the fungus is due to its metabolites. The toxins play an important role in the death of the insect, and may be produced at a particular fungal cell density, at particular times during hyphal development, or are produced

constitutively and gradually accumulate until death. If accumulation occurs, it would be reasonable to expect a rate of degradation to occur as well, and this degradation would require energy and would thus also account for a loss in hemolymph protein concentration. This loss in protein concentration would be exacerbated by the metabolic demands of the growing fungus, and when these and the latter effects are combined, the rate of protein depletion may exceed the rate of nutrient conversion by the fat body, and explain the observed decrease in total protein concentration.

The MALDI TOF studies targeted molecules in the mass range similar to that found in other insect immuno-peptide studies, 500 to 16000 Daltons (Uttenweiller-Joseph et al.,1998). By comparing blood samples before and after challenge with the fungus, trace amounts of peptides were detected that were very similar if not identical in mass to peptides isolated from other insect orders. Novel peptides yet to be characterized were also present, but to confirm the identity of any peptide, purification and partial sequencing is required to accompany the MALDI data (Lowenberger, 2001).

To improve this study, pooling of hemolymph from approximately 100 wireworms per treatment would provide sufficient hemolymph to identify peptides of interest. Hemolymph should be purified by reverse phase HPLC, and fractions of interest should be analyzed by Edman degradation (sequence analysis), and through MALDI-TOF mass spec analysis of both pure and trypsin digested samples. These data would indicate the exact mass of the peptide, the partial amino acid sequence would indicate the peptides identity if a similar peptide was already in the database, and a trypsin digest data would act as a biochemical fingerprint.

Hemocyte studies. The other major division of insect immunity lies with the cellular fraction that maintains a sterile, debris-free hemocoel during development (Tanada and Kaya, 1993). The absence of hemocytes at or very near the time of death suggest that toxins produced *in vivo* are able to alter the hemocyte proportions directly or indirectly, and also cause the death of the wireworm. It is peculiar that most wireworms die with scant evidence of fungal cells, whereas others can tolerate extreme parasitizations. Regardless, in wireworm the only documented immune response to *M. anisopliae* was the aggregation of plasmatocytes around hyphal bodies in the area of fungal invasion (McCauley and Zacharuk, 1968). This response lacked the specificity normally observed with cellular interactions, and thus may have been due to a passive association rather than an immune response. This result is interesting because it supports my observations of a marginal increase in the proportion of plasmatocytes in the hemolymph after treatment with *M. anisopliae.* Furthermore, McCauley and Zacharuk (1968) reported that no phagocytosis occurred, probably because fungal hyphal bodies can be considerably larger than hemocytes. Here I report the same result, with the additional observation that no nodulation, or encapsulation of large bodies occurred in a natural infection. All of this evidence points to an active immuno-modulatory effect of the fungal invasion that could occur either through the avoidance of recognition by the immune response, or through the production of compounds that actively retard the efficacy of either recognition or response mechanisms.

During the cell-to-cell interaction studies with the conidia, changes in the morphology of hemocytes occurred as early as 60 minutes after exposure to the

conidia suspension. This response was interesting since the hemocytes would never encounter the fungus as conidia in nature, and thus were able to recognize these 'unnatural' cells *in vitro.* However, the lack of reaction to hyphal bodies indicates that during the pathogenic phase, the cells are not recognized as being foreign. Perhaps the fungus produces anti-rejection compounds that allow undetected growth in the hemocoel. Research into the evolution and genetic peculiarities of many entomopathogens has shown that species richness has been underestimated (Curran et al., 1994), and that specific factors of virulence determine which isolate is pathogenic to which host. A pathogen such as *M. anisopliae* consists of numerous strains that may be members of a species aggregate. Given this variability, it was thought that isolates taken from locally diseased wireworm would possess the necessary adaptations to be a more successful pathogen. This assumption is true with regards to mortality, in that the wireworm collected from UBC were most susceptible to isolates of fungi acquired from local larvae. However, since the assays never exceeded 65 days, it is not known if the seemingly harmless isolates required longer incubation periods such as the 3-4 months required in other studies with *Agriotes* species larvae (Rockwood, 1951).

