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Ellen Klinger

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SUSCEPTIBILITY OF ADULT COLORADO POTATO BEETLE

(*LEPTINOTARSA DECEMLINEATA*) TO THE FUNGAL

ENTOMOPATHOGEN *BEAUVERIA BASSIANA*

By

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SUSCEPTIBILITY OF ADULT COLORADO POTATO BEETLE (*LEPTINOTARSA DECEMLINEATA*) TO THE FUNGAL ENTOMOPATHOGEN *BEAUVERIA BASSIANA*

By Ellen Klinger

Thesis Advisor: Dr. Eleanor Groden

Factors influencing the susceptibility of adult Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say), to the fungal entomopathogen, *Beauveria bassiana* (Bals.), were studied. In an assay comparing trials between a): laboratory reared, non-diapausing beetles and b): field collected, post-diapausing beetles, survival of *B. bassiana* treated beetles was higher for non-diapausing adults, however, control mortality was higher for post-diapausing adults. In a similar assay with field collected pre- and post-diapausing beetles, survival of *B. bassiana* treated individuals was higher for post-diapause adults and control mortality was higher for post-diapausing adults than pre-diapausing beetles. In a third experiment, the effect of time from eclosion from the pupal case on susceptibility of laboratory-reared, non-diapausing adults was evaluated. A trend of increasing susceptibility up to 60 degree days (base

10° C) was be seen, and a significant decline of mortality occurred after 125 degree days. The effect of sublethal and lethal doses of *B. bassiana* were evaluated on the survival of overwintering beetles. *B. bassiana* dose had no effect on the proportions of beetles sporulating or dying.

Behavior and infection of newly emerged adult CPB in the presence of *B. bassiana* infected cadavers was studied to determine the likelihood of transmission of disease as beetles emerge from the soil and colonize host plants. In 2001, arenas were constructed to accommodate potted greenhouse grown potato plants surrounded with soil to simulate the field environment. *B. bassiana*-killed, sporulating adult beetles were placed in varying patterns surrounding a release point for healthy beetles in the center of the arena. Laboratory reared, newly eclosed beetles were buried just below the soil surface at the release point and were observed for 30 minutes as they emerged and colonized one of four plants. The study was replicated in 2002 using a similar grid in a potato field. In both the arena and field, emerging beetles showed no preference for movement in any cardinal direction, and direction was not affected by the presence or absence of *B. bassiana* sporulating cadavers, nor did the presence of cadavers affect the time taken to colonize a plant or the distance traveled by a beetle. Relative humidity (RH) was a significant factor for distance traveled to the plant, with longer travel distances as the RH declined. The plant colonization behavior of newly emerged Colorado potato beetles does not appear to be altered by the presence of *B. bassiana* cadavers in the immediate environment. The likelihood of emerging adults contacting sporulating cadavers on the soil surface was quantified at different cadaver densities. A curvilinear relationship exists between density of cadavers on the soil

surface and the square root number of encounters, with encounters increasing with increasing density. Proportional mortality and sporulation also have a curvilinear relationship with density, both values increasing with cadaver density. Thus, beetles show no avoidance behavior to sporulating cadavers. Density of sporulating cadavers on the soil surface is an important factor in the horizontal transmission of *B. bassiana* from cadavers to healthy, post-pupation adults.

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CHAPTER 1: LITERATURE REVIEW

Colorado Potato Beetle

History

Since its introduction to the cultivated potato, the Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) has developed resistance to most insecticides used for control and has evolved into the most destructive insect pest of potato in the northeastern United States (Huang *et al.* 1995, Stewart *et al.* 1997, Zhao *et al.* 2000). Native to the western United States and ancestral to Mexico, the CPB moved from feeding on wild solanaceous plants to cultivated potato when potato production was introduced into its range in Iowa (Casagrande 1985). When the US populations of CPB were introduced to the western-spreading cultivated potato, *Solanum tuberosum*, a population switched to feed on potatoes. By 1912, CPB was found feeding almost everywhere potatoes were grown in the U.S. (Gauthier *et al.* 1981). Since then, many different control methods have been attempted. Unfortunately, in the effort to control this pest, it has developed resistance to many insecticidal compounds, first to DDT and at present to many other chlorinated hydrocarbons, organophosphates, carbamates, and some pyrethroids (Gauthier *et al.* 1981, Forgash 1985, Casangrande 1987, Grafius 1997).

Life Cycle

The life history of CPB is composed of 4 developmental stages: egg, larva, pupa and adult. Larvae and adults cause damage to potato plants through feeding on the foliage and occasionally the tubers. In the spring, adults emerge from the soil, colonize potato fields and either lay previously fertilized eggs or mate and lay eggs (Weber and Ferro 1994, Noronha *et al.* 2002). These eggs are typically laid on the underside of potato leaves. The eggs hatch and the larvae progress through four instars, differentiated by size and shape of the head capsule (Boiteau and LeBlanc 1992). Once the fourth instar has completed development, it arrests feeding, and the prepupa climbs off the plant and burrows into the soil to pupate. The pupa remains buried several centimeters under the soil surface for several days until the new adult ecloses and crawls to the soil surface. In Northern Maine, these "summer adults" feed on potato leaves to prepare for overwintering and may also mate. Diapausing summer adults may migrate out of the field to surrounding sites where they burrow into the soil until the following spring (Voss and Ferro 1990a). In northern Maine's potato growing region, there is typically only one generation of beetles per year, whereas in more southern states, and in some southern portions of Maine, there can be a partial second generation (Drummond and Groden 1996).

Post-diapause Movement

In spring, the stimulus that triggers emergence from overwintering sites has been shown to be a combination of temperature and soil surface moisture content (Tauber *et al.* 1994). When there is sufficient moisture present in the soil, emergence corresponds to

accumulation of heat units. Declining moisture causes emergence to slow or cease, and it only resumes after a rainfall. Tauber *et al.* (1994) found that adequate soil moisture is necessary to stimulate beetles to initiate digging, as opposed to drier soil interfering with the efficiency of digging. Beetles, hence, would not emerge when conditions are unfavorable for their host plant. Overwintering emergence occurs simultaneously between male and female beetles (Alyokhin and Ferro 1999).

Once beetles have emerged from the soil, they colonize nearby potato fields. In Massachusetts, this migration is usually accomplished by flight (Voss and Ferro 1990a). Specific orientation cues for this movement are not known, although it has been documented that spring-emerging beetles placed in an unfamiliar habitat had significant orientation to the northeast, independent of other environmental factors (Ng and Lashomb 1983). This orientation is thought to succeed in moving beetles to warmer, southern facing slopes to enable flight to suitable habitats as beetles cannot fly at temperatures below 15°C (Caprio and Grafius 1990). Depending upon food availability within the field, beetles then either take part in short- or long-distance flight or walking (Caprio and Grafius 1990, Voss and Ferro 1990a, Alyokhin and Ferro 1999). Long-distance flight is characterized by beetles climbing at high angles and flying higher than 15 meters for distances too long to track by the unaided eye (Voss and Ferro 1990a). It has been shown that starved beetles more readily take part in this long-distance flight as compared to beetles that have been fed post-diapause (Caprio and Grafius 1990, Ferro *et al.* 1991, Ferro.1999). The objective of this flight is to move beetles to a more suitable habitat. Mating status has no effect on the occurrence of long-distance flight, suggesting that spring mating is not as important as is finding a suitable environment (Alyokhin and

Ferro 1999). Beetles have been shown to survive 29 days without feeding immediately following emergence (Ferro *et al.* 1991).

For well-fed beetles, movement is characterized by "local flight" which involves short distance and flight close to the ground (Voss and Ferro 1990a). Local flight initiation seems to be related to ambient air temperature in that no flights seem to occur below 15°C and most beetles will fly at 20°C (Caprio and Grafius 1990). High light intensity (50-80%) increases flight occurrence, but it is unclear if this is an isolated effect or is in some way related to raising beetle body temperatures, thus increasing occurrence of flight (Caprio and Grafius 1990).

Walking is also utilized for dispersal within and from potato fields. When released in fallow fields, male beetles were noted walking from the field more often than females (Weber and Ferro 1994). This discrepancy between the sexes is thought to be related to the necessity of males to immediately locate a suitable mate, where females may have less desire to mate when they can lay eggs fertilized in the previous season (Ferro *et al.* 1991). Likewise, it has also been noted that within the potato field, males are more likely to be found on the soil and the females are more often found on the plants (Weber and Ferro 1994).

Post-diapause beetles begin mating within 24 hours of emergence (Alyokhin and Ferro 1999). Host plants are not needed as a stimulus for mating, and many beetles mate directly in the overwintering site. However, male post-diapause beetles are again, more motile than their female counterparts, initiating flight more frequently (Voss and Ferro 1990b). Mating is not a necessity in the spring, since females are able to lay eggs that had been fertilized in the previous summer. The fecundity of mated and unmated female

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beetles does not differ, but the viability of eggs is higher in females that mate in spring (Ferro 1999). Feeding does affect reproduction. Unfed females do not lay eggs compared to female beetles that have fed post-emergence (Ferro et al. 1991). The offspring of these overwintering beetles largely determines the size of the following 1st generation (Voss and Ferro 1992).

Once in the new host plant field, CPB can move considerable distances by walking. These can exceed 0.65 km in 5 days (Follett et al. 1996). Post-diapause beetles characteristically aggregate on host plants, possibly attracted by either an intrinsic coordinated movement towards one direction or some other long-range aggregatory signal (Caprio and Grafius 1993).

Summer Emergence of Adults

Between July and early August, CPB prepupae burrow into soil to pupate. After pupation the new adults crawl up through the soil and emerge on the soil surface. Cumulative degree-days are correlated with departure from the pupation site (Alyokhin and Ferro 1999). Once emerged, many beetles migrate to new fields after 24 hours, if sufficient food or space is not available (Williams 1988, Alyokhin and Ferro 1999). Beetles aged 0-6 days post emergence usually move by walking and beetles aged 7-13 days post-emergence account for most of the local and migrational flight events (Voss and Ferro 1990b). Colonization of host plants by summer beetles is apparently non-aggregational and not governed by anemotactic behavior (Caprio and Grafius 1993). Field studies involving releases of newly emerged summer beetles illustrate that dispersal from an aggregation of beetles on a few plants is slow and occurs once nearby plants are

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defoliated (Williams 1988). The same study also indicated a significant orientation of dispersing beetles to the east and northeast direction, which may have been influenced by the nearby location of a forest. Movement and migration from potato plots takes the form of flight and is carried out more frequently and is maintained for longer periods of time by unmated male beetles, probably in search of mates (Alyokhin and Ferro 1999).

Pre-diapause Movement

Once beetles have emerged and dispersed, their behavior is mediated by environmental conditions. If the foliage is sufficient in both quantity and quality, and temperatures optimal for beetle activity, then beetles feed to increase energy reserves. If the foliage is not adequate, but temperature and photoperiod are still in the optimal range of 15 hours at 25°C, beetles may undertake local movements to find sufficient food (de Kort 1990, Hoy *et al.* 1996). However, if temperatures fall and photoperiod is shortened (14 hours, in Massachusetts) or if foliage begins to senesce, adult CPB may initiate diapause (Voss *et al.* 1988). When entering diapause, many CPB migrate to overwintering sites, usually woody areas that are adjacent to potato fields (Weber & Ferro 1993). Woody areas are not the only overwintering sites that have been chosen by the CPB. French *et al.* (1993) found that in the absence of woody vegetation, CPB chose to overwinter in a nearby ditch that consisted of high grasses. CPB can fly to these overwintering sites. Voss and Ferro (1990a) described this flight as a "direct" flight from field to edges of the surrounding woods, with no noticeable climb in flight angle. However, Noronha and Cloutier (1999) observed that much of the emigration from the potato field was accomplished through *en masse* walking events. It is not known what

causes beetles to orient towards these overwintering habitats. Dispersal patterns are integral to the CPB life cycle because location of protected habitats influences the probability of surviving the winter. Winter mortality rates can be quite high for the CPB in the northeastern United States (Voss and Ferro 1992).

In the overwintering sites, adult beetle burrows into the soil. Some beetles do not burrow and eventually die (Lashomb *et al.* 1984). Noronha (1998) suggests that the soil type plays an important role in the ability of the CPB to burrow, with smooth, hard-packed soil hindering the initiation of digging and leading to mortality of beetles. Beetles dig until an optimum depth is reached for the soil size and density (Noronha 1998). Moisture and temperature also impact the numbers of beetles that initiate digging from the soil surface and the depth to which the beetles burrow, with maximum occurance of digging at 16°C and at soil moistures of 50-55% (Noronha 1998). Overwintering beetles burrow to depths ranging from 7.6 and 12.7 cm in New Jersey sandy loam soils and beetles remain underground for the duration of the winter months (Lashomb *et al.* 1984). Death during overwintering has been suggested as the greatest source of mortality for CPB populations (Voss and Ferro 1992).

Beauveria bassiana

History

Beauveria bassiana (Bals.) Vuill., the causative agent of white muscardine disease, was first described in 1835 by Bassi de Lodi who successfully demonstrated the fungus's pathogenic nature against silkworm, *Bombyx mori* (Tanada and Kaya 1993). It

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was his discovery of *B. bassiana*'s infectious nature that led to the development of the germ theory of disease (Kendrick 2000). *B. bassiana* is categorized as a muscardine fungus, due to the characteristic mycotic stage exhibited on hosts after death when white, powdery-appearing hyphae cover the insect cadaver.

Beauveria species are found in the artificial class of Mitosporic fungi, class Deuteromycetes. Within the *Beauveria* genus, species are characterized by conidia borne singly on a zig-zag or denticulate rachis (Tanada and Kaya 1993, Humber 1997). Several species of *Beauveria* have been described, including *B. bassiana*, *B. tenella*, *B. brongniartii*, *B. amorpha,* and *B. velata* (Tanada and Kaya 1993). Strains of the same species have been shown to exhibit variations in virulence and pathogenicity towards insect hosts (Todorova 2000). *B. bassiana* can be distinguished from other *Beauveria* species by its globose conidia, which are usually larger than 3.5 µm in diameter (Humber 1997). *B. bassiana* hyphae grows optimally at 23- 25 °C and conidia require a relative humidity of 92% or higher to germinate.

Infection Cycle

The entomopathogenic nature of *B. bassiana* is quite extensive as it possesses one of the largest host lists of entomopathogenic, imperfect fungi (Tanada and Kaya 1993). This wide host range has enabled *B. bassiana* to become one of the most widely used fungal biological control agents. The infective unit of imperfect entomopathogenic fungi such as *B. bassiana* is the conidium (Gillespie 1988). Infection will usually occur through the integument, but infection has also been observed through the gut and the oral cavity (Broome *et al.* 1976).

Conidia of entomopathogenic Deuteromycetes will attach to an insect cuticle mainly through high hydrophobic forces between the conidial rodlet layer and the insect cuticle (Boucias *et al.* 1988). Binding of recognition proteins to substances on the insect cuticle and production of enzymes (esterase, lipase, *N*-acetylglucosaminidase) by the pre-germinating conidia may also be factors in attachment of conidia to the insect cuticle (Boucias and Pendland 1991). Once attached for a sufficient time and if conditions are suitable, a germ tube grows along the cuticle. Germinating conidia do require a useable source of carbon as well as a nitrogen source for hyphal growth, and the enzymes produced by the conidia may play a role in providing this nutrition (Boucias and Pendland 1991, Tanada and Kaya 1993). Chemical and physical stimuli may help orient a growing germ tube towards an appropriate infection site, but a well developed system of this type is not likely to be found in *B. bassiana*, as it can infect a large range of hosts, and does not necessitate a very specific infection site (St. Leger 1993). It has been shown, however, that conidia of *B. bassiana* that adhere to heavily sclerotized regions grow germ tubes until an easily penetrated area is reached (Pekrul and Grula 1979).

With *Metarhizium anisopliae*, another entomopathogenic deuteromycete, once a suitable penetration site is found, the germ tube differentiates into several specialized penetration structures, including appressoria, penetration pegs and penetrant hyphae. The germ tube forms appresoria, which serve as an attachment site and may provide the "fulcrum" that is needed for the remainder of the penetration process (St. Leger 1993). Appresoria also are the source for cuticle degradation and thus some nutrition for the fungal spore (St. Leger 1993). Formation of appresoria by *B. bassiana* has not been observed, with the exception of Vey and Fargues (1977) who observed formation on the

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cuticle of CPB. A combination of physical and chemical chitin degrading forces most likely allow for the penetration of the fungi into the insect hemocoel (Ferron 1981).

The insect cuticle provides a formidable mechanical barrier to the invading fungal spore. It may also further prevent successful breach through the melanization of cuticle cells surrounding the penetrating hyphae or through the increased production of lysozyme and other antimicrobial proteins (Vilcinskas and Gotz 1999). This penetration event can serve to increase the vulnerability of the insect to bacterial infections, as tissue and cuticle surfaces are breached (Vey and Fargues 1977). Once inside the insect body, the fungus produces hyphal bodies, which multiply by budding and circulate briefly in the hemocoel before invading muscle and fat tissues (Tanada and Kaya 1993). Production of hyphal bodies has been shown to reduce the number of insect hemocytes, thereby immunosuppressing the individual (Hung *et al.* 1993, Pendland *et al.* 1993).