Summary

Given the absence of any systemic immune response, it seems reasonable to assume that the fungus actively controls the microenvironment of the host hemocoel during establishment, or just isn't recognized. The differences in cell type proportion that were detected throughout the course of an infection suggest that the increasing

presence of the fungus affects the wireworm indirectly, or through more subtle mechanisms than those evaluated in this study. The PO and esterase pathways have both been affected by *M. anisopliae* infections in lepidopteran insects (Bidochka et al., 1997; Serebrov, 2001), yet were not found to differ significantly from the control treatments in these elaterid larvae. Changes in the hemolymph proteome are the most likely to identify the mechanisms used by the fungus in penetrating, establishing, proliferating, and ultimately killing the wireworm.

Although extended incubation periods may be deemed impractical by researchers trying to develop a commercially viable product that kills quickly, long-term studies may show that extended incubations are part of a longstanding wireworm management program. Factors such as wireworm species, developmental stage, and rearing or storage methods may all contribute to subtle physiological changes, thus development of balanced nutrient diet and general rearing techniques should be developed before considering immunological studies in this long-lived insect.

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Chapter 4: General Conclusions

Summary. The central theme of this study was to identify factors that contributed to the variance in susceptibility of elaterids to *M. anisopliae.* The wireworm's variability to this fungus infection has been reported in the literature for more than 50 years (Rockwood, 1951), and needs to be understood before an effective and marketable biocontrol product can be developed. For a biological control to be practical, the insect must be infected, and for this to occur consistently there are three major factors a manager should consider. (1) The insect specific factors including behavioral and/or physiological features that are unique to the host species. (2) Pathogen-specific factors, such as its inherent virulence, the level of inoculum present, and its degree of adaptation towards the particular host insect. (3) The environmental factors such as moisture and temperature and soil type. These environmental effects can change the nature of the interaction to such a degree that either the pathogen or the host could be favored when they otherwise would not be.

The identification and characterization of the above factors for wireworm and *M. anisopliae* were attempted in this study. However, despite controlling for the size and feeding status of the larva, the temperature and moisture of the sand, the inoculum level, and to using a potentially adapted isolate of the fungus (local cadaver), variability in the time to death and total mortality occurred. This variability was thought to be due to physiological effects associated with the host immune response. After analysis of the many features associated with the insect

immune response, no significant response was detected in natural infections of wild collected *A. obscurus* or *A. lineatus* wireworm. Indirect host responses included changes in the proportion of hemocytes found in the hemolymph of treated individuals, as well as changes in the protein concentration of affected larvae. Many of the other factors implicated in the immune response to *M. anisopliae* in other insects were not different from the control in these elaterids. Furthermore, after observing the fungus in the hemocoel several times, some distinct phenotypes were displayed from what was presumed to be a clonal colony of *M. anisopliae.* These different strategies suggest that the specific pathology of the fungus is complex, which also means that it is unlikely that a single facet of the immune response is responsible for the wireworm's resistance or tolerance to the invading pathogen. If these differences in fungal phenotype contribute to virulence, then the observation of multiple proliferation strategies suggests that multiple isolates may be more effective at controlling a hardy species of elaterid than a single isolate.

Another objective of this study was to determine if treatment combinations of *M. anisopliae* and biorational insecticides would interact. After screening three pesticides, spinosad was found to improve the transmission and pathogenesis of *M. anisopliae.* This result suggested that the mechanism responsible for the observed effects is most likely multi-factorial and subtle, and thus requires a more sensitive analysis than what was performed in this study.

Critique of research. The long life span and hardiness of the wireworm, and the inability to distinguish the co-established wireworm species all added sources of variability to the subtle interplay that occurred between the fungus and its host. Differences in susceptibility and insect immune response have been shown to be linked to the developmental stage (Goldsworthy, 2002; Rantala et al., 2003), the age (Beetz et al., 2004), the gender (Rolff, 2001) and health of the host (Gillespie, 1997) in other insect orders, but have also been found to differ amongst elaterid species (Zacharuk and Tinline, 1968). The relative influence of each of these factors on the observed variability in wireworm mortality is unknown, and is difficult to quantify in wild-collected elaterid populations.

Regardless of the inherent variability associated with immunological interplay, the most widely reported aspects of the insect immune response were evaluated after exposure to fungus and during the development of disease in wireworms. During these tests only subtle differences were found in immuno-competence, even though wireworms consistently presented symptoms of disease and were thus considered infected. The evidence of a suppressed response would have been strengthened with the inclusion of a positive control. This control would have elicited a potent immune response to compare with the muted responses in the *M. anisopliae* treatments, however no method was found to properly replicate the natural infection process.