Insect hemocytes recognize an invading fungus as non-self propagules and attempt to phagocytose the invading blastospores. This method is usually not effective against highly virulent fungi such as *B. bassiana* (Vey and Gotz 1986). Encapsulation of the invading fungal body may be more successful. Encapsulation occurs when hemocytes attach to the blastospore surrounding it in layers that eventually melanize, preventing the spore from producing further infective hyphae (Vey and Gotz 1986). The fungus may outgrow this barrier and cause subsequent death, or the insect may successfully prevent the infection, and the capsule can remain in the hemocoel for an extended period of time. Studies have shown that fungal hyphal bodies circulating in insect hemolymph may shed parts of their cell wall or lack sugar residues that are integral in the activation of insect immune systems (Pendland *et al.* 1993, Vilcinskas and Gotz 1999). In later stages of

mycosis, hyphal bodies have been found to possess a surface coat that mimics that of host cells, further evading detection by immune systems (Boucias *et al.* 1995). There may also be immunosuppressive properties of *B. bassiana* spores that contribute to reducing spread of hemocytes (Hung *et al.* 1992).

B. bassiana produces secondary metabolites which are also suspected to play a role in their pathogenicity, although conclusive proof of their involvement has not been found. *B. bassiana* produces two families of secondary metabolites, beauverolides and cyclosporins (Vilinskas and Gotz 1999). High levels of beauvericin, a beauverolide could not be found in the hemolymph of *B. bassiana*-infected corn earworms before and leading up to the time of death. Injections of beauvericin did not cause death; therefore Champlin and Grula (1979) concluded that this toxin most likely does not play a role in the virulence of *B. bassiana*. It may, however, serve an anti-microbial function. Bassianolide, another beauverolide has been shown to be toxic when injected into lepidopteran larvae (Boucias and Pendland 1988). Cyclosporins have also not been shown to have consistent insecticidal properties, but play a role in immune suppression in vertebrates (Vilinskas and Gotz 1999).

Insect death from *B. bassiana* can result from hemocyte reduction, soluble nutrient reduction, susceptibility to toxins produced by the fungus, or from bacterial septicemia caused by penetration events. Once the host has expired, the fungus grows saprophytically, mycelium eventually covers the insect body, and conidia are produced within a few days.

Colorado Potato Beetle Susceptibility to *Beauveria bassiana*

Due to its wide-spread pathogenic nature, *B. bassiana* has become a well-known and utilized pathogen for several major economic pests, including European corn borer (*Ostrinia nubilalis*), codling moth (*Laspeyresia pomonella*), Japanese beetle (*Popillia japonica*), chinch bug *(Blissus leucopterus)*, European imported cabbageworm (*Pieris brassicae*), the blueberry flea beetle (*Altica sylvia*) and the Colorado potato beetle (Tanada and Kaya 1993, Drummond and Groden 1996, 2000). *B. bassiana* has been used as a commercial control agent against CPB for 20 years, beginning with the development of Boverin in Russia (Ferron 1981). In addition, *B. bassiana* is found naturally in many of the soils where CPB is problematic, with low levels of natural mycoses occurring. Different growth stages of CPB are characterized with differential susceptibility to *B. bassiana*. Eggs are not susceptible to infection, making this stage an unsuitable target for foliar sprays (Long *et al.* 1998). Larvae are susceptible to infection, but mortality varies between larval instars (Ignoffo 1983, Fernandez 2001, Joergensen 2000) Changes in susceptibility between larval instars may have to do with the amounts of conidia acquired by different sized larvae, differences in immune responses between instars, changes in hemocyte volume, or differences in feeding rates and subsequent nutrition between instars (Seryczynska and Bajan 1974, Logan *et al.* 1985, Bauer *et al.* 1988, Fernandez 2001). Pupae are susceptible to soil inoculations, which can reduce the following adult generation by as much as 74% (Watt and LeBrun 1984). Adults show some susceptibility, but generally much lower than that observed for larval or pupal stages (Fargues 1972, Fargues 1991, Appendix).

Several other physical factors affect *B. bassiana* infectivity including temperature, humidity and UV light. Temperature levels are thought to be the most important external factor in determining an insect's susceptibility to a pathogen, as well as determining the rate of multiplication of the pathogen within the host body, B. Bassiana growing optimally at 25°C (Waitanabe 1987). *B. bassiana* needs a relative humidity above 92.5% in order to germinate and begin the infection process (Walstead *et al.* 1970). UV radiation from sunlight has also been shown to cause conidia to lose viability more quickly than those that are protected from the sun (Daoust and Pereira 1986, Joergensen 2000).

The objective of my thesis is to examine in further detail, the susceptibility of adult Colorado potato beetles to *Beauveria bassiana*. In my first manuscript, I have investigated the effect of physiological age and state on susceptibility of adult beetles, focusing on pre-diapause and post-diapause beetles, as well as overwintering beetles. In my second manuscript, beetle behavior was studied to determine if adult beetles possess mechanisms that allow them to detect and avoid *B. bassiana* sporulated cadavers on the soil surface. I also looked at the relationship between density of sporulated cadavers and the resulting likelihood of emerging adult infection. The information obtained from this thesis will be incorporated into an existing simulation model of primary and secondary transmission of *B. bassiana* in the potato ecosystem.

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CHAPTER 2: ADULT COLORADO POTATO BEETLE SUSCEPTIBILITY TO *B. BASSIANA*

Abstract

The susceptibility of non-diapausing, pre-diapausing, and post-diapausing adults of the Colorado potato beetle to *Beauveria bassiana* was studied. In an assay comparing trials between a): laboratory reared, non-diapausing beetles and b): field collected, post-diapausing beetles, survival of *B. bassiana* treated beetles was higher for non-diapausing adults, but control mortality was higher for post-diapausing adults. In a similar assay with field collected pre-diapausing and post-diapausing beetles, survival of *B. bassiana* treated individuals was higher for post-diapause adults and control mortality was higher for post-diapausing adults. In a third experiment, the effect of time from eclosion from the pupal case on susceptibility of laboratory-reared, non-diapausing adults was evaluated. A trend of increasing susceptibility up to 60-degree days (base 10° C) can be seen, and a significant decline of mortality occurs after 125-degree days. These changes in susceptibility may be related to the tanning process of the cuticle and the likelihood of *B. bassiana* conidia attaching at different stages of cuticle formation, as well as the cuticle's ability to prevent infection. The effect of sublethal and lethal doses were evaluated on the survival of overwintering beetles, and it was found that *B. bassiana* dose had no effect on the proportions of beetles sporulating or dying.

Introduction

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say), is the most destructive insect pest of potatoes in the northeastern United States. Problems with insecticide resistance, environmental contamination, and worker and public health have generated interest in developing more ecologically sound methods for managing this pest. The entomopathogenic fungus *Beauveria bassiana* (Deuteromycetes) has been utilized as a commercial control agent against CPB in some parts of the world for the past 20 years (Ferron 1981, Tanada and Kaya 1993, Feng *et al.* 1994). Different growth stages of CPB exhibit differential susceptibility to *B. bassiana* with larval stages being the most vulnerable (Watt and Lebrun 1984, Fargues 1991, Long *et al.* 1998). Assessment of *B. bassiana* efficacy against adults has received less attention because adults are generally considered to be much less susceptible to infection than larvae, however adult mortality can be a key factor to reducing future populations (Anderson *et al.* 1988).

As the overwintering stage, adult CPB progress through several different physiological states (Leather *et al.* 1993). In the northern Maine potato-growing region, CPB usually experience one complete generation per year (Drummond and Groden 1996). In the midsummer, pre-diapause adults emerge from pupation sites in the soil and feed heavily on potato leaves to prepare for overwintering. Individuals may mate, and within a week of emergence from pupation sites, burrow into the ground to overwinter. In the spring, these beetles (now post-diapause) emerge from the soil and colonize new potato fields. Through pre-diapause and post-diapause stages of development, different physiological processes are occurring: the newly emerged adult sclerotizes its cuticle, builds up fat bodies, and reduces the proportion of water in the body to successfully

overwinter (Chapman 1971). Overwintering beetles must maintain cold-hardiness (including the potential of super cooling) and preserve an appropriate water balance (Lee 1991). The post-diapause adult must successfully regain water balance, develop its reproductive organs, and resume active behavior and mating (Chapman 1971). Each physiological stage also exhibits a repertoire of behaviors that are characteristic of that stage. Some of these behaviors lead to increased opportunity for natural interactions with *B. bassiana* in the soil. Changes in beetle physiology combined with changes in behavior may influence their susceptibility to *B. bassiana*. In this study, we examined whether changes in physiological state of adult CPB impacts their susceptibility to *B. bassiana*. We also investigated the effect of different *B. bassiana* treatments on survival of overwintering beetles.

Materials and Methods

Insects and Foliage

Colorado potato beetle adults were either reared from a laboratory colony, which was supplemented annually by field collections from the University of Maine's Rogers Farm in Stillwater, ME, or collected directly from fields at the same farm. Potato fields used for beetle collection received 7 weekly sprays of the copper hydroxide fungicide Champ Formula 2 ® (Agtrol Chemical Products, Houston, TX) for *Phytophtora infestans* control and no other pesticides. All adult beetles were maintained in paper cups (300ml) at 25±1°C and a 16:8 light-dark cycle and fed fresh greenhouse grown potato foliage

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(*Solanum tuberosum* L., variety Russet Burbank or Kennebec) daily. For laboratory

reared experimental beetles, egg masses were collected daily from cups. At hatch,

approximately 20 larvae were placed in paper cups (300 ml) and raised under the same

environmental conditions as adults. At the end of the fourth stadium, when larvae

stopped feeding (entered the prepupal stage), they were either placed in a metal tin filled

with approximately 6 centimeters of moistened Fafard 3-B professional formula soil

(Agawam, MA) into which they were allowed to burrow (diapause and overwintering

assays), or, if exact age from pupation needed to be known, were placed in petri dishes

lined with pieces of moist paper toweling (age susceptibility assay). Pupation containers

were checked daily for the presence of new adults.

B. bassiana Applications and Assay Procedures

Beauveria bassiana strain GHA was obtained as dried conidia from Mycotech,

now Emerald BioAgriculture (Butte, MT) and plated on Sabouraud dextrose agar (SDA)

(Difco Laboratories, Detroit, MI). Colonies were sub-cultured on SDA, and single, axenic

subcultures (grown for 10 days at 25±1°C, stored at 4±1°C for not more than 60 days)

were utilized for these experiments. Conidia were harvested from these plates with a

sterile loop and suspended in an aqueous solution of 0.1% Tween 20 (JT Baker,

Phillipsburg, New Jersey). Concentrations of conidia were determined using a

hemocytometer, and solutions were diluted as necessary with 0.1% Tween 20. Beetles

were sprayed with either *B. bassiana* or control (0.1% Tween 20 alone) solutions using a

Burkhard Computerized Spray Apparatus® (Hertfordshire, England) at 55.2 kPa with a

diaphragm setting of 6. Two plates of SDA were sprayed at the same time as *B. bassiana*

treated beetles to obtain accurate rates of conidial density and viability at the time of the spray. For all three experiments, each replicate treatment of beetles was treated at the same time with the same *B. bassiana* solution. In all experiments, ≥98% of conidia had formed germ tubes after 18h at 25°C, indicating viability.

After spray treatments in pre- diapause and post-diapause and age susceptibility studies, each beetle was then put in an individual 60 x 15 mm petri dish with moistened filter paper and maintained at 25±1°C and a 16:8 light-dark cycle for 14 days. At 24-hour intervals, each beetle was checked for survival, and if alive, fed fresh greenhouse-grown potato foliage. Dead beetles were placed in a container at 100% RH and monitored for signs of *B. bassiana* sporulation. Sporulation was determined by observance with the unaided eye of *B. bassiana* hyphal growth on the outside of the insect. Occurrence of mortality, time (days) to death, sporulation, and time (days) from beetle death to sporulation were recorded.

Susceptibility of Non-, Pre- and Post-diapause Adults to *B. bassiana*

Experiment A

A 2x2 complete randomized design (parametric survival analysis) was used to compare *B. bassiana* susceptibility between post-diapause and non-diapausing adult CPB with 57 beetles per treatment combination. Post-diapause beetles were collected from the field between June 6 and 11, 2001. Non-diapausing beetles were raised from eggs produced from the laboratory culture. Non-diapausing beetles are considered as such due to their maintenance at a constant temperature and photoperiod that does not initiate diapause. Beetles from each group (non- and post-diapause) were assigned to either a

control or *B. bassiana* treatment and placed 10 per dish in 6 petri dishes (9 cm diameter) per treatment. No formal randomization protocol was used to delegate which beetles in each of the cups were assigned to treatments, but each cup contributed an equal number of beetles to each treatment. Control beetles were sprayed with a 0.1% Tween 20 solution and *B. bassiana* treated beetles were sprayed with a solution of conidia in 0.1% Tween 20 (654.4 conidia/mm^2). Beetles were treated with *B. bassiana* using 2 sprays of the sprayer, but all beetles were treated at the same time and with the same solution. Beetles were censored at 15 days.

Experiment B

A 2x2 complete randomized design was used to compare *B. bassiana* susceptibility between pre- and post-diapause adult CPB with year as a covariate in parametric survival analysis. Six hundred post-diapause field beetles were collected from the University of Maine's Roger's Farm between June 6 and 11, 2001 and on June 3, 2002. Beetles were stored in 300 ml paper cups with lids (25 per cup), given field foliage and maintained at 10±1°C in order to slow physiological development. Approximately 3 weeks after initial collection, post-diapause beetles were warmed by placing them at 14±1°C for 3 days, followed by 19±1° C for 3 days, before moving to 25±1°C. Between July 17 and 19, 2001 and July 8 and 10, 2002, 600 newly emerged pre-diapause adults were collected from the same field as the post-diapause beetles. Two hundred ninety-five beetles of each physiological stage (pre- and post-diapause) were randomly allocated to either a control or *B. bassiana*. Control beetles were sprayed with a 0.1% Tween 20 solution, and *B. bassiana* treatment beetles were sprayed with conidia in 0.1% Tween 20

(632 ± 41.6 conidia/mm^2). Each year, eight consecutive runs of the sprayer were used to spray all *B. bassiana* beetles. All beetles were sprayed with the same stock *B. bassiana* solution. This experiment ran for 28 days, and beetles surviving the duration of the study were censored at 29 days.

Data Analysis

Beetles that died and sporulated within 4 days of the spray (2 in the control and 3 in the *B. bassiana* treatments) were excluded from analyses in 2002. Based on previous assays, these beetles died and sporulated too quickly to have resulted from experimental treatments. In addition, the outward morphology of the *B. bassiana* was quite different from that of the laboratory strain used. For each assay, parametric survival density functions were fit using the Weibull distribution ($\alpha=0.05$) (JMP 2001) and used to detect changes in survival with physiological state, *B. bassiana* treatment, and in Experiment B, year was incorporated as a covariate. Using this analysis, survival is measured by the proportion of beetles dying and incorporating the time to death of these beetles to determine overall survival. Nominal logistic regression ($\alpha=0.05$, JMP) was used to detect differences in proportion sporulation between beetle groups, and two-way analysis of variance using treatment and age as factors ($\alpha=0.05$) was used to determine time to sporulation differences between beetle groups. Sporulation for this and subsequent analyses is calculated as the proportion of dead beetles that showed signs of sporulation. For proportion sporulation, in instances where control groups showed no mortality, Fisher's Exact Test ($\alpha=0.05$) was used to detect significant differences between treatments.

Impact of Age on Susceptibility of Non-diapausing Adult Beetles to *B. bassiana*

Beetle Treatment

The impact of age on susceptibility of non-diapausing beetles was assessed with a 2x2 complete randomized block design with 6 replicates over time treated as blocks in parametric survival analysis. Rearing protocols were developed that allowed for staggered production of adult beetles from the laboratory colony over a 10-day period of time such that beetles representing a range of known ages from adult eclosion were available to be treated at a set time. When the desired age range in adult beetles was achieved, beetles were placed into 15-degree day age groups, (this was the shortest interval at which beetles were collected) and within these groups, were randomly allocated to either the control (0.1% Tween 20) or *B. bassiana* treatment group (0.1% Tween 20 and *B. bassiana* (374.13 ± 85.21 conidia/mm^2)). A lower concentration was used in this experiment compared to earlier experiments, because although preliminary data showed that the LC_{50} level for 195-degree day old beetles was ca. 630 conidia/mm^2 (Appendix), it was hypothesized that younger beetles would be more susceptible to *B. bassiana* treatments. The concentration was therefore lowered in an effort to maintain ca. 50% mortality across all age classes. Beetles were placed in petri dishes for spraying with a maximum of 15 beetles per dish. Variable numbers of sprayer runs were used between replications, as the number of available beetles for treatment varied between replicates, but beetles in each replicate were treated in the same order (youngest- oldest) and with the same *B. bassiana* solution within each replicate. Beetles surviving to the

end of the assay were censored at 20 days. This experiment was replicated six times with 112-632 beetles per trial, with a total of 1875 beetles across all trials.