A positive result of this study was the discovery that the transmission of *M. anisopliae* to wireworm can be increased. The compound enabling this increased

pathogenicity, spinosad, is low risk and perhaps other combinations of *M. anisopliae* and low risk compounds can be explored as well. The evidence of complimentary interactions reported in this thesis supports the growing body of literature advocating the use of biological control synergists in modern agriculture.

Suggestions for further research. Given the lack of a measurable systemic immune response and the occurrence of a synergistic interaction that consistently improved the infectivity of the fungus, a molecular factor is likely to be involved. The prevalence of cryptic *M. anisopliae* infections combined with the lengthy incubations required to kill larvae, show that wireworm can tolerate fungal cells in their hemocoel for extended periods. Furthermore, the detection of induced molecules in hemolymph samples, as well as the detection of destruxin-sized molecules in the extracts of fungal metabolites suggests that a molecular interaction occurs between the two organisms.

There are two major themes addressed in this study, the first investigates the aspects of immune response involved during infection of wireworms, the second involves the identification of combination treatments to improve the pathogenicity of *M. anisopoliae* to wireworms. Since the wireworms are long-lived and can co-exist with a pathogenic fungi, this model system may provide insight into the dynamics of transmission, tolerance and resistance to disease. Furthermore, compounds may be identified from either organism that can have useful applications in other fields of human health or agriculture as is currently being explored by many. However, due to the wireworm's long life cycle this study

is only suitable for researchers with the time to rear the larvae and the willingness to develop molecular tools to identify the exact species of wireworm and pathogen being tested. The second major theme involves screening pesticides for their ability to improve the pathogenicity of *M. anisopliae* to wireworm. Although understanding the mechanism for synergistic interactions may require molecular tools, the practical result of changed mortality is sufficient is assessing whether a combination should be tested at the field scale. The existence of combination treatments and their efficacy is becoming popular amongst pest managers because it can involve the reduction in total pesticides applied. A second benefit of combination treatments is the reduced potential for resistance as long as the agents used act via different mechanisms or on different biological systems. Since understanding the mode of action is not required to identify useful combination treatments, many varieties can be tested, and could include combinations of biological agents of different species, combinations of two insecticides, or combinations of both insecticide and biological controls.

Overall significance to field of study

Spinosad has been approved for use on organic farms in the U.S., and Canada, and *M. anisopliae* is registered in the U.S. If the laboratory success can be replicated in the field, then large-scale initiation of wireworm epizootics may prove to be a useful management strategy. By understanding the mechanism responsible for the interaction, a more suitable synergist compound may be discovered by targeting the physiological process, through the development of a particularly virulent isolate

of *M. anisopliae,* or through genetic improvements of the fungus (St. Leger et al 1996). The suppression of immuno-competence has been reported in only one study involving elaterids, and the focus was mainly on the fungus and not the host response. Here, the synergy trials showed that combinations of reduced risk and biological agents could be used to provide greater control than each alone. Although similar types of synergy have been demonstrated numerous times in the literature, mostly by combining biological agents with low-levels of synthetic chemistries, the results reported here are the first demonstrations of a synergy with spinosad. Thus the evidence presented in this thesis provides information that offers hope that combination treatments might improve the control of wireworms. This represents a small contribution to an agricultural problem that has been plaguing farmers for many years.

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Appendix 1: The mortality effects of combined treatments of reduced-risk pesticides and *M. anisopliae*.

The two reduced risk insecticides that were tested for interactions with *Metarhizium anisopliae,* and did not justify further investigation were the essential oil blend EcoExempt G (EcoSMART Inc), and the synthetic Coleoptera-specific insect growth regulator halofenozide (DOW Agrosciences).

Figure A.1.1 The proportion mortality from individual and combined treatments using the commercial formulation of thyme and clove oil blends (EcoSmart Inc.). Observed (grey markers) and predicted mortality (black markers) are compared for both high (H) and low (L) label rates. Sand concentration was, high rate 39ppm clove and 8ppm thyme, whereas the low rate was 16ppm and 3ppm active ingreadient respectively.

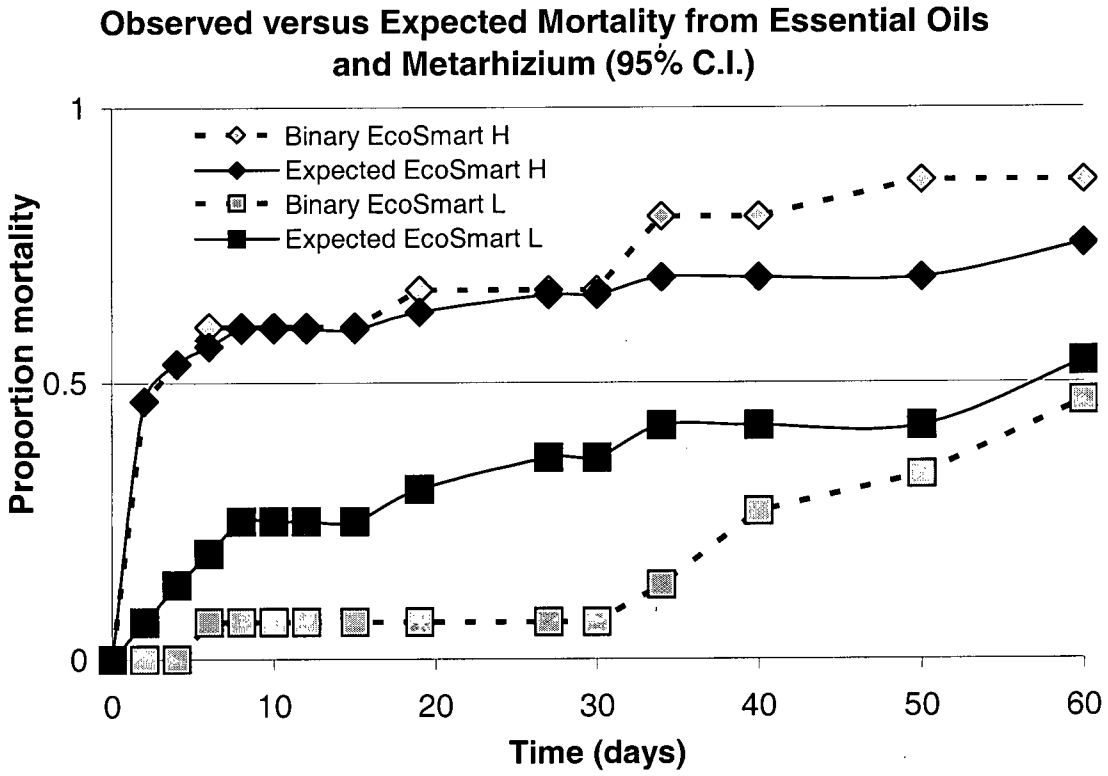
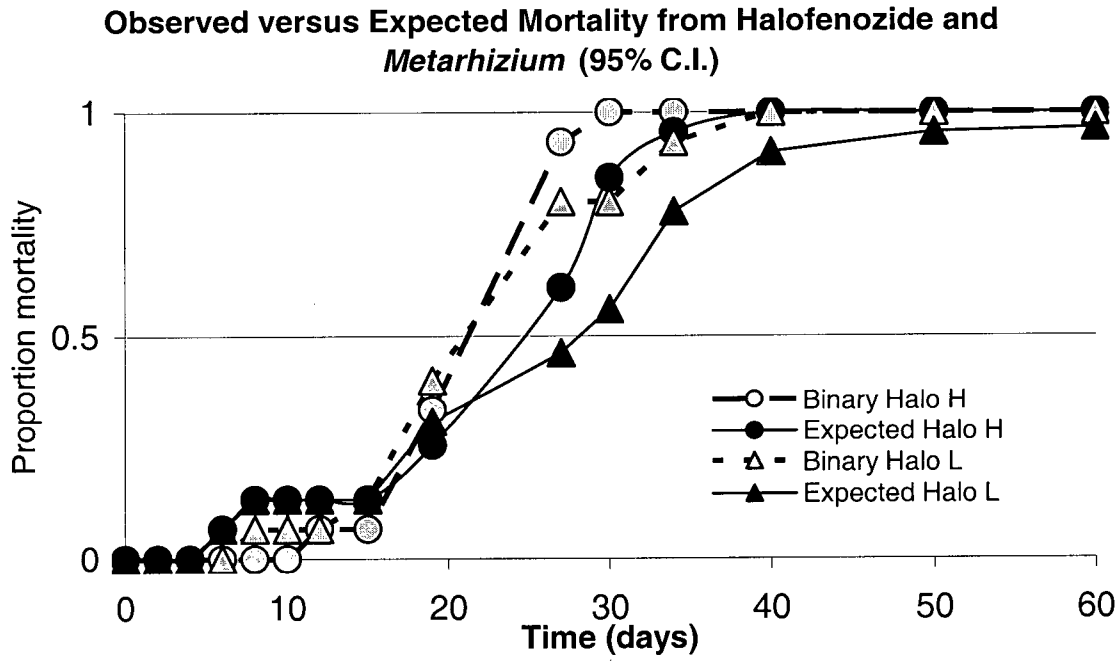