Data Analysis

Parametric survival analysis based upon the Weibull distribution (α=0.05) (JMP 2001) was used to detect changes in survival with beetle age, *B. bassiana* treatment, and replicate as factors. Nominal logistic regression (α=0.05, JMP) was used to detect significant differences in proportion sporulation between beetle ages. Two-way analysis of variance using *B. bassiana* treatment and age as factors (α=0.05) were performed to detect differences in days to sporulation for age groupings.

Effects of Sublethal and Lethal Doses on Overwintering Adult Colorado Potato Beetles

Beetle Treatment

A 3x3 complete randomized block design was used to assess the impact of *B. bassiana* treatment and stage in the overwintering process on adult CPB mortality. Six hundred seventy-five beetles were collected from the field August 21 and 22, 2002, and randomly assigned to one of three treatment groups: control (0.1% Tween 20), sublethal *B. bassiana* concentration (11.2 conidia/mm^2 in 0.1% Tween 20), or lethal *B. bassiana* concentration (627.2 conidia/ mm^2 in 0.1% Tween 20). All beetles in each treatment group were treated with the same solution at the same time. Following treatment with the appropriate solution on August 28, 2002, beetles were randomly allocated to one of three blocks of field cages with one cage for each of three collection times: fall, spring, or

summer within each block, resulting in 25 beetles per cage in three replicate cages per treatment combination (*B. bassiana* dose x collection date). Beetles were then released into appropriate cages and stalks of field grown senescing potato foliage in water-filled test tubes (30 ml) were placed in each cage to provide beetles access to food until they burrowed into the soil. Cages consisted of a 13 cm deep section of metal stovepipe, 25.5 cm in diameter, pushed 3 cm into the soil and covered with mesh screening. Cages were monitored every two days and foliage was replaced as needed until all beetles had successfully burrowed into the soil or died. Beetles that died on the soil surface were taken to the laboratory and placed in a high humidity chamber to monitor for sporulation. Cages were left intact throughout the winter.

Fall and spring beetles were collected from the soil (described below) between November 8 and 13, and May 22 and 23, 2002, respectively. Summer beetles were allowed to emerge naturally between May 23 and June 25, 2002. Overwintering cages were replaced with larger emergence cages (91 x 73 x 15 cm). These cages had mesh tops and metal flashing sides and were designed to capture beetles as they emerged. Cages were pushed 5 cm into the soil. Beakers with water and potato foliage were placed in the cages to ensure that any emerging beetles had appropriate food supplies. Live beetles emerging within the cage were collected every 3 days beginning when the first emerging beetle was observed on June 3. When no additional emergence had been observed in any cage for one week, the soil under and around the cages was sampled to retrieve any additional live and dead beetles.

To retrieve beetles from the soil in fall, spring and after natural emergence, the soil in a 37.16 square meter area surrounding the original circular cage was collected to a

depth of 30.48 cm and sifted with a 5 mm screen to recover beetles and beetle parts. Both

dead and live beetles and beetle parts were taken back to the laboratory. Dead beetles

were checked for signs of *B. bassiana* sporulation, which if not evident were then held at

approximately 100% RH, 25°C and monitored regularly for *B. bassiana* growth. Live

beetles from the fall collection were kept in a sealed Tupperware® tub (38 x 26 x

13.5cm) lined with moistened paper towels at 10°C to simulate overwintering conditions.

These beetles were checked on a monthly basis for death and sporulation. Live beetles

collected from the soil in spring and summer were held and monitored as described above

for bioassays.

Data Analysis

Differences between *B. bassiana* treatments and collection dates on proportion

mortality (as a percent of the number of beetles burrowed: 25 minus the number that died

on soil surface) were analyzed with a two-way weighted ANOVA, weighted by sample

size ($\alpha=0.05$). A similar analysis was performed to determine significance between

treatments for the proportion mortality as a percentage of the number of beetles actually

recovered. Data for these analyses was transformed using the square root of the arcsine

of the proportion. Two-way weighted ANOVAs were also performed to determine any

significant differences between treatment effects and the number of dead beetles before

burrowing into the soil, the total number of beetles recovered, and the number of beetles

that sporulated.

Results

Susceptibility of Non-, Pre- and Post-diapause Adults to *B. bassiana*

Experiment A

B. bassiana treatment resulted in a significant overall decrease in beetle survival, and in an increase in sporulation ($\chi^2_4>9.49$, p<0.001 for both response variables) (Fig. 2.1 A, B). Physiological state of the beetle (non- or post-diapause) alone did not significantly impact survival (measured as a combination of proportion mortality and days to death), however, there was a significant interaction between *B. bassiana* treatment and physiological state on survival ($\chi^2_4>9.49$, p=0.006). Post-diapause beetles experienced less *B. bassiana* induced mortality than non-diapause beetles (Fig. 2.1A). In addition, a significantly higher ($\chi^2_4>9.49$, p=0.02) percentage of the *B. bassiana* treated non-diapause beetles that died sporulated (97.1%) than was observed for similarly treated post-diapausing beetles (89.4%) (Fig 2.1 B). Non- and post-diapause beetles did not differ in regards to the time it took them to die after *B. bassiana* treatment, but control treated non-diapause beetles died more quickly than did post-diapause beetles (Fig. 2.1 C). No sporulation was observed in any control group.

Experiment B

B. bassiana treatment significantly decreased beetle survival and increased proportions of dead beetles sporulating, (Fig. 2.2, Fig. 2.3 A) ($\chi^2_4>9.49$, p<0.001 for both response variables). There were significant differences (regardless of physiological state)

A

Proportion mortality

0.6

0.4

0.2

0.0

c

b

a

a

Non-diapause Post-diapause
(lab) (field)

B

Control
B. bassiana

Proportion Sporulation

1.0

0.8

0.6

0.4

0.2

0.0

a

b

Non-diapause Post-diapause
(lab) (field)

C

Control
B.bassiana

Days to death

14

12

10

8

6

4

2

0

b

b b

a

Non-diapause Post-diapause
(lab) (field)

D

Control
B.bassiana

Days to sporulation

14

12

10

8

6

4

2

0

a

a

Non-diapause Post-diapause
(lab) (field)
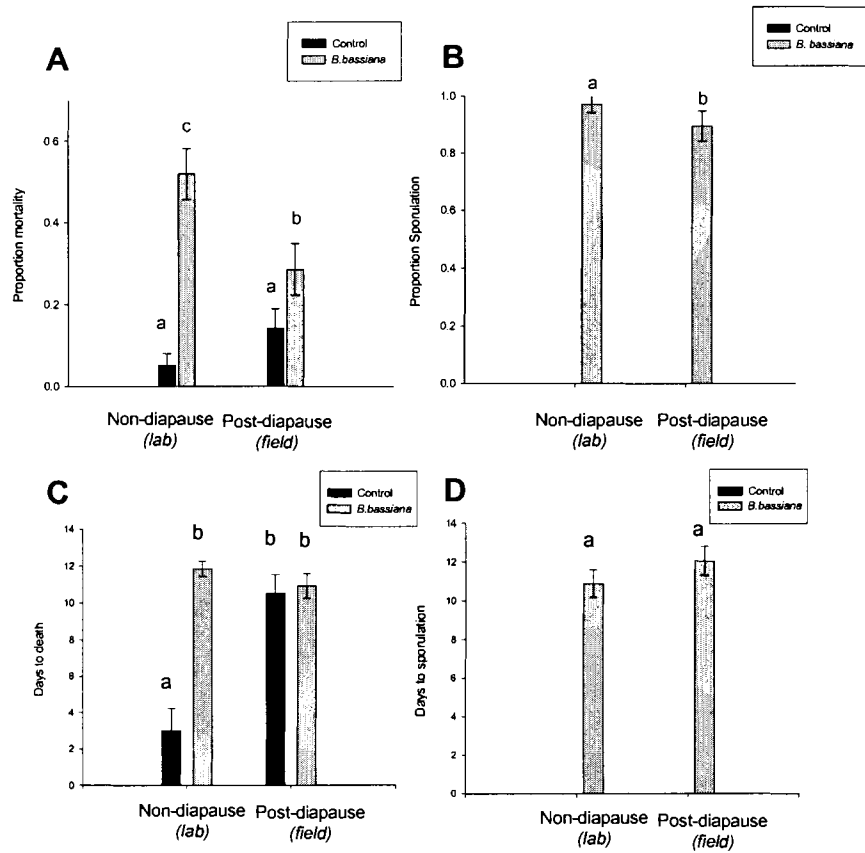
Figure 2.1 Non- and post-diapausing beetle responses (± SE) to *B. bassiana* treatments, including proportion mortality (**A**), proportion of dead beetles sporulated (**B**), days to death (**C**) and days from death to sporulation of dead *B. bassiana* treated beetles (**D**). Letters in A and C correspond to logistic pairings at a Bonferroni adjusted level of $\alpha=0.025$.

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between years in survival ($\chi^2_4 > 9.49$, $p < 0.001$), proportion sporulated ($\chi^2_4 > 9.49$, $p = 0.000$) and days to sporulation ($F_{(1,4)} = 64.1403$, $p < 0.001$), with higher proportions of dead and sporulated beetles in 2002 than 2001, and longer times to death and sporulation in 2002 than 2001. There were significant interactions between *B. bassiana* treatment and physiological state on survival ($\chi^2_4 > 9.49$, $p < 0.0001$), however responses differed between years (Fig. 2.2). In 2002, mortality was significantly greater in both control and *B. bassiana* treated post diapause beetles than in the control and treated pre-diapause beetles for that year, and greater than either group of beetles in 2001. However, mortality did not differ between pre- and post diapause beetles in 2001 and *B. bassiana* treatment mortality did not differ between either of the 2001 beetles and pre-diapause 2002 beetles. In 2002, pre-diapause beetles had significantly greater times to death (17.48 ± 6.85 days) than post-diapause beetles (13.41 ± 7.29 days) (Fig. 2.3 B).

No control beetles sporulated in either pre-diapause or post-diapause beetle groups in 2001, although low levels of sporulation were observed in the control beetles that died in 2002 (Fig. 2.3A). In 2002 pre-diapause beetles had a significantly higher occurrence of dead beetles sporulating ($\chi^2_4 > 9.49$, $p < 0.001$) and took longer to sporulate than post-diapause beetles ($F_{(1,4)} = 11.9508$, $p < 0.001$)(Fig 2.3A), however, no differences were observed between 2001 treated beetles.

The response of pre-diapause field beetles to *B. bassiana* (Experiment B, both years) was compared to responses of non-diapausing lab beetles (Experiment A). Because beetles in Experiment B were censored at 28 days, and beetles in Experiment A were censored at 14 days, data from Experiment B was used only to 14 days, after which beetles were considered censored. Non-diapausing laboratory beetles experienced

significantly more mortality, more sporulation of dead beetles, a shorter time to death than did pre-diapausing field beetles ($\chi^2_4 > 9.49$, p<0.001 for all variables).

Impact of Age on Susceptibility of Non-diapausing Adult Beetles to *B. bassiana*

Due to high control mortality in trials 1 and 2, beetles aged 0-30 degree days were excluded from analyses of data. *B. bassiana* treatment significantly decreased survival and increased sporulation of beetles ($\chi^2_8 > 15.5$, p<0.001) (Fig. 2.4, Fig. 2.5), and beetle age significantly affected beetle survival ($\chi^2_8 > 15.5$, p=0.0062) (Fig. 2.4). However, there was a significant age by *B. bassiana* treatment interaction on survival ($\chi^2_8 > 15.5$, p=0.0446). Control mortality generally decreased with age but the same trend was not evident in *B. bassiana* treated beetles. Mortality did not change with age class, with the exception of an obvious decrease in mortality at 125+ degree-days for *B. bassiana* treated beetles. (Fig. 2.4). *Post hoc* groupings were used to analyze this decline in beetle mortality after 125-degree days. Proportion mortality was significantly lower in beetles who were 125 degree days or older ($\chi^2_8 > 15.5$, p=0.03). Significant quadratic relationships exist between beetle age and days to death and days to sporulation (Fig. 2.6). As beetle age increased, the time it took for beetles to die decreased until beetles were 60-degree days old, then time to death subsequently increased ($F_{(1,7)}$=8.269, p= 0.026). Time to sporulation showed a similar trend of decreasing, then increasing with time ($F_{(1,7)}$=20.165, p=0.004). Of the youngest beetles, 22.9% of the dead beetles sporulated, 32.3% of the middle aged-beetles that died sporulated, and 33.8% of the oldest beetles sporulated.