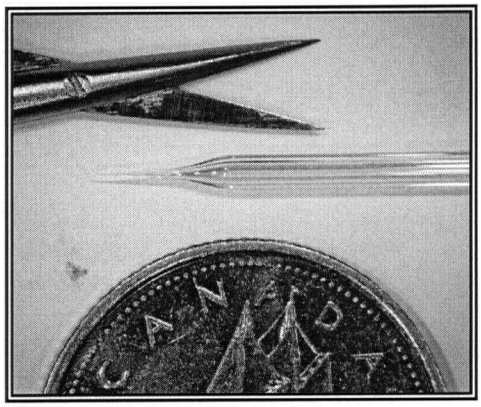
Figure A.1.2 The proportion mortality from individual and combined treatments using the synthetic growth regulator halofenozide. Observed (grey markers) and predicted mortality (black markers) are compared for both high (H) and low (L) label rates. Final sand concentration for high rate was 12ppm, and the low rate was 6ppm active ingredient .



Appendix 2: A description of the apparatus and protocol for the microinjection of wireworm.

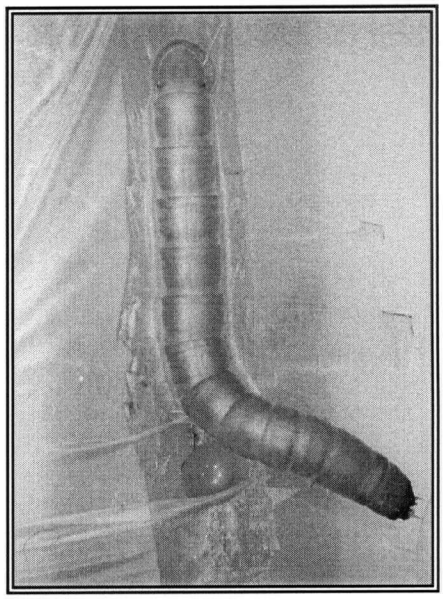
Equipment design: Microsyringes were made by pulling borosilicate capillary tubes (O.D. 1.05mm, I.D. 0.86mm) within a Sutter P-87 Micropipette Puller. These tips were polished and visually checked for tip quality to ensure needle-point range between 20- 30 μm. These micro-syringes were fitted to the end of a repeatable syringe with a 50μL capacity that was calibrated to deliver 1μL injections with the push of a button.

Figure A.2.1 Tools used in bleeding and injection procedures, with a dime included for scale.



A Plexi-glass platform was constructed from a solid block (1.8cm X 5.2cm X 12.2cm) in such a way as to allow a vacuum to hold a wireworm to the surface. A groove was made to accommodate a wireworm, and then tiny holes were drilled into the groove so that they reach the pre-drilled shaft. A vacuum hose is attached to the shaft end and air is drawn through the holes in the groove thereby creating sufficient suction to safely hold the wireworm still for injection, and to reduce the unintentional injury caused by the

Figure A.2.2 Wireworm affixed to vacuum platform, with parafilm used to maintain vacuum and reduce all movement.

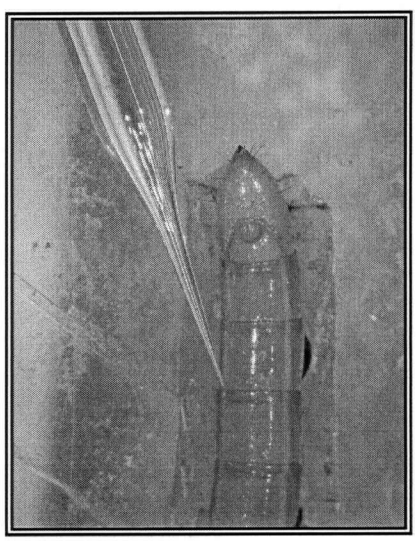


procedure.