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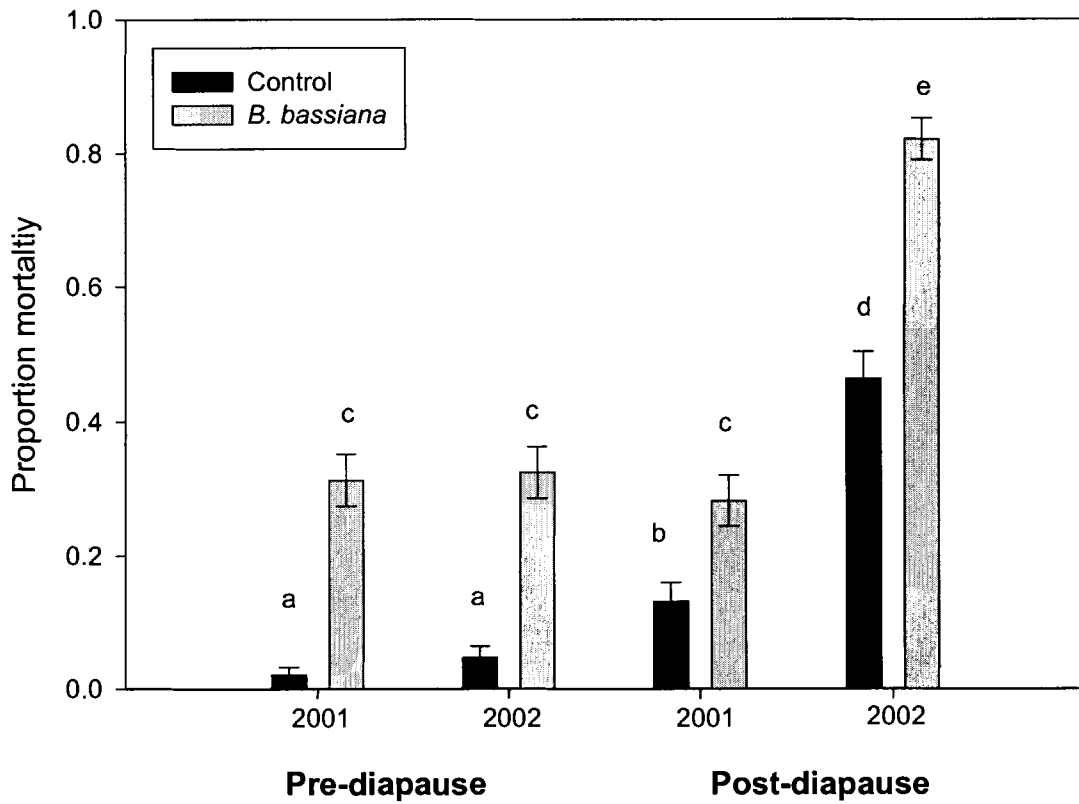
Figure 2.2 Proportion mortality (± SE) of pre-
and post-diapause Colorado potato beetles
(field populations) treated with *B. bassiana*.
Letters correspond to results of logistic
regression pairings at a Bonferroni adjusted
level of α= 0.017.
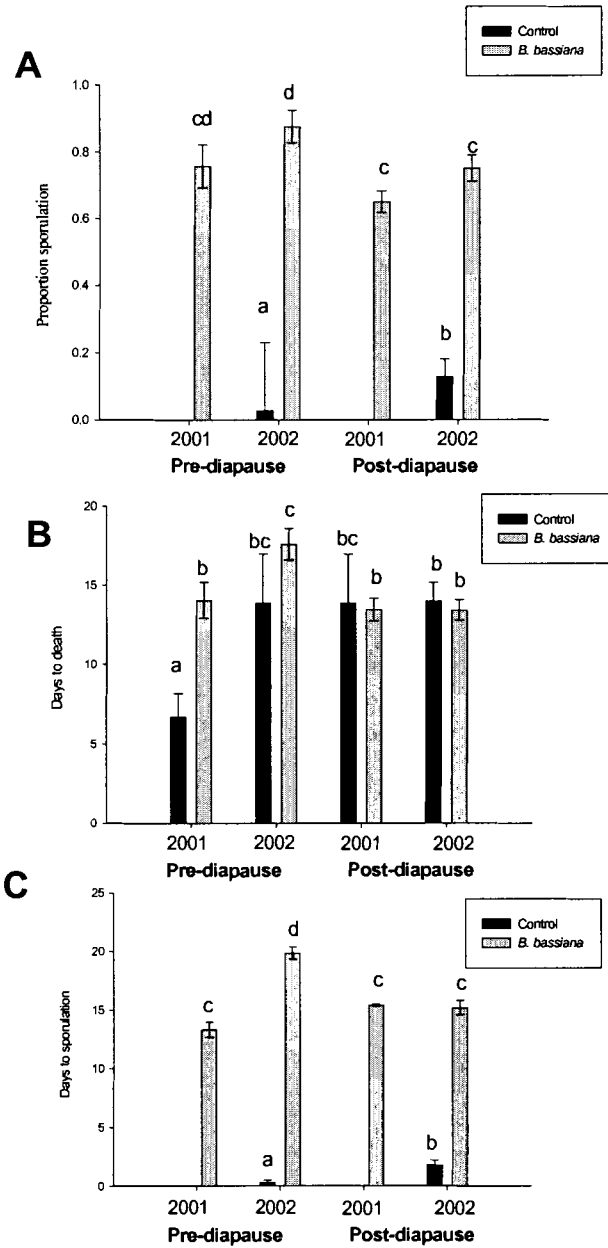
Figure 2.3 Pre and post-diapausing beetle responses (± SE) to *B. bassiana* treatments, including proportion of dead beetles sporulated **(A)**, days to death **(B)** beetle sporulation of *B. bassiana* treated beetles **(C)**. Letters correspond to results of logistic regression pairings and ANOVA pairings at a Bonferroni adjusted level of $\alpha=0.017$.
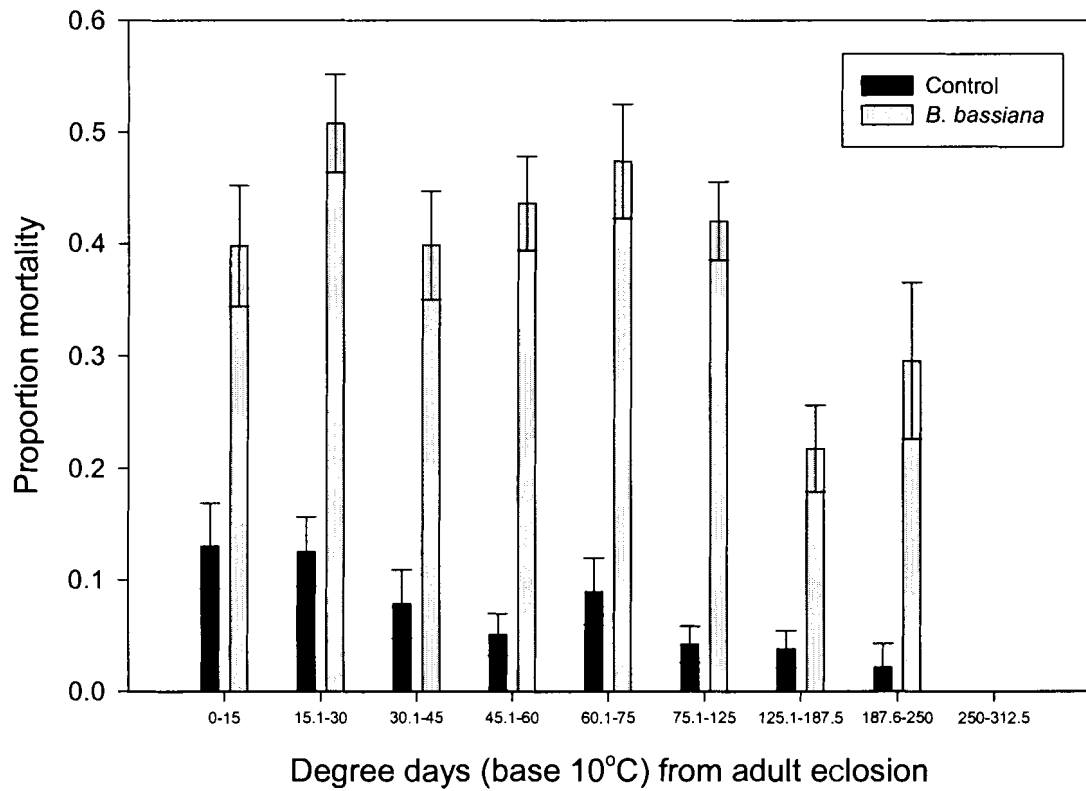
Figure 2.4 Proportion mortality (± SE) in control versus. *B. bassiana* treated Colorado potato beetle adults relative to age (degree days,base 10°C) from adult eclosion.
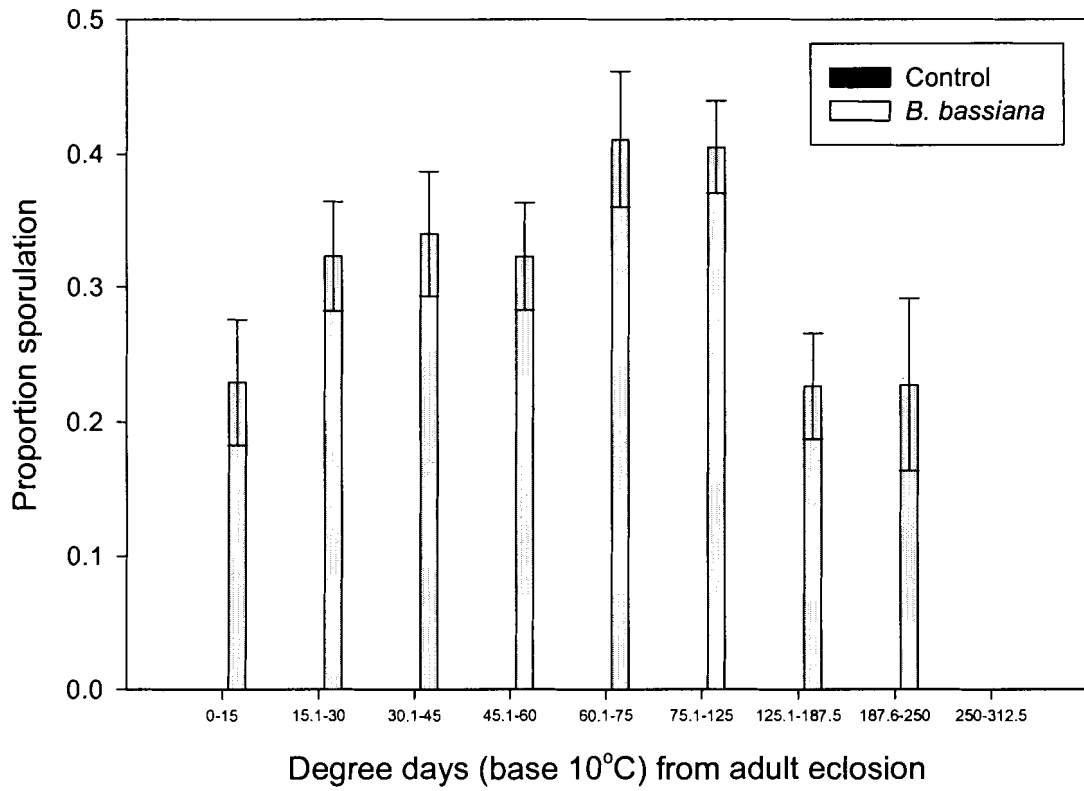
Figure 2.5 Proportion sporulation (± SE) in control versus. *B. bassiana* treated Colorado potato beetle adults relative to age (degree days, base 10°C) from adult eclosion.

Effects of Sublethal and Lethal Doses on Overwintering Adult Colorado Potato Beetles

B. bassiana treatment (control, sublethal, lethal) had no significant effect on the proportion of dead beetles, as a fraction of either total beetles recovered ($F_{(2,10)}$ = 0.1595, p=0.8529) or as a fraction of total beetles burrowed ($F_{(2,10)}$ = 0.1372, p=0.8721) (Fig 2.7A, B). B. bassiana treatment also did not significantly affect the number of beetles that died on the soil surface ($F_{(2,10)}$ = 0.2266, p=0.7998), the total number of beetles recovered ($F_{(2,10)}$ =227.1284, p=0.6817) or the total number of beetles that sporulated ($F_{(2,10)}$ =1.3699, p=0.2824). There were significant effects of time on proportion mortality ($F_{(2,10)}$ = 19.6463, p=0.002) and sporulation ($F_{(2,10)}$ = 38.514, p=0.0431) with the highest levels of mortality in the spring and highest levels of sporulation in the fall (Fig. 2.8). There were no significant interaction effects on any variable for the treatment and time interaction.

Discussion

Decreased insect resistance to disease can be a result of several factors. Stressors in the insect environment can result in a decrease in insect immunity, which could subsequentially increase insect susceptibility to disease (Baines 1992). Low food quality and quantity is a stress factor that has been shown to increase insect susceptibility to B. bassiana (Ramoska and Todd 1985, Furlong and Groden 2003). In addition, other factors such as temperature, mechanical forces, and some insecticides can have significant effects on the susceptibility of insects to pathogens (Brey 1994, Quintela and McCoy 1998,
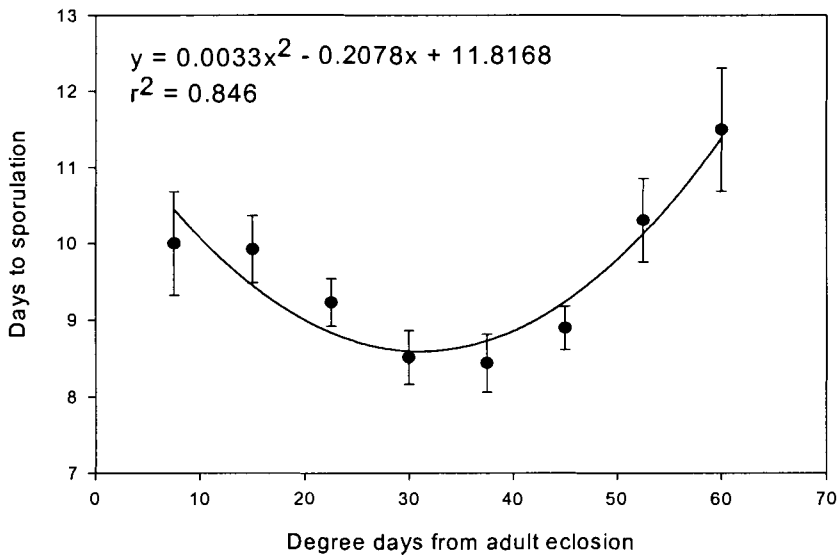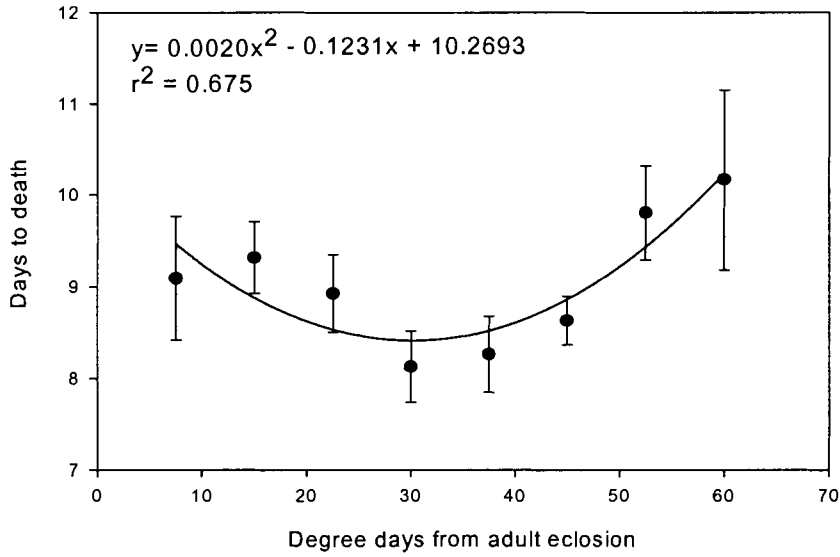
Figure 2.6 The influence of adult Colorado potato beetle age (degree days, base 10°C, from adult eclosion) and *B. bassiana* treatment on days to death **(A)** and days to sporulation **(B)**.
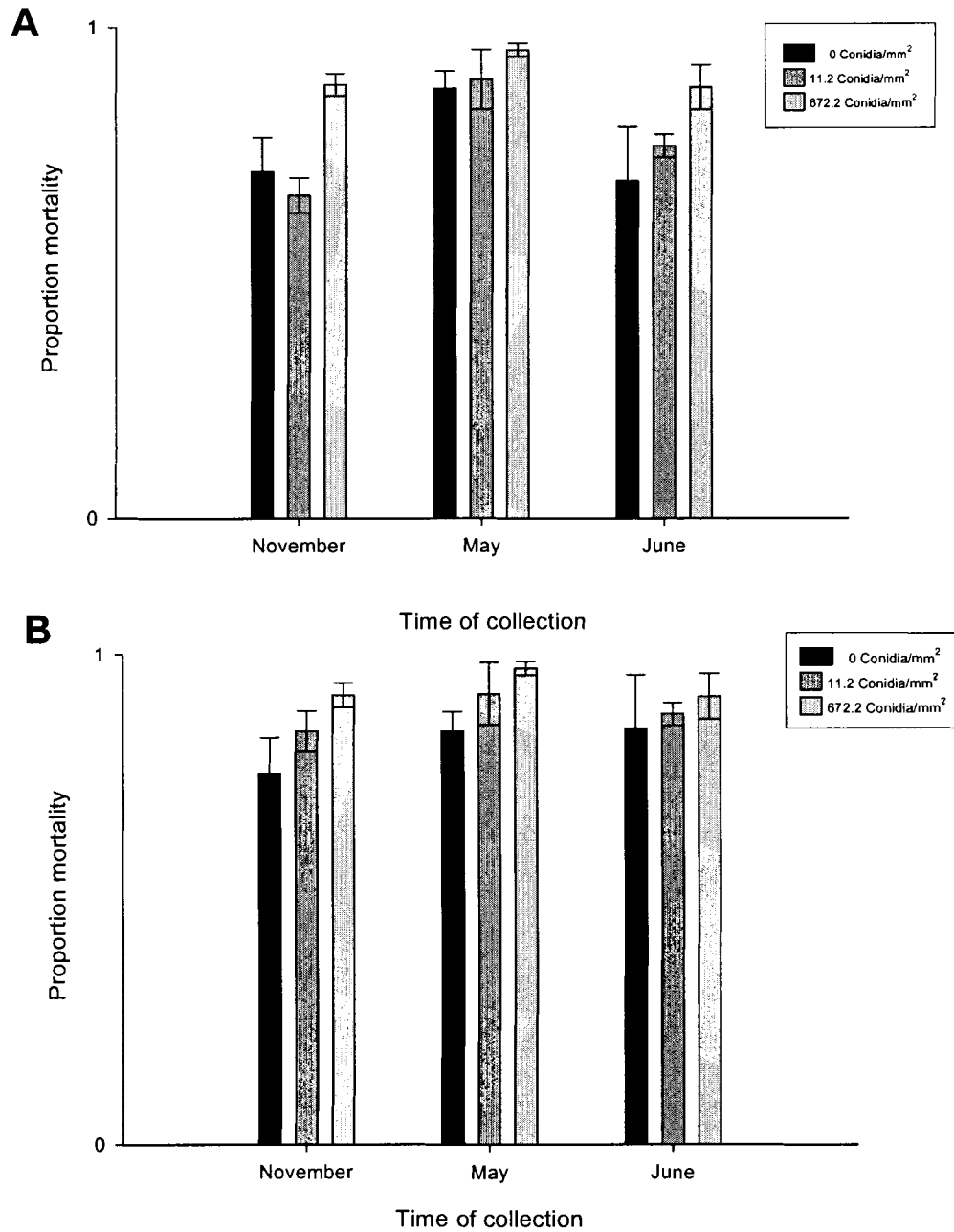
Figure 2.7 Pre- and post-winter mortality of overwintering Colorado potato beetles, expressed as proportion of total collected **(A)** and proportion of total beetles that burrowed at each site (number released - number of dead on soil surface)**(B)**.
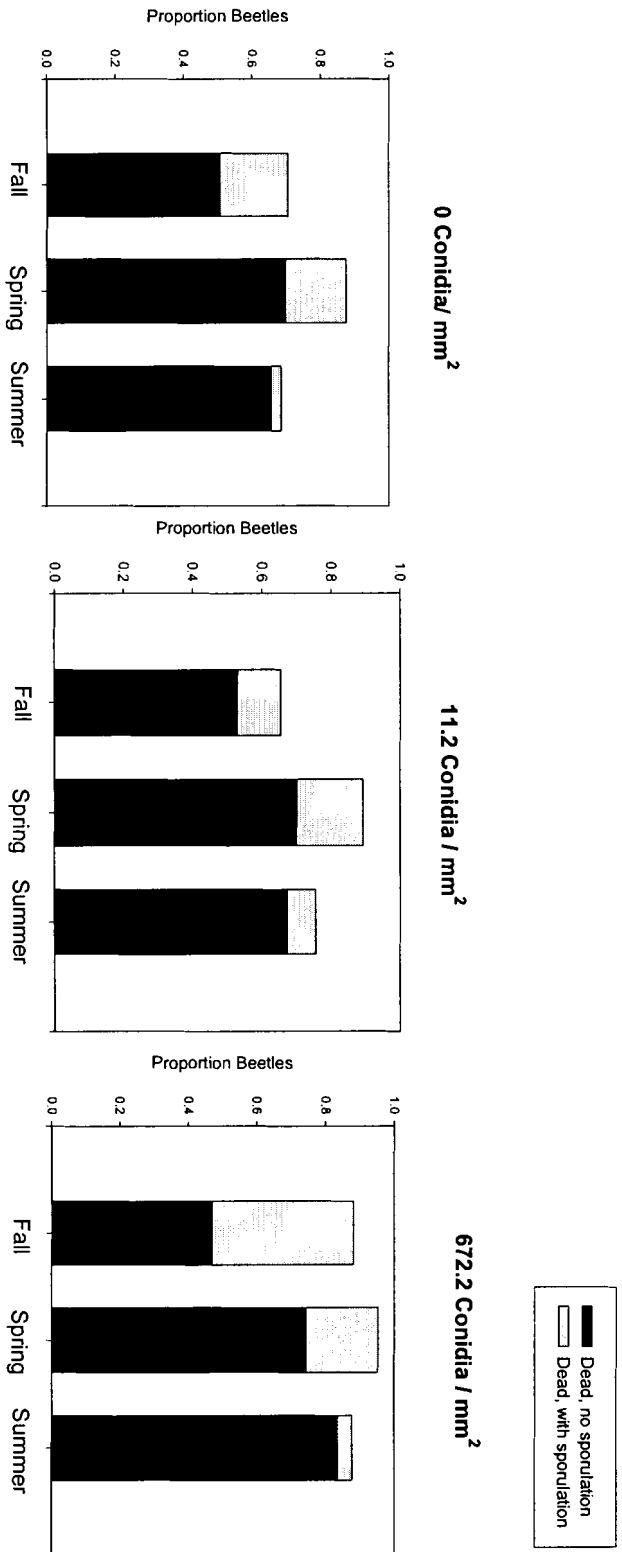
Figure 2.8 Proportion of recovered *B.bassiana* treated Colorado potato beetles which died and sporulated pre- and post-winter diapause.

Furlong and Groden 2001).

This study focused on physiological state and age as factors that may affect susceptibility of adult Colorado potato beetles to *B. bassiana*. The sensitivity of the same insect species to a pathogen may depend on the developmental stage (Vey and Gotz 1986). In the first set of experiments, although post-diapause beetles experienced significantly more *B. bassiana* induced mortality than pre-diapause beetles in 2002, the overall effect of the *B. bassiana* treatment relative to the control was greater for pre- and non- diapause beetles as compared to post-diapause beetles. Post-diapause beetles experienced much higher control mortality in both experiments than either the non- or pre-diapausing beetles. This high level of control mortality is probably associated with the stress that can accompany recovery from the overwintering state and recovery from low temperatures (Bale 1991). Non- and pre-diapausing beetles did not experience high control mortality, and the relative effects of *B. bassiana* were greater. In experiment A, not only did non-diapause beetles have higher proportion mortality, they also had a higher occurrence of sporulation, also indicating a higher susceptibility in non-diapause beetles. In experiment B, proportion mortalities varied between stages between each year. Overall, 2002 exhibited greater proportion mortalities than 2001, as well as greater proportions of sporulated beetles. Post-diapause beetles also showed greater variation in mortalities between years than pre-diapause beetles did.

In comparison to 2001, 2002 was a wetter spring and summer (20.68 inches of precipitation in March-August, as compared to 19.11 inches in 2001). In addition, during beetle collection in 2002, several diseased sporulated cadavers were noted on the soil surface, and small proportions of control group beetles sporulated, showing probable

evidence of exposure to disease in the field. Beetles already having been exposed to higher levels of inoculum experienced greater levels of mortality and sporulation in this year. The greater variability observed between year in control mortality and *B. bassiana* treatment in post-diapause than pre-diapause is likely due to a greater sensitivity to environmental interactions with pathogens.

In cellular defense, insects must produce whole blood cells to either phagocytize or encapsulate a foreign particle, and depending upon the numbers of blood cells available at one time, the defense may be overwhelmed by infection (Salt 1970). Post-diapause beetles most likely vary in the amount of resources they possess when they emerge from overwintering, and energy is needed to produce hemocytes to fight infections. Since they are able to feed directly on foliage, pre-diapause beetles may have more reserves and are better able to produce infection fighting hemocytes, thus making their response to *B. bassiana* in this experiment less variable.

The insect cuticle is an important barrier to the invasion of fungal pathogens. In the experiment evaluating the impact of adult beetle age on susceptibility of non-diapause beetles, we hypothesized that different ages exhibiting different levels of cuticle maturation would differ in their susceptibilities to *B. bassiana*. At pupal case emergence, the beetle elytra are soft and unpigmented and gradually over the course of 4-7 days gains rigidity and color (personal observations). Tanning is the process by which the new insect cuticle sclerotizes. This process involves the transformation of water-soluble arthropodin to insoluble protein (Chapman 1971). This change in hydrophobicity could affect the attachment of *B. bassiana* conidia to the cuticle, as hydrophobic attraction of conidia rodlets to the normal insect epicuticle is an important factor in *B. bassiana*

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pathogenesis (Boucias *et al.* 1988). The tanning and subsequent additional cuticle deposition can take up to three weeks, but is usually shorter (Chapman 1971).

Although proportion mortalities did not significantly increase or decrease with age up to 125-degree days, sporulation levels increased with beetle age to approximately 60-degree days, after which they decline. During the time period between 0 and 60 degree days beetles may show increased susceptibility because their cuticle is tanning and becoming more hydrophobic, but the full cuticle has not been deposited. Follett and Hilbeck (1995) found full elytral hardness in CPB after 3 days at 28°C or 54-degree days, thus, beetles in this study most likely had fully tanned cuticles at 60 degree days. After tanning, cuticle development is characterized by the addition of material between the existing cuticular layers (Chapman 1971). After 60-degree days, we most likely see a decrease in overall susceptibility as additional material is added, providing a increased mechanical barrier against the invasion of fungal pathogens.

However, there are other factors that may be influencing these beetles's susceptibility to *B. bassiana*. Cuticular waxes, which are deposited at ecdysis, and then continually during the intermolt period of an insect's life, contain chemicals that inhibit the growth and penetration of microorganisms (David 1967). Changes in wax deposition, or more consistent deposition after 60-degree days may attribute to the decrease in susceptibility of adult CPB to *B. bassiana*, but does not explain the lower susceptibility of younger adults, as wax deposition is more likely to be incomplete soon after molting (Chapman 1971).

Differences between sexes in susceptibility may also explain these trends. Male and female insects have been found to have differential responses to control methods,

such as nematode treatments and insecticides (Bovin and Belair 1989, Abd-Elghafar *et al.* 1990). Development of reproductive organs of the adult CPB, which occurs between 34 and 51-degree days may also be related to the decrease in susceptibility after 60-degree days in this study (Alyokhin and Ferro 1990).

The trend of changing susceptibility with age revealed in our study suggests that the ages during which adult beetles would encounter *B. bassiana* in its natural soil reservoir are the ages when they are the least susceptible to disease. Our laboratory observations have shown that newly eclosed beetles remain in the soil until approximately 22.5-degree days post-eclosion. Once on the soil surface, these beetles find plants usually within minutes (pers. obs.). Feeding on the plants, beetles are least likely to come into contact with conidia in the soil, and this time period corresponds with the period of highest susceptibility. Once beetles return to the soil to overwinter, they are usually older than 60-degree days. (Beetles in the age assay were exposed to a lower concentration of conidia than were beetles in the diapause studies, so mortality results are not comparable across experiments.)

Bauer *et al.* (1998) found a decrease in susceptibility of *Pieris brassicae* to parasitization by *Cotesia glomerata* with increasing instar age. It was hypothesized that this reduction in susceptibility with age could be due to the altered ability of hemocytes to encapsulate the egg due to either differences in cell differentiation between ages of insect, or due to total number of hemocytes available, as hemocyte volume increases with increasing instar. Additional hemocyte volume with increasing ages, or a more efficient immune system could also explain changes in susceptibility with age.

Surviving infections by fungal pathogens has been reported to reduce an insect's resistance to cold temperatures, increasing overwintering mortality (Ferron 1978). In this study, *B. bassiana* treatment of overwintering beetles had no effect on mortality of adults at any time. Cantwell (1986) also found that soil applications of *B. bassiana* had no significant effect in increasing the mortality of overwintering CPB adults. However, Watt and Lebrun (1984) did reduce pupal and the following adult populations by as much as 74% with soil applications of *B. bassiana* conidia. Studies by Watt and Lebrun were not conducted with overwintering adults, as this and that of Cantwell were, thus the difference in physiological state of the beetle may account for the differences in results. Overall mortality, regardless of treatment was highest in the spring for experimental beetles, and although the total mortality values for summer were less than spring, decomposition of beetles may have resulted in an underestimate of the number that were recovered in the summer. The amount of beetles that sporulated decreased as time progressed, suggesting that those infected with *B.bassiana* in the fall are more likely to die during the fall and winter and not after emergence in the spring.

Beauveria bassiana based pest management strategies and spray plans for CPB frequently focus on targeting larvae. These results indicate that there are differences in susceptibility between physiological stages of the adult beetle and these should be considered when developing management strategies with this pathogen. If adults are to be targeted, our results suggest that treatments should be applied to the newly emerged pre-diapause beetles within 125 degree days of emergence from the soil, as they show a consistent high level of mortality to *B. bassiana* when compared to mortalities of other ages. Early treatment of newly emerged adults would also be most effective for reducing

oviposition. Based upon this study, treatment of overwintering beetles with *B. bassiana* does not appear to be a viable control plan.

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CHAPTER 3: HORIZONTAL TRANSMISSION OF

BEAUVERIA BASSIANA BETWEEN CADAVERS

AND ADULTS OF *LEPTINOTARSA DECEMLINEATA*

Abstract

Behavior and infection of newly emerged adult Colorado potato beetles

(*Leptinotarsa decemlineata*) in the presence of *Beauveria bassiana* infected cadavers

were studied to determine the likelihood of transmission of disease as beetles emerge

from the soil and colonize host plants. In 2001, arenas were constructed to accommodate

recessed potted potato plants surrounded with soil to simulate the field environment. *B.*

bassiana killed and sporulated adult beetles were placed in varying patterns surrounding a

release point for healthy beetles in the center of the arena. Laboratory reared, newly

eclosed beetles were buried just below the soil surface at the release point and were

observed for 30 minutes as they emerged and colonized one of four plants. Beetle

movements were recorded relative to a superimposed grid consisting of 5 x 5 cm squares.

The study was replicated in 2002 using a similar grid in a potato field. In both the arena

and field, emerging beetles showed no preference for movement in any cardinal direction,

and direction was not affected by the presence or absence of *B. bassiana* sporulating

cadavers, nor did the presence of cadavers affect the time taken to colonize a plant or the

distance traveled by a beetle. Relative humidity (RH) was the only significant abiotic

factor affecting the distance traveled to the plant, with longer travel distances as the RH declined.

The likelihood of emerging adults contacting sporulated cadavers on the soil surface was quantified at different cadaver densities, and a curvilinear relationship exists between density of cadavers on the soil surface and square root number of encounters, with the number of encounters increasing with increasing cadaver density. Proportion mortality and sporulation also have a curvilinear relationship with density, both values increasing with cadaver density. Thus, beetles show no avoidance behavior to sporulated cadavers, and density of sporulated cadavers on the soil surface is an important factor in the horizontal transmission of *B. bassiana* from cadavers to healthy, newly emerging adults.

Introduction

Beauveria bassiana is a fungal pathogen of many insects, and its development as a biological control agent has been of considerable interest as problems with insecticide resistance become more prevalent in today's agroecosystems (Huang *et al.* 1995, Stewart *et al.* 1997, Olson *et al.* 2000, Zhao *et al.* 2000). Applications of *B. bassiana*, an indigenous soil fungus, to agricultural systems have been shown to reduce pest populations, such as those of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Campbell *et al.* 1985, Hajek *et al.* 1987, Anderson *et al.* 1988). Since its first noted adaptation to the cultivated potato in 1859, the Colorado potato beetle (CPB) has developed resistance to most insecticides, which makes it the most destructive insect pest of potatoes in the Northeastern United States, including the state of Maine.

A discrete application of *B. bassiana* in a potato field can reduce CPB

populations, however, because *B. bassiana* conidia are sensitive to UV light and rainfall,

and because temperature and humidity play an important role in *B. bassiana* virulence,

the direct effects of the primary spray can be short lived (Walstead *et al.* 1970, Daoust

and Pereira 1986, Inglis *et al.* 2000). In contrast, secondary infection of healthy CPB

individuals by dead, sporulating cadavers on the soil surface can be an important source

of infection, and horizontal infection from cadavers has been illustrated in other

insect/entomopathogen systems (Brown and Nordin 1982, Thomas *et al.* 1995, Furlong

and Pell 2001, Long *et al.* 2000a). Mathematical models demonstrate that without

transmission of a disease, infection prevalence approaches a level of zero, illustrating the

necessity of transmission if a fungal disease is to be sustained in the environment

(Anderson and May 1981).

To determine how significant horizontal transmission is in reducing pest

populations, the likelihood of healthy insects contacting diseased insects needs to be

assessed. Long *et al.* (2000a) found that CPB prepupae were more likely to come in

contact with sporulated cadavers on the soil surface as cadaver density increased, and

subsequent mortality and sporulation increased with cadaver density. The adult stage,

while typically not as susceptible as the larval stages (Fargues 1972, Appendix) can

acquire infections from contact with sporulating cadavers. *B. bassiana* sprays that have

targeted larval stages produce sporulating cadavers on the soil surface. These cadavers

can coincide with the emergence of new adults from pupation sites in the soil. Once the

adults emerge, they travel along the soil surface in order to find an acceptable host plant.

The purpose of this study was to investigate the relationship between cadaver density and contact and subsequent infection of newly emerged adult CPB, a potentially important factor in horizontal transmission of the disease in CPB populations. This study also attempted to discern if there is any avoidance behavior exhibited by adult beetles when exposed to sporulated cadavers on the soil surface.

Methods

Insects and Foliage

Colorado potato beetle adults were reared from a laboratory colony which was supplemented annually by field collections from the University of Maine's Rogers Farm in Stillwater, ME. Potato fields used for beetle collections received seven weekly sprays of the copper hydroxide fungicide Champ Formula 2 [®] (Agtrol Chemical Products, Houston, TX) for *Phytophtora infestans* control and no other pesticides. All adult beetles were maintained in paper cups (300 ml) at 25±1°C and a 16:8 light-dark cycle and fed fresh greenhouse grown potato foliage (*Solanum tuberosum* L.) (variety primarily Kennebec, with the exception of Delta Gold for density studies) daily. For laboratory reared experimental beetles, egg masses were collected daily from cups. On hatch, approximately 20 larvae were placed in paper cups (300 ml) and raised under the same conditions as adults. When larvae reached the fourth instar and stopped feeding (entered the prepupal stage), they were placed in petri dishes lined with pieces of moist paper toweling. Pupation containers were checked daily for the presence of newly metamorphosized adults.

Cadaver Production

Beauveria bassiana strain GHA was obtained as dried conidia from Mycotech, now Emerald BioAgriculture (Butte, MT), and plated on Sabouraud dextrose agar (SDA) (Difco Laboratories, Detroit, MI). Colonies were sub-cultured once on SDA, and for each experiment one of these single, axenic subcultures (grown for 10 days at $25\pm1^\circ$C, stored at $4\pm1^\circ$C for not more than 60 days) were utilized. Conidia were harvested from these plates with a sterile loop and suspended in an aqueous solution of 0.1% Tween 20 (JT Baker, Phillipsburg, New Jersey). Concentrations of conidia were determined using a hemocytometer, and solutions were diluted as necessary to achieve 2×10^7 conidia/ml in 0.1% Tween 20. Beetles were sprayed with either *B. bassiana* or control (0.1% Tween 20 alone) solutions.

To produce cadavers for these experiments, either adults or late second instar larvae from the laboratory culture were sprayed with a *B. bassiana* solution using a Burkhard Computerized Spray Apparatus® (Hertfordshire, England) at 55.2 kPa with a diaphragm setting of 6. Two plates of SDA were sprayed at the same time as *B. bassiana* treated beetles to obtain accurate rates of conidial density and viability at the time of the spray. In all experiments, $\geq$98% of the conidia had formed germ tubes after 18h at $25\pm1^\circ$C, indicating viability. Sprayed beetles were held in a Tupperware® container (38 x 26 x 13.5 cm) lined with moist paper toweling, and fed fresh potato foliage on a daily basis. Once sporulated, cadavers were removed from this arena, and stored in a petri dish with moistened filter paper over which a layer of cheesecloth was laid. The cadavers were placed on the cheesecloth to prevent their direct contact with the moistened filter paper. Cadavers were stored at $4\pm1^\circ$C and used within 3 days of sporulation. At the end

of each day of observations, at least one cadaver of the cadavers used in the experimental

arena was sampled for viability of conidia. A sterile needle was touched to the side of the

cadaver and streaked onto an SDA plate. The plate was held at 25±1°C for 18 hours to

ensure that the cadaver's viability was not less than 70%.