Injection Procedure: A repeatable Hamilton syringe fitted with the micro-syringe glass needle was loaded with 50μL of injection solution. Injection solution was one of two treatments; saline blank, or saline-spore suspension that was delivered in 1μL inoculations. The injections were made at a shallow angle, in any ventral, inter-segmental cuticle region that is below the medial plane yet two segments above the anus to avoid damage to the alimentary canal (Figure A.2.3).

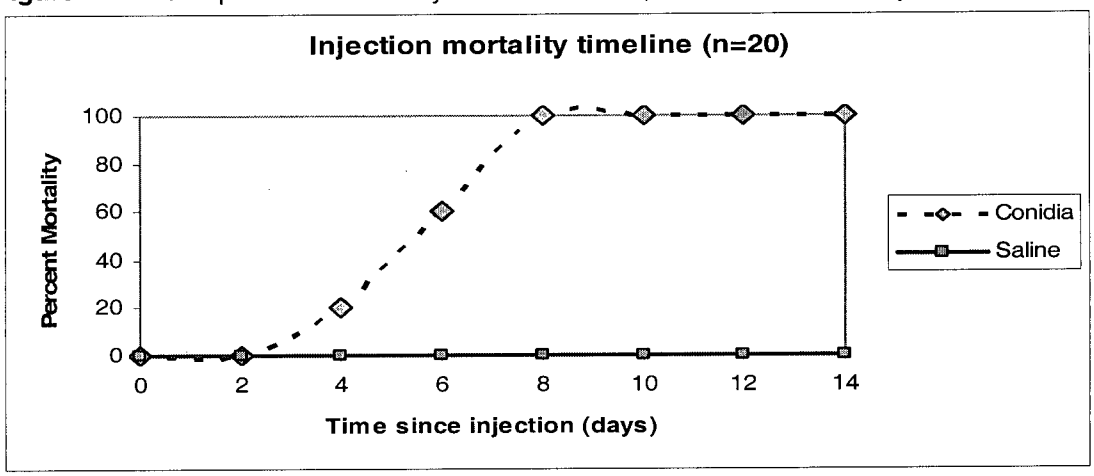
After delivering a 1μL injection, the needle is slowly removed and the wound is allowed to coagulate and clot slightly before normal circulatory pressure is restored. The wireworm is then placed in a solo-cup filled with moist Kim wipes to heal until a scab forms (15-30 min). After healing, wireworms were placed back onto soil.

Figure A.2.3 Micro injection with glass needle.



To assess mortality caused by the injection procedure, 1μL of conidia 1.43 x 10^7 /mL was injected into 10 individuals, and 1μL of phosphate buffered saline was injected into 10 control individuals. The wireworm were kept at room temperature, in moist cups of sand, under complete darkness, and were checked every day for mortality. (Figure A.2.4)

Figure A.2.4 Comparison of mortality in saline-conidia, and saline-control injections



As indicated by the above chart, 100% of individuals injected with conidia died within 8 days, whereas no saline-injected individuals died from the injections. This result suggests that the safe injection of insects with hard cuticles is possible, and that mortality can be induced by the injection of conidia.

Appendix 3: Recipes used in this study for the culture of *Metarhizium anisopliae.*

Metabolite studies. All recipes were prepared at 25% of the recommended concentration to stress the fungus and possibly maximize metabolite production. NOTE: The amounts reported in the recipe have been reduced to 25%.

Bandoni –MYP

- Malt 3.75 g/L
- Yeast extract 0.125 g/L
- Peptone 0.625 g/L

Soy Trypticase broth

Potato dextrose broth

Injection studies: One goal of these studies was to test the effects of injected hyphal bodies and blastospores on wireworm mortality. However, the fungus preferentially grows as a filamentous fungi even in submerged culture. The following recipe was used to induce blastospore and hyphal body growth in submerged culture.

Potato dextrose broth supplemented with trehalose.

- Potato dextrose broth 6g/L
- 0.05 % Triton X-100
- Trehalose 1g/L

Synergy studies: For these studies *M.* anisopliae was grown on Saborauds dextrose agar supplemented with yeast extract (SDAY) to produce enough the infectious conidia for use in the experiments. 30g/L SDAY.