Determining Avoidance Behavior of Adult Colorado Potato Beetles to *B. bassiana*

Infected Cadavers

Beetle behavior was studied in a controlled environment in 2001, in which beetle

movements were recorded in a wooden arena, designed to replicate a field setting. The

same behavior was studied in the summer of 2002, but in a field setting with a modified

version of the previous year's design.

2001

In June 2001, arenas were constructed that consisted of a wooden box with an 80x

65 cm² plywood bottom and 14 cm high sides, elevated on 30 cm legs such that the box

sat off of the ground to accommodate recessed potted potato plants. Four 19.5 cm

diameter holes were cut into the plywood bottom of the box, and 19 cm plastic plant pots

were attached with silicon caulking. Contact paper was placed on the bottom of the box,

to provide an easily sterilized surface. Metal flashing was attached to the inside sides of

the box with Liquid Nails® (Macco, Cleveland, OH), to deter beetles from climbing this

surface (Fig. 3.1).
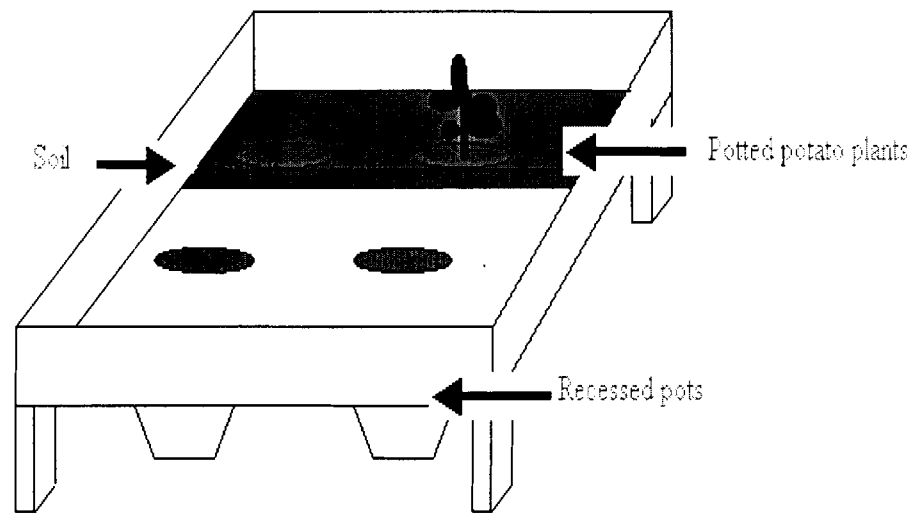
Figure 3.1 Diagram of behavioral arenas used for study in 2001.

Every 3-5 days of the assay, new greenhouse-grown potato plants were placed in each of the four recessed pots. A layer of sieved (1 cm^2 mesh) Caribou gravelly loam collected from the University of Maine's Aroostook Farm in Presque Isle, Maine, was placed in the bottom of the box to a depth of approximately 5 cm, to create an even surface with the top of the recessed pots such that there was no barrier to beetle movement. The soil was moistened with water from a spray bottle before each assay. North-south oriented pots were spaced at a distance of one foot to mimic distances between plants in a conventional potato field. Each plant center was located 30 cm from the center of the arena, the average distance that prepupae travel before burrowing into soil in the field (Long *et al.* 2000b). A 5.08 cm square grid made of cotton string was superimposed over the top of the arena to provide a framework for quantifying beetle movements.

Individual beetle movement was monitored within the arenas relative to the potted potato plants and the presence (and proximity) or absence of sporulating cadavers. Temperature and relative humidity were monitored using a HOBO® Pro Series Datalogger (Onset Computer Company, Bourne, MA) placed underneath the arena. A beetle that had eclosed primarily within the previous 2-5 days (250 beetles between 1 and 5 days, 4 between 6 and 10, and 50 between 30 and 50) was placed just under the soil in the center of the arena and was observed by either a human observer or a video camera. In the case of human observation, the observer remained directly overhead of the beetle as it traveled, and, using a stopwatch and a grid diagram, followed the path the beetle took to colonize a host plant. Measurements of the number and location of that the beetle passed through to reach a plant, the time the beetle took to reach a plant, the amount of time

spent in each square, and which of the four plants the beetle colonized were recorded. Beetles were considered to have colonized a plant when its entire body was on any part of the plant. Beetles that did not move from the starting point after 20 minutes, who left the arena by climbing up the side, and those that, once started moving, ceased movement for more than 20 minutes were removed and were not used for the analyses of total time for plant colonization or specific plant colonized. Observations made with the video camera were corrected for parallax error so that the upper grid lines corresponded with the actual spacing on the bottom of the grid. The video camera taped the trial for at least 20 minutes, after which, if no movement was observed, a new trial was begun.

Arenas were rotated between individual beetle trials, so that any orientation to a cardinal direction could be monitored. After a beetle had reached a plant it was then put in an individual 60 x 15 mm petri dish with moistened filter paper, fed fresh greenhouse-grown potato foliage and maintained at 25±1°C and a 16:8 light-dark cycle for 21 days. At 24-hour intervals, each beetle was checked for mortality, and if alive, fed fresh foliage daily for 28 days. Dead beetles were placed in a container at 100% RH and monitored for signs of *B. bassiana* sporulation. Sporulation was indicated by the observance with an unaided eye of *B. bassiana* hyphal growth on the outside of the insect. Occurrence of mortality and sporulation, and time (days) to death and time (days) from beetle death to sporulation were recorded.

Control runs of the experiment consisted of the arena with the potato plants and no sporulating cadavers. For *B. bassiana* treatments sporulated cadavers were arranged in u-shapes surrounding the beetle release point, so that there was one possible path for a beetle to travel if travel was to be completed in a *B. bassiana* free square (Fig 3.2). The

opening in the U-shaped pattern was rotated between assay runs to avoid bias from

beetles traveling in one direction. Sixty-seven adult beetles were exposed to the control

treatment in 2001, and fifty-three were exposed in the *B. bassiana* treatment. Of them, 27

observations were not fully utilized because of the lack of beetle movement in the pre-

established time limit.

2002

A similar design was utilized for the field study in 2002 as was used for the arena study in

2001. However, in 2002, arenas consisted of a 121.92 x 91.44 cm bottomless frame that

was placed over two plants in two adjacent rows of a potato plot at the University of

Maine's Roger's Farm, Stillwater, ME. The frames accommodated a similar grid as the

2001 arenas with 391 5.08 x 5.08 cm squares per frame. Assay runs were performed and

environmental conditions were monitored as described in 2001, however all observations

were made by human observers in 2002, and beetles were not placed under the soil at the

start position, as the soil was too compact. A total of 125 beetles were observed for the

control treatment, and 59 were observed for the *B. bassiana* treatment. Of these, 20

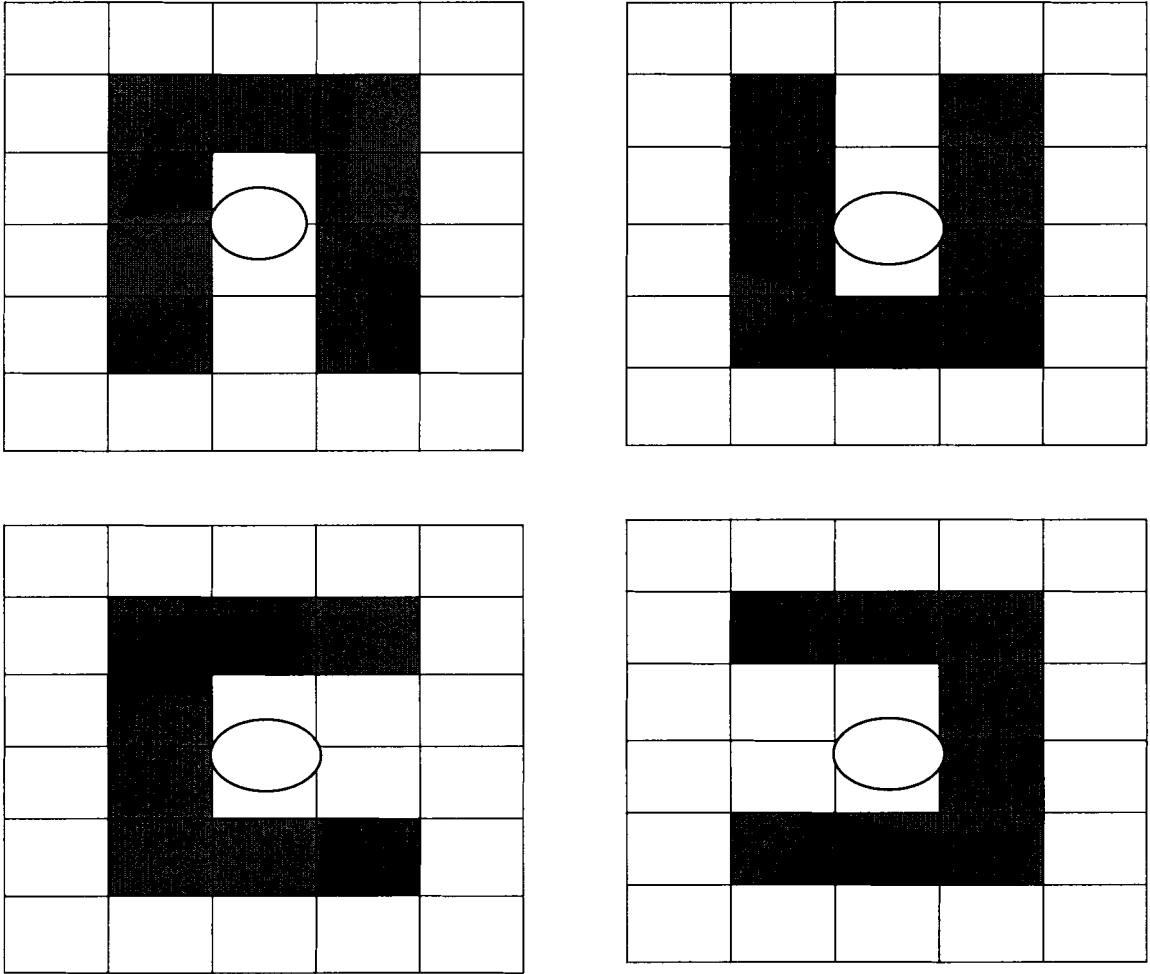observations were not utilized due to lack of beetle movement.

Figure 3.2 Diagram of cadaver placement surrounding release point in arena trials. Circles represent release point of adult beetles, shaded squares represent cadaver placement.

<u>Data Analysis</u>

To test for beetle preference in colonizing host plants, a chi-square analysis was performed to determine if beetles were colonizing one of the four plants in a percentage significantly different than 0.25. To test if beetles were avoiding areas with *B. bassiana* cadavers present, the number of beetles passing through *B. bassiana* occupied squares relative to unoccupied squares was compared. In 2001, 10 grid squares surrounded the release point, and in 2002, 12 grid squares surrounded the release point (the 2002 grid was larger). A probability of the beetles randomly passing through a *B. bassiana* filled square was calculated, based on the number of squares in each trial that contained a *B. bassiana* sporulated cadaver. Chi-square analysis was used to determine if the actual frequency of beetles passing though *B. bassiana* filled squares deviated significantly from this frequency. Two way ANOVAs were performed to determine if there were any significant differences between beetle age, temperature, relative humidity and *B. bassiana* treatments in relation to total time for beetles to colonize plants (entire duration of trial) and in relation to total number of grid squares the beetle passed through to colonize a plant (a measure of directness). In both ANOVAs, data was log-transformed and analysis was conducted at an alpha level of 0.05.

The Relationship Between Cadaver Density and *B. bassiana* Infection

<u>Density Studies</u>

To determine the relationship between cadaver density on the soil surface and adult beetle infection, the same arenas used in the behavioral assays in 2001 were used.

A 693 cm^2 area surrounding the beetle release point was marked out for cadaver placement in the appropriate densities. Sporulated larval cadavers were placed on the soil surface at densities of 0, 0.0173, 0.0289, 0.0606, and 0.2352 cadavers per cm^2. Non-sporulated larval cadavers were also placed in corresponding densities for control trials. An adult beetle was placed in the center of the arena and was allowed to colonize one of four potato plants. The number of cadavers encountered by the beetle was recorded and each encounter was also given a numerical rating. A rating of "1" was determined by a contact with a cadaver that lasted less than one second and involved either a leg or antennae touching the cadaver only. A "2" contact lasted between 1 and 2 seconds and involved an adult beetle coming in contact with a cadaver on the underside of its body. A rating of "3" was given to beetles that had contact that lasted longer than 2 seconds and involved sustained contact with some part of the beetle's head, thorax or abdomen. Duration of encounter was also recorded, along with temperature and relative humidity. All beetles were kept in 60 x 15 mm Petri dishes and held for 2 weeks to check for subsequent death and sporulation.

Data Analysis

Linear regression was utilized to determine relationships between cadaver density and square root transformed number of encounters. Linear regression was also utilized to determine relationships between log10 transformed cadaver density and proportion mortality and sporulation. Two-way ANOVAs were used to determine significant factors impacting time to plant colonization as well as factors impacting the average rating of contact in any given trial.

Results

Determining Avoidance Behavior of Adult Colorado Potato Beetles to *B. bassiana*

Plant Colonization

One hundred ninety-two beetles were exposed to the control treatment in assay

runs in both years, and 112 were exposed to *B. bassiana* treatments. In 2001 and 2002,

neither beetles in the control treatment, nor beetles in *B. bassiana* treatments colonized

one of four plants at a frequency significantly different from an expected 0.25 ($\chi2$, 3df

=7.00; $\chi2$, 3df = 4.06, respectively for control, $\chi2$, 3df =0.08; $\chi2$, 3df =1.54 respectively

for *B. bassiana* treatment), indicating no impact of cardinal direction on beetle

movement.

In both 2001 and 2002 beetles did not demonstrate any avoidance of sporulating

cadavers. In 2001, the calculated probability of a beetle passing through a grid square

surrounding the release point was 0.10. The calculated probability of a beetle passing

though a *B. bassiana* square was 0.22. Beetles did not significantly deviate from the

expected frequency for the *B. bassiana* treatment ($\chi2$, 1 df = 5.25). In 2002, the

calculated probability of a beetle passing though a grid square surrounding the release

point was 0.08. The calculated probability of a beetle passing though a *B. bassiana*

square was 0.15. Beetles again did not significantly deviate from this frequency ($\chi2$, 1

df= 0.08,).

<u>Beetle Activity</u>

In 2001, both adult and larval cadavers were used. In 2002, only larval cadavers were used. In regards to the area of each grid square (25.81 cm^2), adult cadavers occupy approximately 2.52% of the square area, and larval cadavers occupy approximately 1.19% of total area within the grid square. Out of 112 beetles that were exposed to cadavers in both 2001 and 2002, 21 beetles had contact with at least one sporulated cadaver. Of those 21, 28.6% died, and 19% of the dead subsequently sporulated. Of the 91 beetles that did not encounter a cadaver, 15.4% died and none sporulated.

Analysis of environmental impacts on beetle movement revealed significant effects of relative humidity and year on the distance traveled ($F_{(1,6)}$ = 11.1698, p=0.0010 and $F_{(1,6)}$ = 10.6732, p=0.0015 respectively), and the time traveled from emergence from the soil to colonizing their host plant ($F_{(1,6)}$ = 9.3998, p=0.0024 and $F_{(1,6)}$ = 36.4134, p<0.0001 respectively). However, regression revealed the relationship between relative humidity and distance and time traveled to be weak, with RH explaining only 3.3% of the variation in distance traveled ($F_{(1,255)}$=12.112, p=0.002, r^2= 0.033) (Fig 3A), and less than 1% of the variation in the time spent traveling ($F_{(1,255)}$= 7.080, p=0.073, r^2 0.009) (Fig 3.3B). The time a beetle spent within control squares as opposed to *B. bassiana* filled squares was analyzed and presence of *B. bassiana* did not significantly alter the amount of time a beetle spent in a square ($F_{(1,6)}$ = 0.0982, p=0.09).

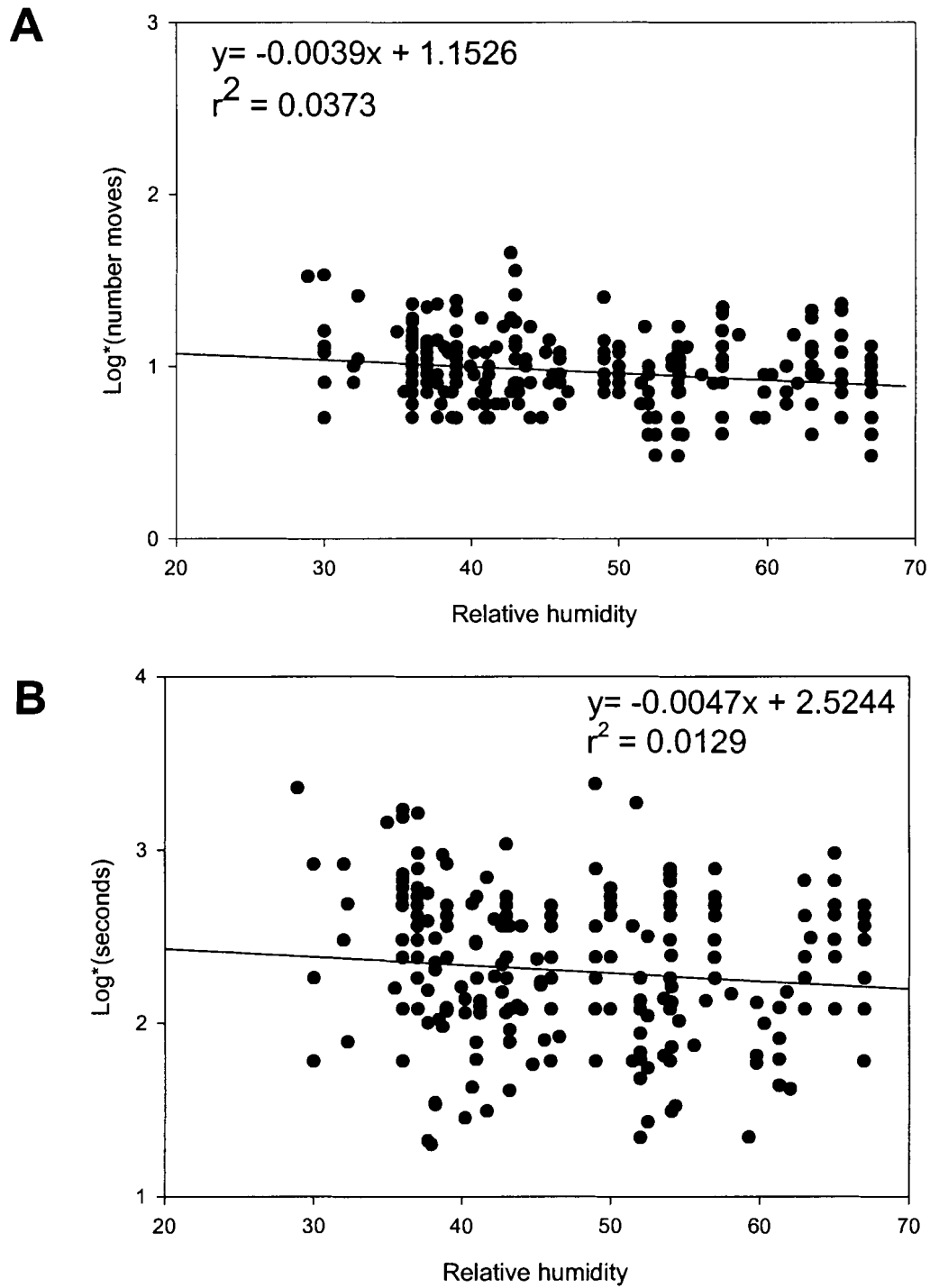**Figure 3.3** Relationship between relative humidity and log-tranformed number of moves (**A**), and log-transformed seconds to plant colonization (**B**) for adult Colorado potato beetles in relationship to one of four possible plants.

More beetles were eliminated from trials for failing to move within 30 minutes in 2001 than in 2002. Both RH and treatment significantly (at the α=0.1 level) impacted the number of beetles that exhibited no movement. More beetles were eliminated at higher relative humidities and lower temperatures ($\chi^2_6 > 12.6$, p=0.07, p=0.09, respectively).

Cadaver Density Study

The number of encounters of adult beetles with sporulated cadavers on the soil surface prior to plant colonization significantly increased as density of cadavers increased ($F_{(1,65)}$ = 56.957, p<0.0001), and this relationship is best described by the linear regression between the square root transformed number of encounters and the log transformed density of cadavers (Fig. 3.4). Proportion mortality of adults increased as cadaver density increased ($F_{(1,65)}$ =19.468, p<0.001) and proportion sporulation increased in the same manner ($F_{(1,65)}$ =15.979, p>0.001). These relationships are best described by the regression between the proportions and the log transformed density of cadavers on the soil surface (Fig. 3.5,3.6). Presence of *B. bassiana* did not impact the total time it took for a beetle to colonize a plant ($F_{(1,6)}$ = 0.9769, p=0.3787), but total time was affected by age ($F_{(1,6)}$ = 9.1169, p=0.0038), with older beetles taking more time to colonize plants. Increased densities of cadavers on the soil surface resulted in increased time to colonize plants, but only at the 0.10 alpha level ($F_{(1,3)}$ = 2.6394, p=0.0994). The average numerical rating of contact increased with increased cadaver density to 0.0606 cadavers/cm^2, after which it dropped slightly ($F_{(1,3)}$ = 7.6706, p=0.0015) (Table 3.1).
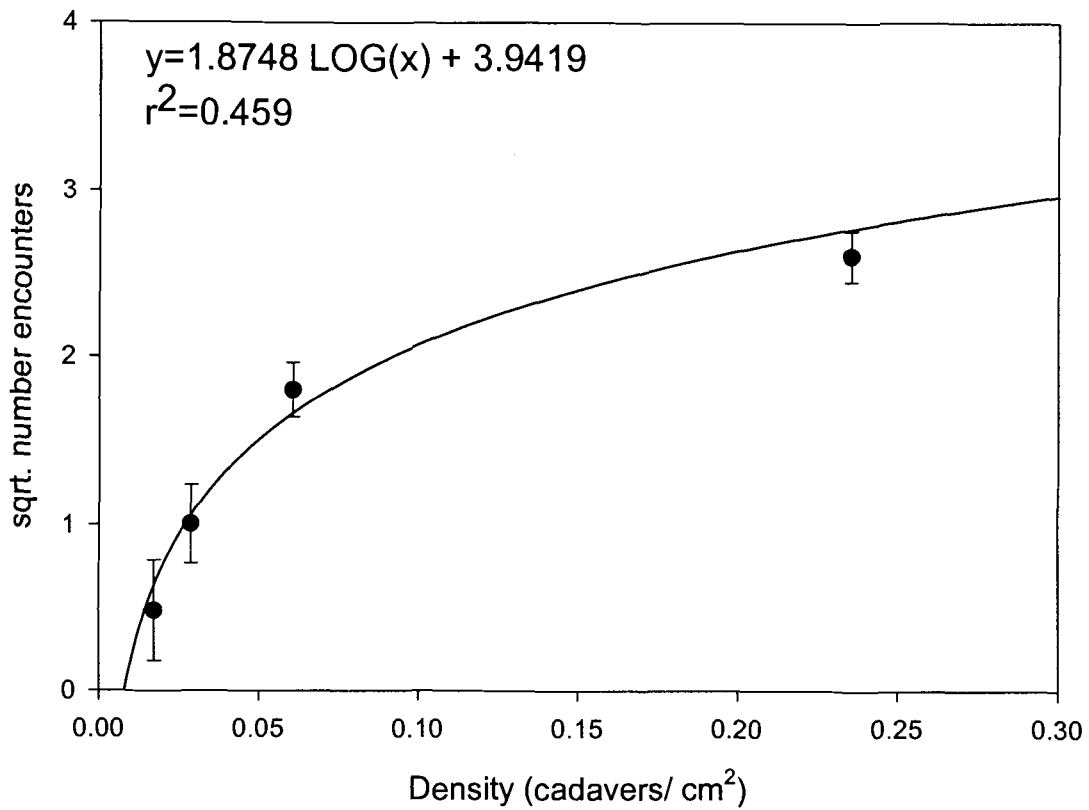
Figure 3.4 Relationship between log transformed density of
second instar Colorado potato beetle sporulated cadavers
and square root transformed number of encounters by adult
Colorado potato beetles. Regression equations and r^2 values are fit to
individual data points. Figures depict mean ± standard errors.

Figure 3.5 Relationship between log transformed density of sporulated second instar Colorado potato beetle cadavers and log transformed proportion mortality of adult Colorado potato beetles. Regression equations and r^2 values are fit to individual data points. Figures depict mean ± standard errors.

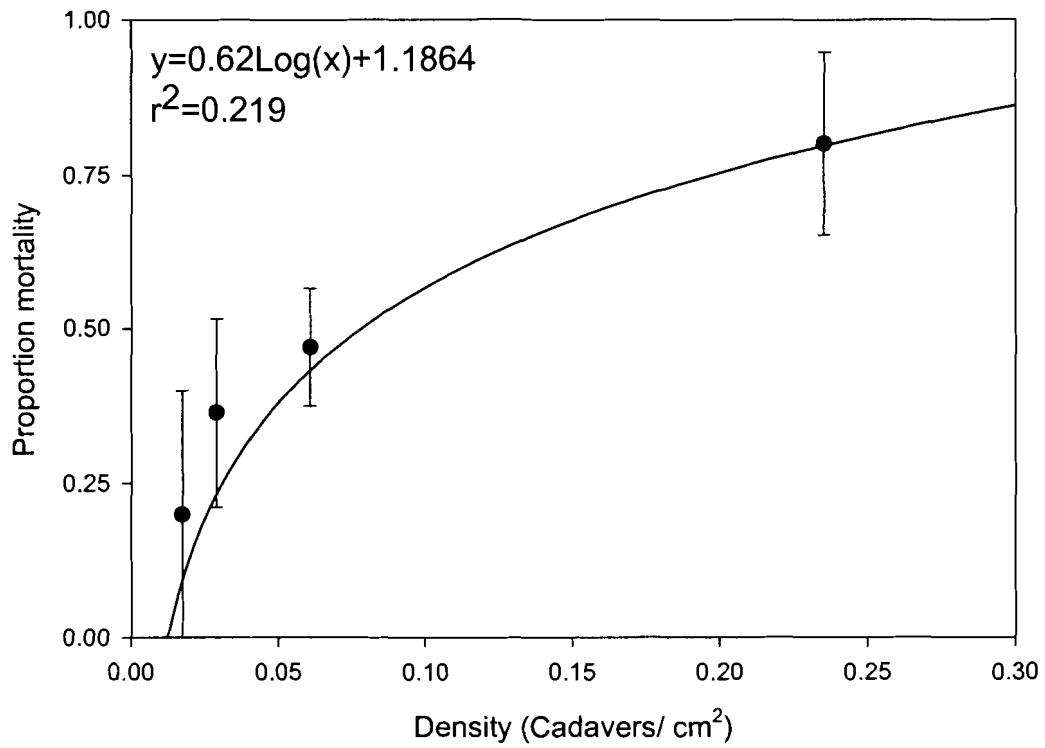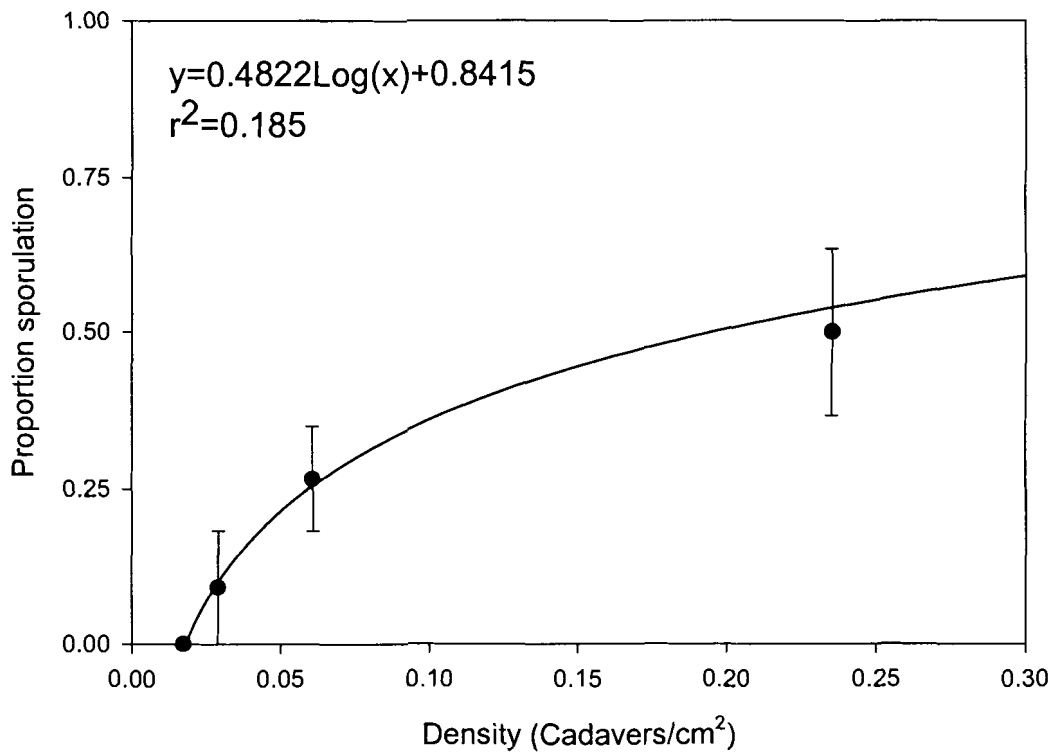**Figure 3.6** Relationship between log transformed density of sporulated second instar Colorado potato beetle cadavers and log transformed proportion sporulation of adult Colorado potato beetles. Regression equations and r^2 values are fit to individual data points. Figures depict mean ● standard errors.

Table 3.1 Average rating of contact between adult Colorado potato beetles and *B. bassiana* sporulated cadavers at varied cadaver densities.

Density (cadavers/ cm^2)	Average Rating
0.0173	0.1176 ± 0.33
0.0289	0.8182 ± 0.57
0.0606	1.3973 ± 0.69
0.2352	1.2283 ± 0.23

Discussion

In both the controlled arena experiment in 2001, and in the field experiment in 2002, beetles did not show any strong evidence of avoidance to *B. bassiana* sporulated cadavers in the environment. This is evidenced in part by the fact that beetles in both years did not preferentially travel to and colonize any of the four available potato plants regardless of the presence of sporulating cadavers between their emergence point and the plant. Likewise, beetles in control and *B. bassiana* treatments settings did not orient to a compass direction as they search for a host plant.

Beetles also did not travel at an increased frequency through grid squares that did not contain a sporulated cadaver. The fact that 21 beetles came into contact with a sporulated cadaver on the soil surface when the area of a grid square that a cadaver occupies is less than 3%, demonstrates that beetles most likely do not have a mechanism for detecting diseased cadavers on the soil surface. Avoidance of 3% of a grid square

would not necessitate a large movement. Avoidance of sporulated cadavers has been reported for some insects, but not others. Termites show avoidance of dead sporulated cadavers, but do not avoid live diseased individuals (Kramm *et al.* 1982). Villani *et al.* (1994) found that Japanese beetle grubs burrowing through soil actively avoided areas where *Metarhizum anisopliae* conidia had been added. However, Lord (2001) found that *Cephalonomia tarsalis*, a parasitoid of sawtoothed grain beetles *(Oryzaephilus surinamensis)* do not distinguish between *B. bassiana* infected and non infected beetles, although the wasps are susceptible to the disease. Wasps attack their hosts when they are still alive, and thus do not encounter sporulating cadavers. Wasps were also found to enter environments with conidia present at the same frequency as clean environments.

Although relative humidity was the only abiotic factor that played a statistically significant role in beetle movement, its impact was minor in this study. It has been reported that decreases in humidity result in increased movement of insects (Fraenkel and Gunn 1940). In this study, similarly, beetle movement was slightly higher at lower relative humidities. Beetles were also excluded from trials due to lack of movement more frequently at high than low relative humidity levels.

In the arena trials, 19% of beetles that had contact with a sporulated cadaver died and exhibited subsequent sporulation, showing evidence of *B. bassiana* horizontal disease transmission. In the cadaver density studies the frequency of encounters of sporulated cadavers increased as density increased, in a curvilinear fashion. This trend supports the theory that the density of infected individuals in the environment is dependent upon the density of diseased individuals (Anderson 1981, Brown and Nordin 1982, Kelly-Tunis *et al.* 1995, Thomas *et al.* 1995). These results illustrate a relationship that appears to differ

from that described by Long *et al.* (2000a) for the relationship between the density of *B. bassiana* cadavers and encounters with burrowing prepupae. In this study adults had, overall, more contact with cadavers as they emerged from the soil and colonized their host plant than was found for prepupae climbing off the plant and burrowing into the soil to pupate (prepupae averaged between 0.01 and 2.25 encounters per individual, whereas adults between 0.25 and 6.25 encounters per individual). Long *et al.* (2000a) found a linear relationship between number of encounters and cadaver density, which could have resulted from the lower numbers of overall encounters in that study. Adult CPB are generally considered more motile than prepupae and this motility appears to increase their chance of contacting a diseased individual on the soil surface. The curvilinear trend indicates that perhaps there is a saturation point of encounters possible between a healthy CPB and diseased cadavers on the soil surface. In this study it is most likely that densities above 0.30 cadavers per cm^2 would not yield an increase in encounters.

In the relationship between mortality and cadaver density, mortality increased in a curvilinear fashion as density increased. The same trend can be seen in the relationship between cadaver density and proportion of dead individuals that sporulated. Overall mortality and sporulation values are similar to the findings of Long *et al.* (2000a), with, on average, prepupae sporulating 36.8% more than adult beetles. This is most likely due to the decreased susceptibility of adults to *B. bassiana* than mature larvae (Fargues 1972, Appendix).

This study produced no evidence of adult CPB actively avoiding *B. bassiana* sporulated cadavers on the soil surface, and increased densities of sporulated cadavers result in increased numbers of encounters between adults and cadavers, as well as

increased proportion mortality and sporulation. There are many other factors, however, besides cadaver density that plays a role in disease transmission. Environmental factors such as temperature and humidity (Fernandez 2000), as well as rainfall (Furlong *et al.* 2003) can play a part in disease transmission. Higher temperatures may cause more beetle movement, but may fall out of the range of optimal *B. bassiana* growth. Long *et al.* (2000a) found infection rate increased with decreased temperatures, and temperature could not explain differences in cadaver encounters, time to burrow and depth of burrowing, suggesting that temperature has a greater impact on fungal growth than it does on beetle behavior. In fact, temperature is thought to be the most important external factor in determining insect susceptibility and the multiplication of a microbial pathogen within its host (Waitanabe 1987).

Humidity has been shown here to affect beetle movements, but weakly. Humidity may play a more important role in the successful growth of *B. bassiana* within the ecosystem. For successful *B. bassiana* growth and germination, relative humidity must be above 92.5% (Walstead *et al.* 1970, Fernandez 2001). Depending where in the field a cadaver is placed (underneath the potato canopy or on exposed soil), the humidities at the soil surface can fluctuate below the optimal RH range, hindering the infectivity of cadavers.

The effect of radiation may also have an effect on the transmission of *B. bassiana* within the agroecosystem. Conidia exposed to sunlight lose viability much more quickly than those protected from the sun (Daoust and Pereira 1986, Joergensen 2000). However, viability of cadavers found underneath the potato canopy have been found to have a positive relationship with solar radiation, most likely due to microclimate changes

associated with the change in radiation and not the direct sunlight (Fernandez 2001). Although these environmental factors may serve to reduce the amount of infective conidia on sporulated cadavers, it has been shown that an 86.1% reduction of conidia on cadavers did not significantly decrease the infection rate of CPB prepupae coming in contact with them (Long *et al.*2000a).

Horizontal infection of adult CPB by *B. bassiana* is not influenced by the beetle's ability to detect and avoid sporulated cadavers on the soil surface, and is more likely to occur as the density of cadavers on the soil surface increases. A management practice aimed at maximizing horizontal transmission should increase the number of cadavers on the soil surface but the motility of the targeted life stage also needs to be considered. However, overall susceptibility of the targeted life stage to *B. bassiana* also needs to be considered. Although in this study, adults are more motile, the overall effect of encounters is higher for prepupal stages, due to their higher susceptibility. It may be more effective to target those stages that will have a greater reaction to each cadaver encounter, than to focus on the stages that have the highest number of encounters.

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APPENDIX: LC_{50} FOR ADULT LABORATORY REARED COLORADO POTATO BEETLES

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is one of the most serious insect pests of potatoes in the northeastern United States. The entomopathogenic fungus *Beauveria bassiana* (Bals.) has been utilized as a commercial control agent against CPB in some parts of the world for the past 20 years (Ferron 1981, Tanada and Kaya 1993, Feng *et al.* 1994). Several studies have been published illustrating LC_{50} dosages for most of the stages of the beetle, including eggs (Long *et al.* 1998), larvae (Fargues 1991), and pupae (Watt and Lebrun 1984). However, since the adult stage of the beetle is generally thought to be less susceptible to *B. bassiana* treatments, LC_{50} values have not been published for the adult beetle.

In this study, our objective was to determine LC_{50} levels of *B. bassiana* for laboratory-reared adult Colorado potato beetles, as well as to determine the most appropriate method of treatment. This information was to be used in subsequent laboratory assays.

The experiment was initiated on January 16th, 2001. One hundred and sixty adult beetles (approximately 195 degree days old, base $10\pm1°C$) were used for this study. These adults were reared from a laboratory colony which was supplemented annually by field collections from the University of Maine's Rogers Farm in Stillwater, ME. Potato fields used for beetle collections received seven weekly sprays of the copper hydroxide

fungicide Champ Formula 2 ® (Agtrol Chemical Products, Houston, TX) for *Phytophtora*

infestans control and no other pesticides. All adult beetles were maintained in paper cups

(300 ml) at 25±1°C and a 16:8 light-dark cycle and fed fresh greenhouse grown potato

foliage (*Solanum tuberosum* L.) daily.

Beauveria bassiana strain GHA was obtained as dried conidia from Mycotech,

now Emerald BioAgriculture (Butte, MT), and plated on Sabouraud dextrose agar (SDA)

(Difco Laboratories, Detroit, MI). Colonies were sub-cultured once on SDA, and for each

experiment one of these single, axenic subcultures (grown for 10 days at 25±1°C, stored

at 4±1°C for not more than 60 days) were utilized. Conidia were harvested from these

plates with a sterile loop and suspended in an aqueous solution of 0.1% Tween 20 (JT

Baker, Phillipsburg, New Jersey). Concentrations of conidia were determined using a

hemocytometer, and solutions were diluted as necessary with 0.1% Tween 20.

To determine the best method of *B. bassiana* treatment, beetles were either

sprayed directly (topical application) or exposed to treated potato leaf disks. For topical

applications, *B. bassiana* or control (0.1% Tween 20 alone) solutions were sprayed using

a Burkhard Computerized Spray Apparatus® (Hertfordshire, England) at 55.2 kPa with a

diaphragm setting of 6. Two plates of SDA were sprayed at the same time as *B. bassiana*

treatments to obtain accurate rates of conidial density and viability at the time of the

spray. For leaf disk exposure, 20 mm diameter leaf disks, cut from greenhouse grown

potato plants were placed on the surface of 1% water agar and sprayed with appropriate

solutions. Leaf disks were fed to beetles immediately following treatment. For each type

of treatment (topical or leaf disk), 6 concentrations of *B. bassiana* were tested; 0 conidia/

ml, 2 x 10^5, 6.32x10^5, 2x10^6, 6.32x10^6, and 2x10^7 conidia/ml. In all experiments, ≥98%

of the conidia had formed germ tubes after 18h at 25±1°C, indicating viability. Post treatment beetles were held in individual 60 x 15 mm petri dishes at 25±1° C for 14 days. Proportion mortalities were determined from this data.

Figure A.1 shows the dose response curve for topical applications. However, as the three lowest concentrations did not vary from each other, two of these doses were eliminated, and another curve was refit to the remaining data (Fig. A.2). This curve shows the LC_{50} for topical applications to adult beetles was ca. 2 x 10^7 conidia/ ml. For leaf disk exposures, mortality did not exceed 25% at any concentration, suggesting that this method for treating adult beetles is less efficient than topical applications.
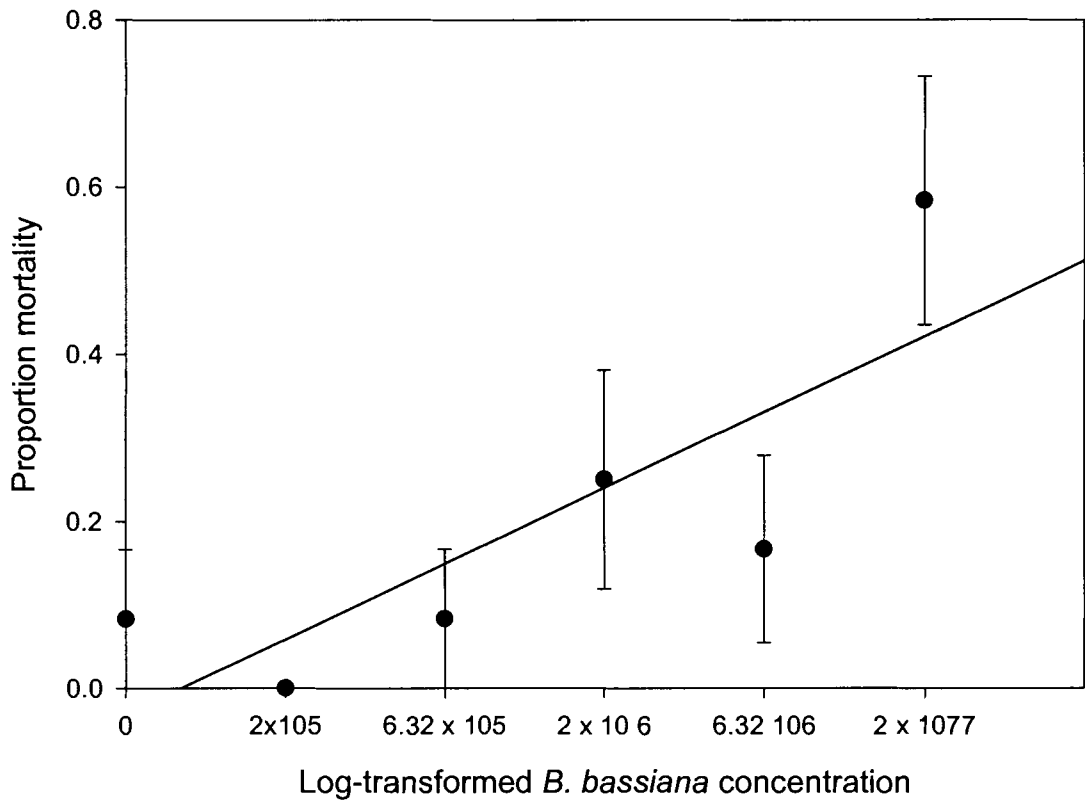
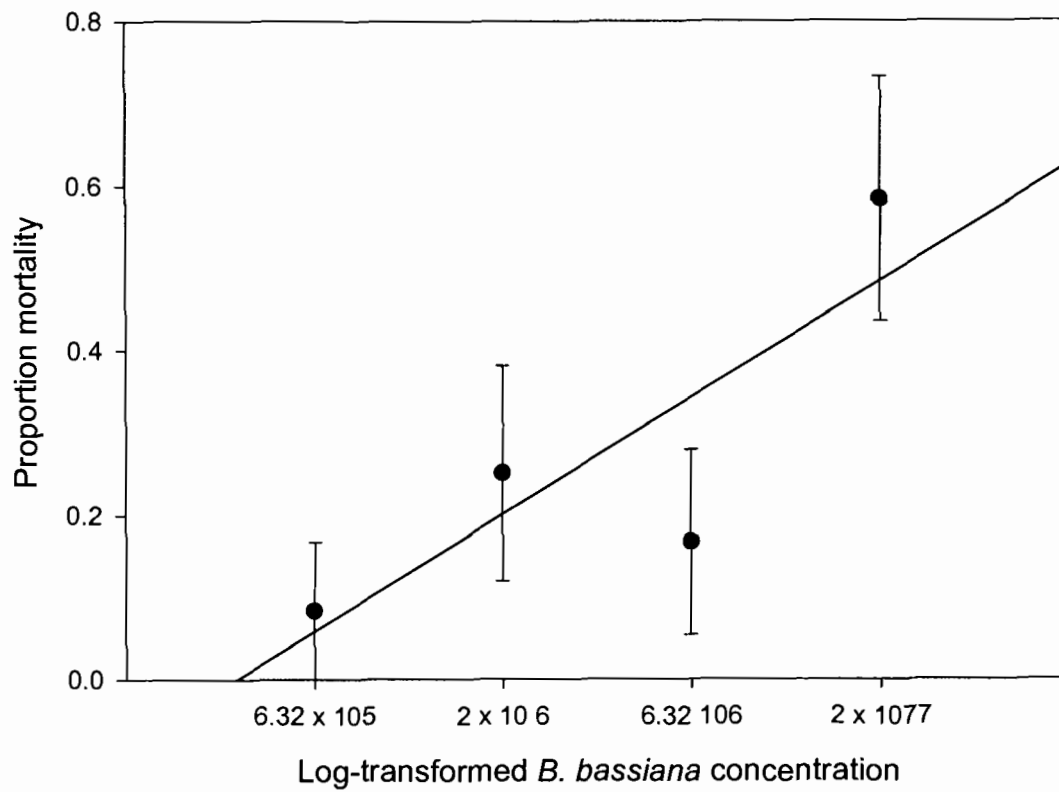**Figure A.1** Dose response of topical applications of *B.bassiana* to adult Colorado potato beetles

Figure A.2 Dose response of higher concentration topical applications of *B. bassiana* to adult Colorado potato beetles.
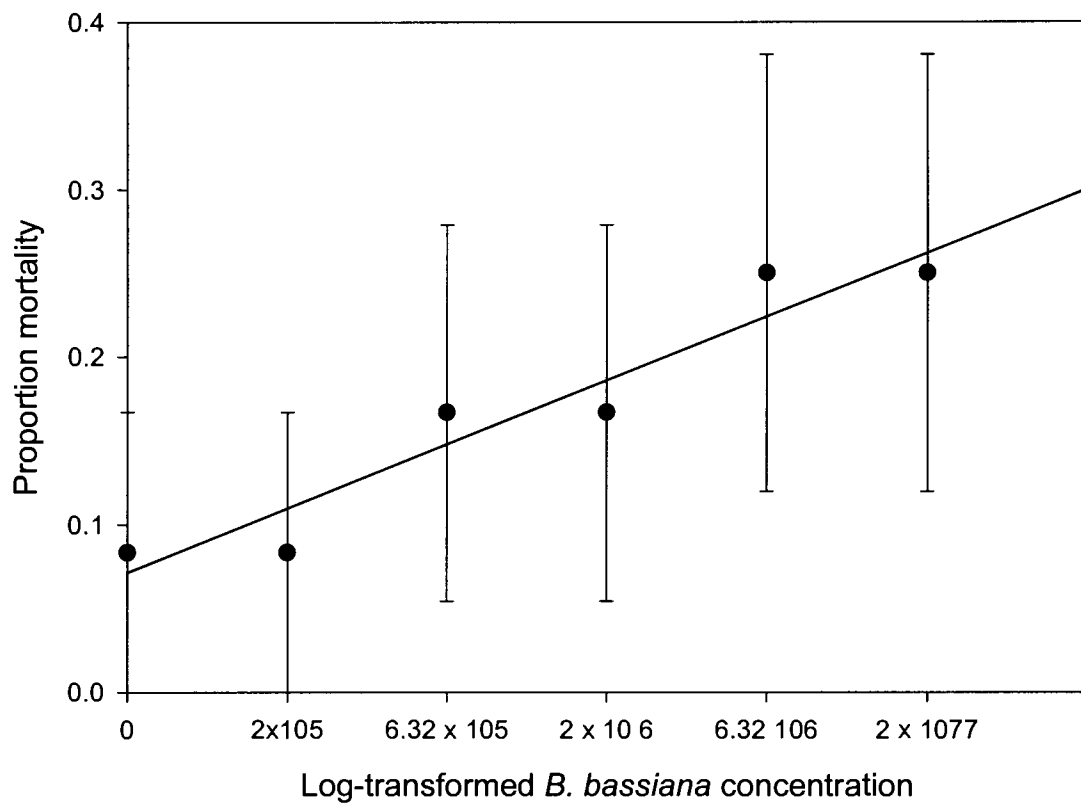
Figure A.3 Dose response of leaf disk *B. bassiana* applications to adult Colorado potato beetles

BIOGRAPHY OF THE AUTHOR

Ellen Klinger was born in Carlisle, Pennsylvania via forceps delivery, which has left a permanent mark under her left eye. Relatively soon after birth, she was taken to the warm, sunny climate of Atlanta, where she grew up without any major incident and successfully avoided obtaining a southern accent. During the ever tumultuous middle school years, she was again moved, this time to Knoxville, Tennessee, home of the amazing Sunsphere (but she won't tell you if it is, indeed, full of wigs). After surviving 3 years in "Volunteer country", she was returned to her birthplace and fortunately graduated from Carlisle High School in 1996, despite an unfortunate incident involving a fire alarm during the last week of her senior year (please see her "permanent record" for further details). She decided to pursue her education at Lycoming College, located in the bustling metropolis of Williamsport, Pennsylvania, home of the Little League World Series. She graduated Lycoming in 2000 *summa cum laude* and with departmental honors with a B.S. in Biology and a minor in Environmental Science. She entered the graduate program in Ecology and Environmental Sciences at the University of Maine in August, 2000. She has since developed a close relationship with many things, including a certain 10 lined beetle, a pathogenic deuteromycete, certain drinking establishments in the greater Orono area, as well as many wonderful people at the University of Maine. She has been a member of the Beta Beta Beta biological honor society, Phi Kappa Phi honor society, as well as an active member of the Entomological Society of America, the Acadian Entomological Society and the Society of Invertebrate Pathology. She is a candidate for the Master of Science degree in Ecology and Environmental Sciences from The University of Maine in August, 2003.